

POTATO EXPRESSING BEETLE-SPECIFIC
Bacillus thuringiensis Cry3Aa TOXIN REDUCES
PERFORMANCE OF A MOTH

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Abstract—Expression of the *Bacillus thuringiensis* beetle-specific toxin Cry3Aa, which renders a genetically modified potato cultivar resistant to the Colorado potato beetle *Leptinotarsa decemlineata*, exerts a deleterious effect on the polyphagous moth *Spodoptera littoralis*. The caterpillars of *S. littoralis* feed less and produce smaller pupae on the genetically modified cultivar (NewLeaf Superior) than on the parental nontransgenic cultivar (Superior). The conversion efficiencies of total dry matter, combustion heat, carbon, and nitrogen from leaves to insect biomass are similar on both cultivars. In spite of similar food utilization and a relatively small difference in the body mass at pupation, female adults that developed from caterpillars fed on NewLeaf Superior lay a mean of 309 eggs compared to a mean of 713 eggs deposited by females that developed from caterpillars fed on Superior. Because of this difference and a simultaneous reduction in fertility (egg hatchability) from 78 to 48%, a pair of adults that fed as larvae on NewLeaf Superior produces only 148 larvae, whereas a pair of adults that fed as larvae on Superior produces 556 larvae. We suggest that small amounts of Cry3Aa that accumulate in insect tissue and persist until the adult stage are responsible for the decline in reproduction.

Key Words—*Bacillus thuringiensis*, *Spodoptera littoralis*, *Leptinotarsa decemlineata*, genetically modified crops, Cry3Aa, Bt applications, insect digestion, pest population growth.

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INTRODUCTION

The gram-positive soil bacterium *Bacillus thuringiensis* Berliner (Bt) produces an array of insecticidal toxins that are denoted with the Greek letters α , β , γ , and δ (Heimpel, 1967). The most important δ -toxins are known as Cry proteins in reference to their crystalline nature (Yamamoto, 2001). More than 150 Cry proteins are produced by different Bt strains (Schnepf et al., 1998), and each of them acts lethally on a certain range of insect species (Zalunin et al., 2004). For example, toxins of the Cry1A class are lethal for certain Lepidoptera, whereas those of the Cry3A and Cry11A classes are lethal for Coleoptera and Diptera, respectively. Toxin crystals formed during bacterial sporulation are dissolved when the spores germinate in the insect gut lumen; the toxin is activated following partial hydrolysis by insect digestive enzymes. Activated Cry proteins bind to receptors in the membrane of midgut cells and affect formation of pores for uncontrolled ion movement (Whalon and Wingerd, 2003). The pH gradient between gut lumen and hemolymph is disrupted, and the affected gut cells are lysed. The insect usually dies of starvation and septicemia rather than as a result of direct Bt toxicity.

The peptidic nature, high activity on specific target species, and relative safety to domestic animals and man make Cry toxins ideally suited for genetic modifications aimed at protecting agricultural crops against insect pests. Potato plants carrying the gene for the Cry3Aa toxin represented the first transgenic crop produced for commercial use (Perlak et al., 1993). This “Bt potato” proved fully resistant to the target pest, the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (CPB) (Whalon and Wierenga, 1994; Arpaia et al., 2000; Reed et al., 2001). Field observations revealed that the predators of CPB and other beneficial insects were not affected (Riddick and Barbosa, 1998; Reed et al., 2001; Duan et al., 2004). However, laboratory studies with maize expressing Cry1Ab and fed to caterpillars showed that the toxin considerably enhanced larval mortality of the lacewing *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae), which had preyed on the caterpillars (Hilbeck et al., 1998a). Lethality of high toxin doses for *C. carnea* was confirmed by adding the toxin to a liquid diet of this predator (Hilbeck et al., 1998b).

The Bt maize cultivar MON810, which was reported to contain 7.9–10.3 ppm Cry1Ab in the leaves (Dutton et al., 2003), has been studied as a food source for the Egyptian armyworm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) (EAW). No deviations from the controls were observed in the performance of EAW on the Bt maize (Hilbeck et al., 1998a), and in experiments with a semiartificial diet, the intoxication of EAW caterpillars was obtained only with a high concentration (100 ppm) of Cry1Ab (Hilbeck et al., 1999). An equally high concentration was needed to affect *C. carnea* when the toxin was administered to the artificial diet of this predator (Hilbeck et al., 1998b). These

data suggest that the lethality to *C. carnea* from prey fed on Bt maize is hardly a result of a massive accumulation of toxin. If this were the case, then the prey itself would be intoxicated. It is more likely that low toxin doses, which the caterpillars receive with their plant food, cause some subtle changes that are not manifested in an increased death rate of the caterpillars, but rather render these larvae less suitable as lacewing prey. A number of other studies have indicated that the interactions between Bt toxins and the nontarget insect species are complex and insufficiently understood (Scriber, 2004). In the present paper, we show that a low expression level of the beetle-specific toxin Cry3Aa in potato affects the physiology of EAW developing on the leaves. The effect is small and not easy to detect, but may alter long-term population dynamics of EAW.

METHODS AND MATERIALS

Potato Cultivation. Minitubers of the transgenic cultivar NewLeaf Superior, which carries a *Cry3Aa* Bt-toxin gene construct, and its parental, non-transgenic cultivar Superior, were obtained from Monsanto Co. (St. Louis, MO, USA). Subsequent potato generations were propagated as plant explants in agar cultures. Rooted explants were transferred to soil in 100-ml pots and later replanted to 10-l containers. Plants were grown in a greenhouse at 20°C and natural light from May to August; artificial illumination (16 hr/d) was used during the winter months.

Insect Handling. The stock culture of the EAW was derived from an Egyptian field population and maintained in the laboratory at 20–23°C and 16:8 hr (light/dark) photoperiod for 10–15 generations. The diet was *Manduca-Heliothis* Premix (Stonefly Industries Inc., Bryan, TX, USA), which is a mixture of wheat germ and agar. The four experiments described in this paper were conducted in July–August and October–November 2003, and in March–May and September–October 2004. Groups of five newly ecdysed caterpillars of the penultimate instar were transferred to potato leaves cut from nonflowering plants that were at least 20 cm tall. Each group received leaves (one per day) cut from the base to the top of a single plant. Unless stated otherwise, each experiment consisted of six groups of larvae. Food consumption, frass production, and insect body weight were measured daily. Newly emerged adults were collected, and groups of two to three males and two females were kept until death in paper cylinders (20 cm high, 10-cm diam) closed on both sides with a Petri dish. The adults were provided with tubes filled with water and diluted honey. Egg masses laid on the paper cylinder were cut out, and the numbers of hatched and unhatched eggs were counted.

Cry3Aa Quantification. The amounts of toxin in the potato leaves, the insect bodies, and the insect frass were measured with Bt-Cry3A ELISA PathoScreen

kit (Agdia Inc., Elkhart, IN, USA). One-gram aliquots of leaves and frass and 290- to 980-mg samples of pupae and adults were crushed in a mortar in 9 ml of extraction buffer provided with the kit. The homogenates were centrifuged at $15,000 \times g$ for 10 min, and 100- μ l aliquots of the supernatants were taken for enzyme-linked immunosorbent assay (ELISA), which was performed according to the manufacturer's instructions.

Food Conversion Analysis. In one experiment containing six groups of five insects on each potato cultivar, samples of the potato leaves, the insects, and their frass were dried, and the powdered dry matter was used for carbon and nitrogen quantification and for combustion calorimetry. Carbon and nitrogen were quantified with the elemental analyzer NC 2100 Soil Analyzer (Thermo-Quest, Milan, Italy). Energy content was measured by burning the samples in oxygen at 30 bars in an IKAC 700T calorimeter (IKA Werke, Janke & Kunkel, Stauffen, Germany). The efficiency of food utilization was evaluated and described according to Slansky and Scriber (1985) based on dry matter analysis from two experiments. The index M , called the metabolic loss ($M = I - F - B$, I = ingested food; F = frass; B = biomass), indicates how much of the digested food was not used for biomass accumulation. Approximate digestibility is the ratio on a percentage basis of digested to ingested food, i.e., $AD = [(I - F)/I] \times 100\%$. Gross growth efficiency or efficiency of ingested food conversion to biomass (ECI) indicates the ratio of body mass increase to the amount of ingested food, i.e., $ECI = (B/I) \times 100\%$. Net growth efficiency of the conversion of digested food to biomass represents the ratio of body mass increment to digested food, i.e., $ECD = [B/(I - F)] \times 100\%$.

Data Presentation and Analyses. Data are presented as mean \pm standard error. Comparisons of the growth and food utilization parameters on the Bt and non-Bt potatoes were performed with a t -test ($\alpha = 0.05$). Female fecundity and fertility were examined in three independent experiments and were analyzed by two-way analysis of variance (ANOVA); correlation of these parameters with the pupal body weight was examined with two-way analysis of covariance (ANCOVA). These analyses were conducted using Statistica 6 (StatSoft Inc., Tulsa, OK; <http://www.statsoft.com>).

RESULTS

Larval Growth Rate and Cry3Aa Turnover. Transfer of EAW caterpillars from the Premix diet to potato leaves caused a reduction of their growth rate. The insects retained on Premix reached a maximal larval body weight around 700 mg (data not shown), whereas those transferred to the potato leaves of either cultivar reached a weight of about 500 mg (Figure 1). The total duration of the penultimate and last larval instars was 11 d on both cultivars. Larval

mortality averaged 1.4% on both cultivars, whereas pupal mortality was 2.2% on the Superior and 4.4% on NewLeaf Superior. The growth of larvae varied among the four experiments, but invariably they grew bigger on Superior than on NewLeaf Superior. In one experiment (Figure 1), the insects feeding on Superior reached a pupal weight of 227 ± 4.9 mg, and those grown on NewLeaf Superior reached a pupal weight of 205 ± 6.5 mg. These two values are significantly different ($P = 0.02$).

The uptake of Cry3Aa was examined in another experiment in which the insects reached a mean pupal weight of 282 ± 9.8 mg on the Superior and 258 ± 8.2 mg on the NewLeaf Superior leaves ($P = 0.015$). In this experiment, the larvae consumed 3856 ± 97 mg Superior and 3307 ± 94 mg NewLeaf Superior leaves per specimen ($P = 0.01$). Given a mean Cry3Aa leaf content of 1.43 ng/g, each caterpillar reared on NewLeaf Superior ingested nearly 5 μ g of the toxin (Table 1). About 1.5 μ g was discarded with the feces, and most of the remainder was apparently degraded to the extent that it could not be detected with our ELISA. However, between 10 and 60 ng detectable Cry3Aa was retained in each female pupa, and approximately 1% of this amount remained in female adults analyzed in the middle of their ovipositional period. No toxin was detected in male pupae. The leaves of Superior contained no Cry3Aa, and the insects feeding on this cultivar were, therefore, not analyzed for the toxin.

Leaf Matter Conversion to Insect Biomass. EAW development on NewLeaf Superior was associated with slightly lower food consumption than it was

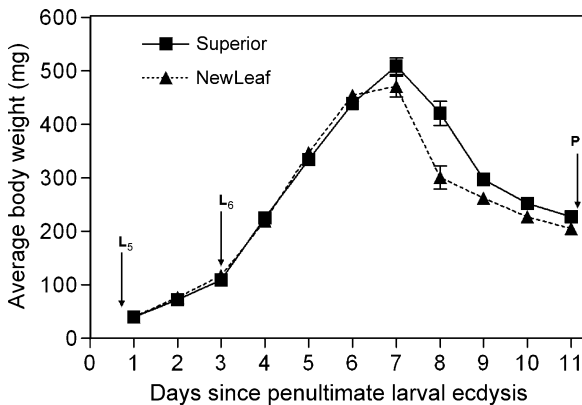


FIG. 1. Growth of *Spodoptera littoralis* from the beginning of the penultimate larval instar (day 1) until pupal ecdysis (day 11) on leaves of the potato cultivars Superior and NewLeaf Superior, respectively. Six groups of five larvae were followed on each potato cultivar (i.e., $N = 6$). Vertical lines represent standard errors. Arrows indicate approximate times (± 6 hr) of ecdyses to the fifth larval instar (L_5), sixth (last) larval instar (L_6), and pupa (P).

on Superior (Table 1), but it was not clear if this alone was responsible for the lower pupal weights attained by larvae grown on the NewLeaf Superior cultivar. Analysis of the conversion efficiency of food dry matter confirmed that caterpillars reared on the Superior cultivar consumed more leaf material ($P < 0.001$) and grew larger ($P = 0.007$) than those feeding on the NewLeaf Superior cultivar (Table 2). The mass of Superior leaves was assimilated more ($AD = 54$) than that of NewLeaf Superior ($AD = 48$), and this difference was marginally significant ($P = 0.051$). Surprisingly, most of the extra matter ingested by larvae from the Superior leaves dissipated as metabolic losses (cf. M values in Table 2). Higher metabolic costs on the nontransgenic potato might have been caused by the intake of a larger amount of food and possibly also by more efficient degradation of allelochemicals from this cultivar. The efficiencies of ingested (ECI) and digested (ECD) food to biomass were not significantly different between the two cultivars, suggesting that the difference in the amount of food consumed was the primary cause of the different growth rates.

Quantitative Analysis of the Dry Matter. Similar conversions of leaf dry matter to body biomass did not exclude differences in the availability and utilization of specific food components. This possibility was examined by analyzing

TABLE 1. GROWTH PARAMETERS AND ACCUMULATION OF Cry3Aa IN TWO POTATO CULTIVARS AND IN *Spodoptera littoralis*^a

Parameters	Fed on		Statistics
	Superior	NewLeaf Superior	
Initial larval weight (mg) [15]	44.3 ± 0.1	43.6 ± 0.1	$t = 1.12; P = 0.289$
Cry3Aa content in leaves (ng/g) [6]	n.d.	1.03–1.65	–
Ingested food per larva (mg) [15]	3856 ± 61	3307 ± 59	$t = 6.21; P < 0.001$
Calculated Cry3Aa uptake (ng/specimen)	–	4729	–
Frass produced per larva (mg) [15]	1735 ± 49	1521 ± 34	$t = 6.65; P < 0.001$
Cry3Aa content in frass (ng/g) [6]	–	0.87–1.11	–
Calculated Cry3Aa excreted (ng/specimen)	–	1520	–
Fresh pupal weight (mg) [15]	282 ± 6	258 ± 5	$t = 3.07; P = 0.012$
Cry3Aa content in ♀ pupae (ng/g) [5]	–	0.03–0.2	–
Calculated Cry3Aa content (ng/♀ pupa)	–	29	–
Cry3Aa content in ♀ adults (ng/g) [5]	–	0.002–0.003	–
Calculated Cry3Aa content (ng/♀ adult)	–	0.38	–

^aSample size (N) is given in brackets (a group of five insects was used for each growth parameter measured, and 1 g of plant or insect material was used for each Cry3Aa measurement). Results are presented as means ± standard errors. Because six attempts to detect Cry3Aa in the leaves of the Superior cultivar were all negative (n.d. = not detected), insects grown on this cultivar were not analyzed for the presence of Cry3Aa. No Cry3Aa was found in male pupae and adults. When appropriate, means from each cultivar were compared with a t -test ($\alpha = 0.05$).

TABLE 2. EFFICIENCY OF CONVERSION OF BIOMASS FROM TWO POTATO CULTIVARS TO BIOMASS IN *Spodoptera littoralis* PUPAE^a

Parameters	Fed on		Statistics
	Superior	NewLeaf	
Penultimate instar dry matter (mg)	4.8 ± 0.16	4.6 ± 0.10	<i>t</i> = 3.07; <i>P</i> = 0.012
Newly ecdysed pupa dry matter (mg)	62.3 ± 6.0	52.3 ± 4.6	<i>t</i> = 3.57; <i>P</i> = 0.005
<i>B</i> , Dry biomass increment (mg)	57.5 ± 6.0	47.7 ± 4.5	<i>t</i> = 3.31; <i>P</i> = 0.008
<i>I</i> , Ingested food dry matter (mg)	426 ± 23	355 ± 35	<i>t</i> = 6.21; <i>P</i> = 0.001
<i>F</i> , Frass dry matter (mg)	196 ± 22	184 ± 29	<i>t</i> = 3.06; <i>P</i> = 0.012
<i>M</i> = <i>I</i> - <i>B</i> - <i>F</i>	173 ± 28	123 ± 39	<i>t</i> = 5.71; <i>P</i> = 0.014
AD = (<i>I</i> - <i>F</i>)/ <i>I</i> (×100%)	54 ± 8	48 ± 16	<i>t</i> = 0.94; <i>P</i> = 0.051
ECI = <i>B</i> / <i>I</i> (×100%)	13.5 ± 2.4	13.4 ± 0.19	<i>t</i> = 0.95; <i>P</i> = 0.783
ECD = <i>B</i> /(<i>I</i> - <i>F</i>) (×100%)	25 ± 0.28	28 ± 0.22	<i>t</i> = 0.89; <i>P</i> = 0.393

^aSix groups of insects (*N*) were analyzed for all parameters. Each group included five insects, but the data (means ± standard errors) are expressed per specimen.

B, body mass increment between penultimate instar larva and newly ecdysed pupa; *I*, mass of food consumed during the experiment; *F*, discarded frass and exuviae; *M*, metabolic loss; AD, approximate digestibility, i.e., ratio of digested to ingested food; ECI, conversion efficiency of ingested food to biomass, i.e., ratio of body mass increment to ingested food; ECD, conversion efficiency of digested food to biomass, i.e., ratio of body mass increment to digested food.

the carbon and nitrogen content and the combustion energy in larvae, leaves, frass, and pupae (Table 3). The penultimate instar larvae were analyzed for all parameters after feeding on the Premix diet and not comparatively against the two cultivars. The carbon content in leaf dry matter ranged from 36.5 to 40.2% in both cultivars; the means were not significantly different. The nitrogen content of leaves ranged from 5.21 to 5.97% in both cultivars; the means were not significantly different. The mean combustion heat values (energy content) in the leaves of each cultivar were also not significantly different. The variation in all three parameters was probably because, in large part, of the leaf age and the physiological status of the plant. Thus, the nutritional value of the Superior and the NewLeaf Superior potato leaves is virtually identical.

In the frass, the carbon content, nitrogen content, and combustion heat were not significantly different when compared across the two cultivars (Table 3). The carbon content of the frass was lower than that of the leaves, whereas the nitrogen content of the frass was about the same as that of the leaves. This suggests that the insects assimilated plant compounds containing nitrogen somewhat less efficiently than the non-nitrogenous organic materials. The combustion heat of the frass was only 85% of that of the leaves, irrespective of the cultivar.

As was the case with leaves and frass, the mean measures of carbon, nitrogen, and energy content from pupal dry matter were not significantly different between the two cultivars (Table 3). However, the carbon content was

TABLE 3. CARBON, NITROGEN, AND COMBUSTION HEAT IN LEAVES OF TWO POTATO CULTIVARS AND IN LARVAE, FRASS, AND PUPAE OF *Spodoptera littoralis*^a

Type of dry matter	Parameter analyzed	Fed on		Statistics
		Superior	NewLeaf Superior	
Larval body	Carbon (%)	44.7 ± 0.2 (8) (start of the penultimate instar)		
Leaves		37.7 ± 0.5 (4)	37.5 ± 0.4 (4)	$t = 0.30$; $P = 0.776$
Frass		34.2 ± 0.5 (4)	35.6 ± 0.6 (4)	$t = 1.86$; $P = 0.115$
Pupal body		51.5 ± 0.8 (9)	52.4 ± 0.1 (9)	$t = 2.44$; $P = 0.095$
Larval body	Nitrogen (%)	9.14 ± 0.09 (8) (start of the penultimate instar)		
Leaves		5.67 ± 0.09 (5)	5.74 ± 0.06 (5)	$t = 0.50$; $P = 0.634$
Frass		5.67 ± 0.22 (5)	5.41 ± 0.14 (5)	$t = 0.88$; $P = 0.414$
Pupal body		8.94 ± 0.18 (9)	8.98 ± 0.11 (9)	$t = 0.33$; $P = 0.745$
Larval body	Combustion	30,414 ± 76 (6) (start of the penultimate instar)		
Leaves	heat (J/g)	15,781 ± 165 (6)	15,897 ± 248 (6)	$t = 0.39$; $P = 0.704$
Frass		13,500 ± 318 (9)	13,517 ± 195 (8)	$t = 0.05$; $P = 0.965$
Pupal body		25,105 ± 286 (9)	24,768 ± 184 (11)	$t = 1.06$; $P = 0.302$

^a Table entries are means ± standard errors (N in parentheses). Newly ecdysed penultimate instar larvae were assayed just prior to their transfer from the semiartificial diet to either of the potato cultivars; pupae were assayed just subsequent to ecdysis from last instar larvae. The carbon and nitrogen contents are given in dry matter percentage; the combustion heat in J/g dry matter.

higher, and the nitrogen content was lower in pupae when compared to newly ecdysed penultimate instar larvae. The combustion heat of pupae fell short of the value found in larvae at the beginning of the penultimate instar.

Mortality and Reproduction of EAW Grown on Potato Leaves. Caterpillars that fed continuously on the Premix diet perished only exceptionally and produced adults yielding 1077 ± 91 viable eggs per female. However, when the newly ecdysed penultimate instars were transferred from Premix to the potato leaves, 3.6% of insects reared on Superior and 5.8% of those reared on NewLeaf Superior perished prior to adult eclosion. Because the mortality varied greatly and the difference was relatively small, it was disregarded in our investigation of the fertility of emerged adults. The fecundity and fertility were measured in three experiments performed several months apart with different generations of EAW and their food plants. Evaluation of the results by two-way ANOVA revealed differences between the three experiments and significant dependence of the level of oviposition (fecundity) and the number of hatched eggs (fertility) on the type of cultivar (Table 4). Interaction between the two factors, cultivar and experiment, was not significant for fecundity but was significant ($F = 6.7$, $df = 2.23$, $P = 0.005$) for fertility. Addition of pupal weight as a continuous predictor in two-way ANCOVA calculations confirmed differences between the experiments and the dependence of female fecundity and fertility on the

TABLE 4. PUPAL WEIGHT AND ADULT FECUNDITY AND FERTILITY OF *Spodoptera littoralis* GROWN ON LEAVES OF TWO POTATO CULTIVARS^a

Experiment	Potato cultivar	Pupal weight	Fecundity (eggs/female)	Fertility (hatched/female)
I	Superior (5)	286 ± 25	784 ± 92	595 ± 85
	NewLeaf	251 ± 14	344 ± 5	182 ± 5
II	Superior (5)			
	Superior (6)	236 ± 16	624 ± 11	424 ± 27
	NewLeaf	233 ± 48	245 ± 3	73 ± 7
III	Superior (4)			
	Superior (5)	231 ± 12	750 ± 34	672 ± 26
	NewLeaf	211 ± 7	330 ± 45	182 ± 35
	Superior (4)			
ANOVA cultivar (Superior/NewLeaf)		$F = 6.6; df = 1, 23; P = 0.017$	$F = 584; df = 1, 23; P < 0.001$	$F = 730; df = 1, 23; P < 0.001$
ANOVA experiment (I/II/III)		$F = 11.8; df = 2, 23; P < 0.001$	$F = 19; df = 2, 23; P < 0.001$	$F = 49; df = 2, 23; P < 0.001$
ANCOVA cultivar (Superior/NewLeaf)		–	$F = 431; df = 1, 23; P < 0.001$	$F = 565; df = 1, 23; P < 0.001$
ANCOVA experiment (I/II/III)		–	$F = 17; df = 2, 23; P < 0.001$	$F = 48; df = 2, 23; P < 0.001$
ANCOVA correlation with pupal weight		–	$F < 0.1; df = 1, 23; P = 0.85$	$F = 0.4; df = 1, 23; P = 0.54$

^aEAW larvae of different generations (experiments I, II, and III, respectively) were transferred from the semiartificial diet to the potato leaves at the start of the penultimate instar. Emerged adults were grouped by two females and three males, and their reproduction was recorded until death (*N* per experiment is given in parentheses). Two-way ANOVA was used to evaluate the effect of potato cultivar and experiment on the pupal weight, fecundity, and fertility. Correlation between pupal weight and female reproductive characteristics was analyzed with ANCOVA.

potato cultivar. The effect of pupal weight on the reproductive potential was not significant.

Mean female fecundity calculated for all experiments combined was 713 ± 13 eggs on the Superior and 309 ± 8 eggs on the NewLeaf Superior cultivars. With mean egg hatchability of 78 and 48%, respectively, the insects grown on Superior produced 556 and those on NewLeaf Superior only 148 viable progeny per female.

DISCUSSION

The results presented in this report demonstrate that the potato cultivar NewLeaf Superior, which contains the *Cry3Aa* transgene, is a less suitable host

plant for EAW than the nontransgenic parental cultivar Superior. Insects feeding on the transgenic cultivar ingest less food, reach a smaller pupal weight, and yield fewer progeny than those on the nontransgenic cultivar. Lower food consumption accounts for reduced body mass increment, but our statistical analysis showed that smaller body size is not the cause of a nearly 74% reduction of the reproductive potential. The decrease in female fecundity and fertility is highly correlated with the potato cultivar and probably due to an unknown effect of Cry3Aa sequestered in the females. The insects assimilate part of Cry3Aa present in the potato leaves at 1.5 ng/g and accumulate up to 0.2 ng/g Cry3Aa in newly ecdysed female pupae. The major impact of dietary Cry3Aa on EAW, i.e., the fertility reduction, is manifested about 10 d after the termination of feeding when the body content of Cry3Aa is nearly undetectable. We have no explanation for the sex difference in Cry3Aa sequestration.

The reduction of energy content and the low nitrogen assimilation during food passage through the digestive tract indicate that the larvae preferentially utilize carbohydrates, and that some of the nitrogenous compounds present in potatoes are not digestible. The relative differences in carbon and nitrogen content between penultimate instar larvae and pupae are probably caused by the accumulation of fat and other reserves and possibly also by the secretion of thick cuticle in the pupae. The relatively lower combustion heat measured in the pupae might be explained by the accumulation of oxygen-rich sugars, including chitin, in the pupae.

The impact of Cry3Aa on EAW resembles the suppression of fertility caused in CPB by Cry3B expressed in potatoes (Arpaia et al., 2000). The beetles readily consumed the genetically modified plants, but laid virtually no eggs. Their sterility was traced to a low vitellogenin level in the hemolymph, but no explanation could be provided for this effect. The low mortality of CPB consuming Cry3B indicated that lethal disruption of the midgut wall, which is typical for the action of Cry toxins (Whalon and Wingerd, 2003), did not occur. It seems more likely that the toxin somehow penetrated into the hemolymph and deranged functions of the body organs involved in vitellogenin production.

The deleterious effect of Cry3Aa on EAW is surprising because the toxin is regarded as specific for Coleoptera (Knowles, 1994). Deml et al. (1999) observed that the presence of more than 10 ppm of a Cry3A preparation in the diet of several caterpillar species caused mortality, affected food utilization, and reduced larval growth considerably. Validity of these data, however, is undermined by the fact that CPB resisted this Cry3A preparation, indicating that it might have actually been a Cry version active primarily on Lepidoptera. Sublethal effects of various Bt treatments on the nontarget species have been described in several reports (reviewed by Scriber, 2004), but our study is the first in which expression of a presumably inactive toxin in a transgenic food plant is correlated with its sequestration in the affected insect herbivore.

Lepidopteran larvae presumably do not possess specific midgut receptors for Cry3Aa, but this does not fully exclude toxin interaction with the EAW cells. Several α -helices that form the first of the three domains of the Cry proteins are conserved between the moth- and the beetle-specific toxins (Li et al., 1991; Grochulski et al., 1995). Helix 5 from Cry3Aa was shown to insert readily in diverse planar lipid bilayers, to bind to the surface of midgut cells in the fall armyworm *Spodoptera frugiperda* (J.E. Smith), and to kill a cell line derived from the latter species (Gazit et al., 1994). Hence, a plausible explanation for the inhibitory Cry3Aa action on EAW is that the toxin binds to and hampers the digestive functions of midgut cells, thereby reducing food intake and larval body growth. The small toxin amounts that are sequestered may also interfere with some of the cells (trophocytes, follicle cells, fat body cells) intimately involved in the process of egg production. The interference probably occurs during the EAW pupal stage when much of the egg formation takes place.

We cannot exclude the hypothesis that Bt potatoes curb the growth and reproduction of EAW by a mechanism that is indirectly related to Cry3Aa expression. For example, transgene insertion could have modified the expression pattern of various endogenous genes and thereby altered the production of defensive substances such as proteinase inhibitors, alkaloids, etc. A deliberate suppression of the production of proteinase inhibitors in potatoes by means of molecular engineering enhanced the weight gain of the beet armyworm *Spodoptera exigua* (Hübner) by 52–63% and the fecundity by 73% (Ortego et al., 2001). A reverse, i.e., an elevated level of the inhibitors, would probably curb the development of EAW. However, it is unlikely that investment into the production of Cry3Aa in the genetically modified potato would be associated with increased output of other defense compounds. We conclude that Cry3Aa itself causes deterioration of the EAW performance on the NewLeaf Superior cultivar (Hussein et al., 2005). It remains to be examined if an interaction of Cry3Aa with other defense mechanisms contributes to this effect. Simultaneous administration of sublethal doses of a Cry toxin with a neem tree extract exhibited synergism (Trisyono and Whalon, 1999), and this might also occur between Cry3Aa and the solanine alkaloids present in the potato plants.

Whatever the mechanism of reduced EAW performance on the NewLeaf Superior potato, it appears that Cry toxins may exert subtle but potentially cumulative side effects on nontarget insects. The widespread adoption of Bt sprays and the rapidly expanding cultivation of Bt crops require refined ecosystem monitoring before their possible environmental impacts can be fully reconciled. Any pest management program bears a risk of negative environmental impacts that must be weighed against the benefits and compared with the alternative programs (Scriber, 2004). The effects of Bt sprays and of Cry expression in genetically modified plants on target pest species are not identical. For example, CPB adults resist topically applied Bt (i.e., foliar formulations)

containing Cry3Aa (Zehnder and Gelernter, 1989), but are readily eliminated on the transgenic potatoes expressing Cry3Aa (Perlak et al., 1993; Whalon and Wierenga, 1994). Thus, the results of the present study can be extrapolated to Bt sprays only with utmost caution. In fact, they accentuate the need for comparative environmental impact studies of diverse pest treatments.

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INTERACTIONS BETWEEN *Euphorbia esula* TOXINS AND BOVINE RUMINAL MICROBES

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Abstract—Cattle generally avoid grazing leafy spurge (LS; *Euphorbia esula*), whereas sheep and goats will often eat it. Understanding metabolism of toxic phytochemicals in LS by bovine rumen microflora may help explain why cattle often develop aversions to LS after initially eating it. Toxicity of LS compounds after *in vitro* fermentation with normal vs. antibiotic-modified bovine rumen digesta was evaluated at different lengths of fermentation. Levels of toxic and aversion-inducing ingenols were determined for fermented and nonfermented mixtures of LS and bovine rumen digesta, and the toxicity of an aversion-inducing extract of LS to rumen microbial species that are common in cattle, sheep, and goats was evaluated. Fermentation of LS with bovine digesta increased the toxicity of extracted compounds. Introduction of neomycin (an antibiotic that preferentially inhibits gram-negative bacteria) into the LS and bovine rumen digesta mixtures did not appear to affect toxicities regardless of fermentation length. Levels of ingenol were observed in LS and bovine digesta mixtures (both fermented and nonfermented) that were consistent with levels of ingenols reported for LS. Finally, a toxic extract of LS had little or no negative effect on the growth of several common species of rumen bacteria. The results indicate that LS is not generally toxic to the ruminal bacteria, but that microbial activity in the rumen may be responsible for enhancing LS toxicity to cattle.

Key Words—Cattle, ingenol, leafy spurge, ruminal fermentation, toxicity.

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INTRODUCTION

Leafy spurge (LS; *Euphorbia esula* L.) is a problematic invasive plant species that was introduced in North America in the 19th century and now occurs in many areas of the western United States and Canada. Grazing of LS by sheep and goats is considered to be an important management tool, but many observations indicate that cattle eat little LS (Lym and Kirby, 1987; Hein and Miller, 1992). Cattle may initially graze the plant, but they appear to develop an aversion (Kronberg et al., 1993a). In contrast, sheep and goats can maintain good productivity with considerable daily intake of LS (Landgraf et al., 1984; Walker et al., 1994; Kirby et al., 1997).

One possible reason for differences in foraging behavior among ruminant species is differences in metabolism of phytochemicals by the host's ruminal microflora (Kronberg, 1999). Ruminal microbes have the capacity to detoxify some phytochemicals and increase toxicity of others (James et al., 1975; Carlson and Breeze, 1984; Pass et al., 1984; Craig et al., 1992; Duncan and Milne, 1992). Among ruminants, there are striking differences in tolerance of plant toxicants (Smith, 1992). If diet selection differences among ruminants are a result, in part, of differential rumen metabolism of phytochemicals (Kronberg, 1999), cattle may be reluctant to graze LS because their ruminal microbes do not metabolize toxic and aversive LS phytochemicals as do ruminal microbes in goats (Kronberg and Walker, 1993). Among potential toxicants in LS, ingenol and some of its esters occur in LS and are known to induce feed aversions in cattle (Halaweish et al., 2002). Moreover, antibacterial agents are known to modify ruminal fermentation (Russell and Strobel, 1988; Wachenheim et al., 1992) and could affect the ability of the ruminal microflora to metabolize compounds in LS to a less or more toxic form. This study had three objectives: (1) to determine if antibiotic addition to bovine rumen digesta would alter the ruminal microflora such that the toxicity of LS to ruminants was decreased or increased at different lengths of fermentation; (2) to quantify ingenol and its esters in fermented mixtures of LS and bovine ruminal digesta; and (3) to determine if an aversion-inducing extract of LS inhibited the growth of several common species of ruminal bacteria, thus indicating that LS could be inducing feeding aversions simply by inhibiting forage digestion.

METHODS AND MATERIALS

Leafy Spurge Collection and Preparation. Leafy spurge was collected in its flowering growth stage in July 2000 near Veblen, SD, USA (ca. 46°N, 97°W). Stems were cut within 4 cm of the ground, and this herbage was air dried and ground in a Wiley mill through a 1-mm screen. Air-dried alfalfa

(*Medicago sativa*) was also ground in a Wiley mill through a 1-mm screen. Air-dried LS was used rather than freeze-dried because the authors have found air-dried LS to be highly aversive to cattle in several feed aversion trials. Alfalfa was used as the control plant material because it is a forb like LS, but in contrast to LS, it is readily consumed by cattle.

Digesta Collection and Preparation of Fermentation Samples. For comparison of the effects of fermentation with normal or modified bovine digesta, ruminal digesta were collected from two ruminally cannulated yearling cattle fed with a diet of alfalfa and grass (mixed cool-season grass species) hay for at least 14 d prior to digesta collection. Whole digesta from the two individuals were combined to avoid the consequences of obtaining atypical digesta from one animal, and 75 ml of mixed digesta were added to 300 ml of buffer solution (Tilley and Terry, 1963) and 25 g of ground LS or alfalfa (control) in a 500-ml Erlenmeyer flask. The flasks were agitated, purged with CO₂, plugged with vented stoppers, and incubated at 39°C with periodic agitation. For the modified cattle digesta treatment, 0.5 mg of neomycin sulfate (an antibiotic that preferentially inhibits growth of gram-negative bacteria) were added to the buffer solution immediately before it was mixed with digesta and plant material to alter the population of microbes during fermentation. Because most conditioned food aversions develop from negative feedback received within 12 hr after ingestion of food (Kronberg et al., 1993b), metabolism of leafy spurge toxins was evaluated at 0, 6, and 12 hr. To stop the fermentation, samples were frozen quickly in thin layers (ca. 1 cm) in plastic bags.

Extraction of Fermentation Samples. Fermentation samples (400 ml each) were removed from the freezer 1–2 d prior to extraction. Samples were thawed at 4°C and extracted five times in a 2-l separatory funnel with 500 ml of petroleum ether (PE; manual shaking for 8 min each). PE extracts were filtered through Whatman 50 filter paper to remove any particles and were then concentrated by using a rotary evaporator at 45°C. Concentrated samples were pipetted into vials and refrigerated for use in the Brine Shrimp Lethality Tests.

Brine Shrimp Lethality Test. Potential toxicity of extracts to ruminants was estimated by using the Brine Shrimp Lethality Test, a general bioassay for toxic plant constituents (Meyer et al., 1982; Anderson et al., 1991). PE extracts of fermentations were completely dried under N₂ gas and were then redissolved in PE to a concentration of 1 mg ml⁻¹. Cytotoxic effects of the extract samples were evaluated at 50 µg of sample per milliliter of seawater. To obtain this concentration, 250 µl of sample were pipetted into each vial, and four vials (replicates) per sample were made. Samples were dried overnight in a hood. The next day, 20 µl of dimethyl sulfoxide (DMSO) were added to each vial to dissolve the samples. Two control vials were also used that contained only 20 µl of DMSO. Up to 50 µl of DMSO may be added per 5 ml of brine before DMSO toxicity affects the results (McLaughlin et al., 1991).

Seawater was prepared in an 18.9-l tank containing 152 g of sea salt (purchased locally) dissolved in 4 l of deionized water at room temperature (22–24°C). Shrimp eggs were added to one side of the tank (separated by a perforated dividing dam), and the tank was placed near a lamp to attract the shrimp. Shrimp eggs were allowed to hatch for approximately 48 hr after setup; hatchlings migrated toward the light and through the perforated dam, where they were collected.

Fermentation extract samples were vortexed, and seawater was added to a volume of 3 ml. Ten shrimp were added to each vial, and the final volume was brought to 5 ml. Vials were placed near a lamp for 48 hr, at which time the number of live shrimp in each vial were counted.

Two toxicity assays were conducted as described above. Assay 1 evaluated shrimp toxic response to LS or alfalfa fermented with bovine rumen digesta containing the antibiotic. Assay 2 evaluated the shrimp toxic response to LS fermented with normal bovine rumen digesta or the digesta containing the antibiotic.

Statistical Analysis. The general linear models procedure of SAS (1988) was used for statistical analysis. For each assay, the model statement included treatment, time of fermentation, and the interaction term as independent variables and the number of live shrimp in each vial as the dependent variable; vial was the experimental unit. Fischer's least significant difference procedure was used to separate treatment means. The threshold *P* value for significance was 0.05.

Isolation of Ingenol. LS was collected, dried, and ground as described above and was then extracted with methanol/water (9:1). The methanol extract was evaporated under reduced pressure to yield a concentrated aqueous sample, which was extracted with PE. The PE extract was removed, and the aqueous layer was freeze-dried and hydrolyzed with 0.5 M methanolic KOH for 2 hr at room temperature to hydrolyze esters, releasing ingenol and other alcohols. The hydrolyzed sample was neutralized to pH 7.0 with 1 M HCl. After neutralization, the sample was extracted with CH₂Cl₂ to yield an ingenol-rich fraction (Upadhyay et al., 1977), which was evaporated to dryness.

The 0- and 6-hr fermentation samples from bovine digesta not treated with antibiotic were analyzed. Because there were no significant differences between the 6- and 12-hr fermentation samples in the toxicity assays, we did not analyze the 12-hr sample. Each fermentation sample was hydrolyzed to yield an ingenol-rich fraction (0.8 and 0.9 g for 0- and 6-hr fermentation samples, respectively). The 0- and 6-hr fermentation samples from the antibiotic-treated rumen digesta were not hydrolyzed, so that we were able to detect potential differences with respect to types of ingenols in the fermentation samples.

Thin-Layer Chromatography/Solid Phase Extraction. For the nonfermented LS aqueous extract, normal-phase preparative thin-layer chromatography (TLC;

TABLE 1. OUTLINE OF SOLVENT SYSTEM FOR SOLID PHASE EXTRACTION

Fraction	% MeOH	% H ₂ O	% ACN
1	100	0	0
2	0	100	0
3 ^a	0	0	100
4	0	100	0
5 ^b	0	50	50
6	0	25	75

^a Leafy spurge sample (2.0 mg 1 ml⁻¹ ACN) was added at this time.

^b High-performance liquid chromatographic analysis confirmed the presence of ingenol in fraction 5. ACN = acetonitrile.

20 × 20 cm plate; 1000 pm) was used to detect ingenol by comparison with an ingenol standard. Sample was applied as a continuous band on preparative TLC plates; ingenol standard was applied as a reference. For this experiment, the ingenol-rich fraction from the CH₂Cl₂ extract (total extract for seven plates was 662 mg) was applied on seven preparative TLC plates. The plates were developed in a chamber containing hexane and isopropyl alcohol (2:1, v/v). They were analyzed and compared to the ingenol standard. The band corresponding to the ingenol standard was scraped off the plate, ground with mortar and pestle, extracted three times with CH₂Cl₂ and once with methanol, then filtered through Whatman 41 paper to remove silica material. This dried extract (2.0 mg) was dissolved in acetonitrile (1 ml) and processed through reversed-phase solid phase extraction (RP-SPE) to remove color and interfering material from samples prior to high-performance liquid chromatography (HPLC). A series of solvents was processed through SPE (Table 1). Each eluate from SPE was evaporated, redissolved in acetonitrile, and analyzed for ingenol by HPLC. Nonhydrolyzed samples were processed in the same manner via SPE. Hydrolyzed fermentation samples were not processed through SPE, but rather a guard column was used during the HPLC process to remove interfering materials.

High-Performance Liquid Chromatography. Each fraction eluted from SPE was analyzed (20 µl per injection) using a reversed-phase HPLC column (Econosil C18; 250 mm × 4.6 mm; 5 µm). The nonfermented aqueous extract of LS was used to optimize HPLC separation of ingenol, yielding the following chromatographic program with gradient elution: solvent A (acetonitrile) at 100% for 5 min, then decreased gradually to 60% in 45 min; solvent B (water) was increased from 0 to 40% in the 45-min gradient elution. Column effluent was monitored at 220–240 nm by using a photodiode array detector. Ingenol (Sigma, 1 mg in 1 ml of acetonitrile) was used as the standard. The nonhydrolyzed samples (0.5 mg each) were redissolved in 100 µl acetonitrile, and 15 µl were injected into the HPLC.

Standard Curve of Ingenol Concentration. A standard curve was generated using the solvent system described by Vogg et al. (1999) to determine if the concentration of ingenol esters was increased or decreased during exposure to rumen microbes. Four ingenol standard concentrations (0.0005–0.0375 mg ml⁻¹) were analyzed and used to construct a standard curve.

Determination of Ingenol Concentration in Samples. Ingenol in fermentation samples was determined by a known addition method. Five microliters of ingenol standard (1 mg ml⁻¹ acetonitrile) were dissolved in 95 µl of acetonitrile; Fifteen µl of this were injected into the HPLC to determine the peak area of ingenol. Next, 5 µl of ingenol standard (1 mg ml⁻¹) and 95 µl of each hydrolyzed sample (3 mg dissolved in 1 ml acetonitrile) were combined. Fifteen microliters of each combined sample (equivalent to 0.04275 mg of fermentation sample) were injected into the HPLC to determine how much of the peak area of ingenol was increased by ingenol in the hydrolyzed fermentation samples. Results from the sample enrichment technique produced average peak areas of ingenol standard alone and fermentation sample-enriched ingenol. The difference in peak areas was calculated to yield the peak area of ingenol in each fermentation sample. This value was then used in the equation generated by the standard curve to yield a value of milligram ingenol per injection (1 injection = 0.04275 mg of fermentation sample).

Effect of Leafy Spurge Extract on Growth of Pure Cultures of Ruminal Bacteria. The effect of an aversion-inducing LS extract on growth of six pure cultures of ruminal bacteria was tested in 96-well microtiter plates in an anaerobic glovebag (Weimer and Abrams, 2001). Six species were selected to represent bacteria capable of various ruminal catabolic activities, including the fermentations of cellulose (*Fibrobacter succinogenes*), hemicellulose (*Butyrivibrio fibrisolvens*), pectin (*Lachnospira multipara*), peptides (*Prevotella ruminicola*), starch (*Streptococcus bovis*), and soluble sugars (all five species plus *Selenomonas ruminantium*). The LS extract was dissolved to 0.1 mg ml⁻¹ in ethanol; the solution was transferred into the glovebag and then dispensed (30 ml) aseptically into wells of a microtiter plate. The covered plate was placed in an incubator (39°C) within the glovebag to accelerate evaporation of the solvent, which typically occurred within 2 hr. Plates were inoculated with diluted pure cultures of the ruminal bacterial species [300 µl per well of a mixture containing 5.4 ml of Modified Dehority Medium (Weimer and Abrams, 2001) supplemented with 0.5% (w/v) glucose or cellobiose + 0.1% (w/v) yeast extract, and 0.6 ml of an overnight culture of the ruminal species grown on the same medium]. Each strain was inoculated into eight wells that contained the dried LS extract and eight wells lacking LS extract, which served as controls. Additional controls were provided by wells that contained uninoculated culture media lacking LS extract to verify aseptic technique. The microtiter plate was incubated at 39°C in a Bio-Tek 808-IW plate reader contained within the glovebag.

The instrument shook the plates at maximum agitation for 30 sec prior to each reading of culture turbidity (A_{540}) at intervals of 15–30 min over a period of 16–18 hr, depending on the individual experiment. Methods for calculation of growth rates and for statistical comparisons have been described previously (Weimer and Abrams, 2001).

RESULTS AND DISCUSSION

Toxicity of Leafy Spurge after Fermentation with Normal or Modified Bovine Ruminal Digesta. In assay 1, LS was more toxic than alfalfa ($P < 0.001$; Figure 1), and its toxicity was increased by exposure to bovine ruminal microorganisms for 6 hr ($P < 0.001$) or 12 hr ($P = 0.008$). Observed toxicity was similar ($P = 0.20$) in 6- and 12-hr fermentations. These results indicate that microbial metabolism of chemicals in LS made them more toxic.

In the second assay, adding neomycin sulfate to the mixture did not affect the outcome ($P = 0.65$; Figure 2). LS toxicity increased following 6 hr of exposure to either untreated ($P < 0.001$) or antibiotic-treated bovine ruminal digesta ($P = 0.02$). The addition of neomycin sulfate to the mixtures was intended

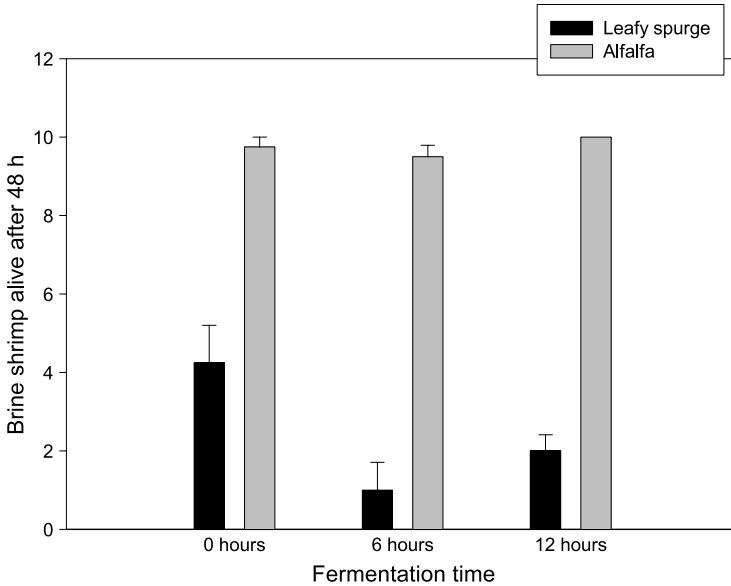


FIG. 1. Number of live brine shrimp in response to extracts of leafy spurge or alfalfa fermented with modified cattle rumen digesta for 0, 6, or 12 hr (assay 1). Bars represent the SE of the means.

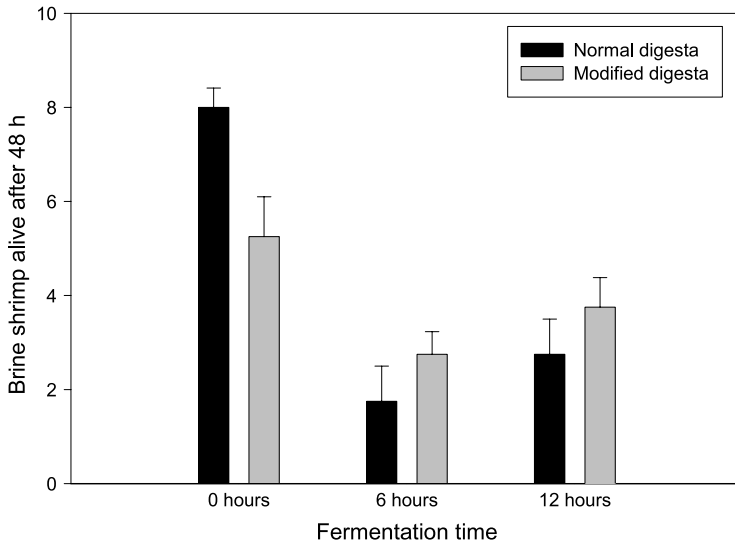


FIG. 2. Number of live brine shrimp in response to extracts of leafy spurge fermented with normal or modified cattle rumen digesta for 0, 6, or 12 hr (assay 2). Bars represent the SE of the means.

to suppress gram-negative bacteria. Assuming that gram-negative bacteria in the digesta were suppressed, this appeared to have no effect on rumen microbial interaction with toxins in leafy spurge. Neomycin blocks protein synthesis in sensitive bacteria, but over the short incubation times used, the bacteria present in the digesta may have already synthesized the proteins they used to metabolize or modify compounds in LS.

Compounds in LS appeared to become more toxic as time of exposure to ruminal microbes increased from 0 to 6 hr. Results from the second assay were consistent with the first in that microbial metabolism of LS compounds appeared to make them more toxic.

This increased toxicity may be caused by ruminal microbes converting compounds in LS into more toxic ones. The ability of ruminal microbes to detoxify or increase the toxicity of plant compounds is well known (James et al., 1975; Duncan and Milne, 1992; Smith, 1992; Wachenheim et al., 1992).

Ingenol Content in Leafy Spurge Following Fermentation with Bovine Rumen Digesta. Retention times from the ingenol standard and the non-fermented aqueous extract of LS were compared, and it was determined that Fraction 5 eluted from SPE contained parent ingenol. The standard curve developed with a HPLC Solvent System described by Vogg et al. (1999) generated a linear detector response with concentration that was used to quantify

ingenol in the hydrolyzed 0- and 6-hr fermentation samples. Results from the sample enrichment technique indicated that the 0-hr fermentation sample contained an average of 0.0024 mg of ingenol/mg sample injected (Figure 3); the 6-hr hydrolyzed fermentation sample contained 0.0011 mg of ingenol/mg sample injected (Figure 4). These values correlated to 0.0074 and 0.0038% ingenols in the original LS and cattle digesta mixture for the 0- and 6-hr fermentation samples, respectively, and they fall within the range of ingenol concentrations found in LS by others. Seip and Hecker (1982) found 0.0005% ingenols in LS, and Upadhyay et al. (1977) found 1.0% ingenols in leafy spurge. The lower concentration of ingenols in the 6-hr fermentation sample could be a result of several factors. Ruminal microbes may have converted ingenols into other toxic compounds, as evidenced by results from the toxicity assays. In each sample, there were peaks that eluted after ingenol, which indicates that these

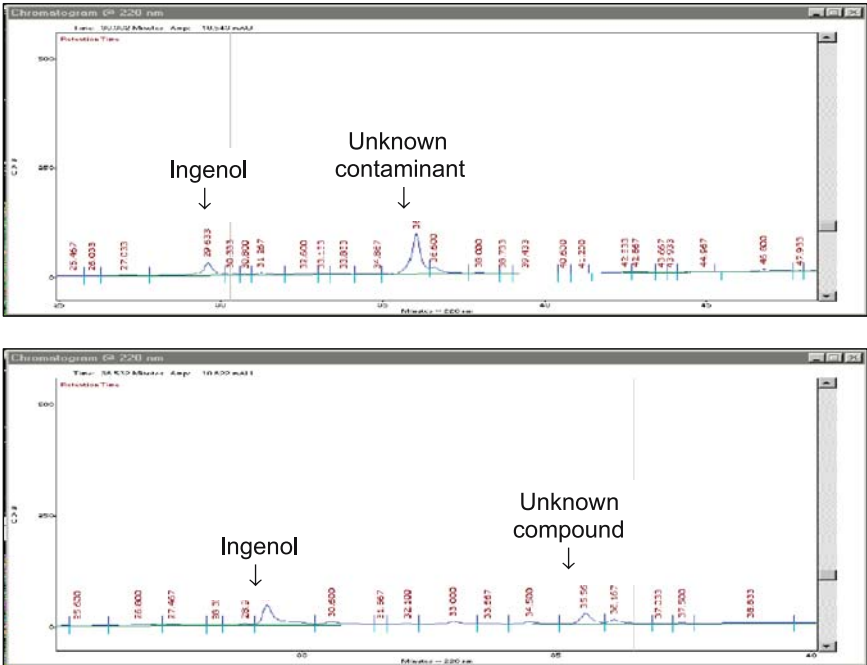


FIG. 3. High-performance liquid chromatogram (HPLC) of ingenol standard: solvent system II; 5 μ l dissolved in 95 μ l of acetonitrile; 15 μ l injected. Average peak area of ingenol = 696,783 AUP; retention time = 29.633 min (top). HPLC chromatogram of ingenol standard (5 μ l) + 0 hr hydrolyzed fermentation sample (95 μ l); 15 μ l injected; solvent system II. Average peak area of ingenol = 948,438 AUP; retention time = 29.300 min (bottom figure).

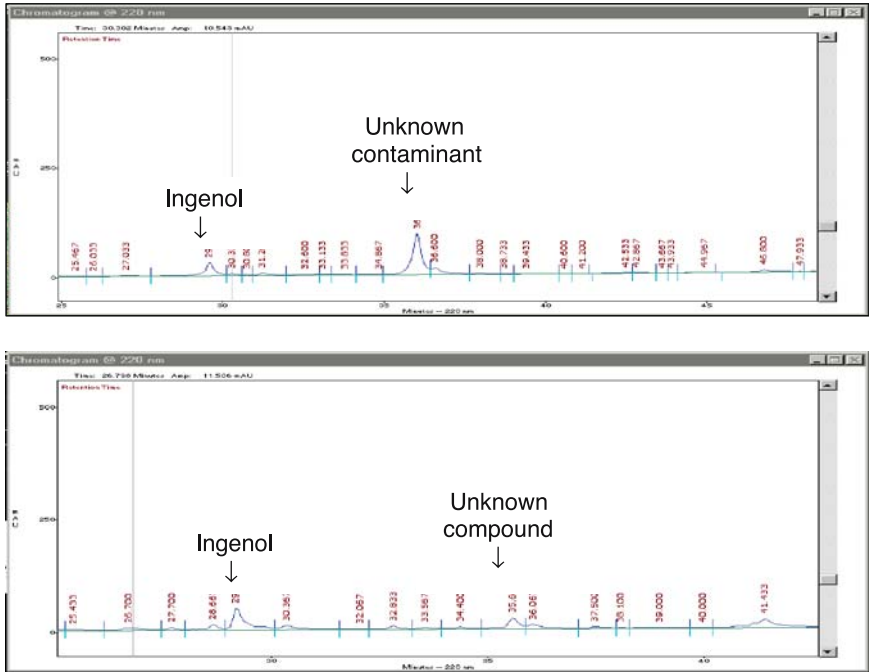


FIG. 4. HPLC chromatogram of ingenerol standard: solvent system II; 5 μ l dissolved in 95 μ l of acetonitrile; 15 μ l injected. Average peak area of ingenerol = 696,783 AUP; retention time = 29.633 min (top). HPLC chromatogram of ingenerol standard (5 μ l) + 6 hr hydrolyzed fermentation sample (95 μ l); 15 μ l injected; solvent system II. Average peak area of ingenerol = 893,311 AUP; retention time = 29.200 min (bottom figure).

unknown compounds are less polar than ingenerol. Rumen microbes may have transformed ingenerol into less polar derivatives. Less polar compounds cross cell membranes easier, and this may explain the higher toxicity of extracts of the fermented mixtures. Alternatively, there may be other toxic compounds in leafy spurge that occur at low levels and are less polar than ingenerols.

For the fermentation samples that were not hydrolyzed, ingenerol esters present in both the 0- and 6-hr samples showed similar retention times (Figure 5). In each of these samples (from SPE Fraction 5), there were two significant esters present. These esters can passively diffuse through hydrophobic mammalian membranes and enter cells where their toxicity would alter cellular activities (Campbell, 1999). Hasler et al. (1992) demonstrated that ingenerol esters activated protein kinase C and were biologically active in cell development, cell-to-cell communication, epidermal growth-factor binding, arachidonic acid metabolite release, and ornithine decarboxylase activity.

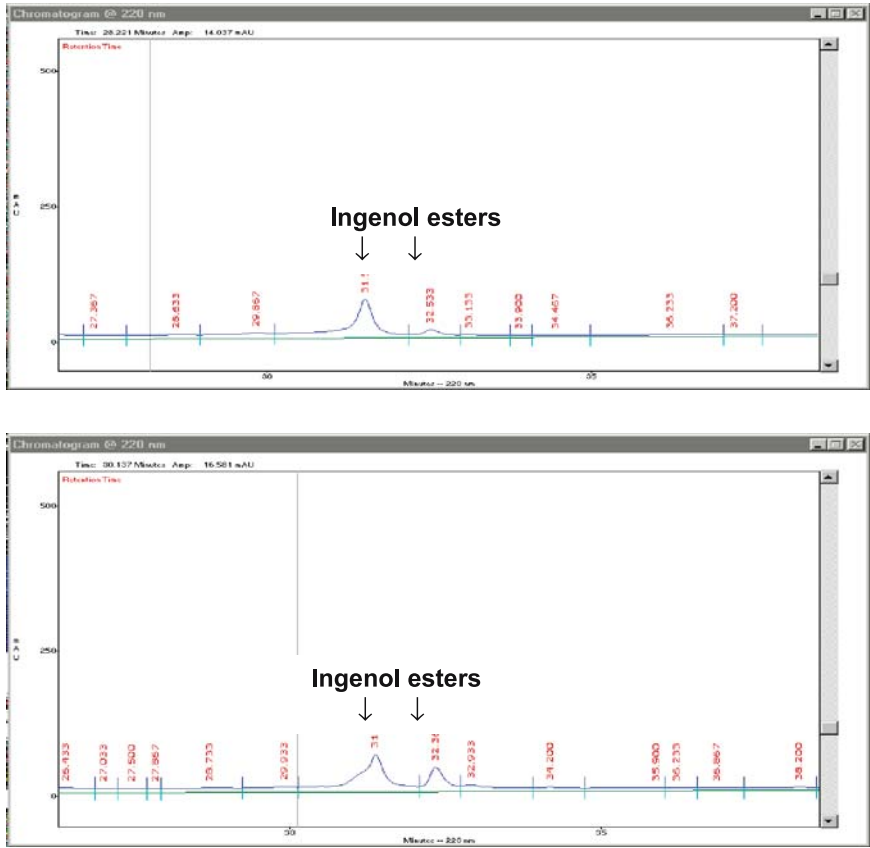


FIG. 5. HPLC chromatogram of 0-hr leafy spurge fermentation sample, nonhydrolyzed (from SPE fraction 5). Five milligrams dissolved in 100 μ l acetonitrile; 15 μ l injected; solvent system II. Ingenol ester 1 retention time = 31.533 min; ester 2 retention time = 32.533 min (top). HPLC chromatogram of 6-hr leafy spurge fermentation sample, nonhydrolyzed (from SPE fraction 5). Five milligrams dissolved in 100 μ l acetonitrile; 15 μ l injected; solvent system II. Ingenol ester 1 retention time = 31.400 min; ester 2 retention time = 32.367 min (bottom figure).

Effect of Leafy Spurge Extract on Growth of Pure Cultures of Ruminal Bacteria. The leafy spurge extract had a small inhibitory effect ($P < 0.05$) on the growth rate of only one of the six rumen bacterial strains tested (*Selenomonas ruminantium*; Table 2). The extract also altered ($P < 0.05$) maximum culture turbidity for this strain as well as for *Streptococcus bovis*, but not for the other four strains of rumen bacteria. It appears that LS does not contain compounds

TABLE 2. EFFECT OF PURIFIED LEAFY SPURGE EXTRACT (LSE; 0.01 mg/ml) ON THE GROWTH OF PURE CULTURES OF RUMINAL BACTERIA

Bacterial strain	Substrate	μ_{\max} (h ⁻¹)			Maximum OD ₆₀₀		
		-LSE	+LSE	<i>P</i>	+LSE	-LSE	<i>P</i>
<i>Butyrivibrio fibrisolvens</i> H17c	Glucose	0.259	0.284	NS	0.932	0.906	NS
<i>Fibrobacter succinogenes</i> S85	Cellobiose	0.230	0.231	NS	1.375	1.374	NS
<i>Lachnospira multipara</i> 40	Glucose	0.655	0.636	NS	1.871	1.923	NS
<i>Prevotella ruminicola</i> B ₁ 4	Glucose	0.587	0.579	NS	1.390	1.370	NS
<i>Selenomonas ruminantium</i> D	Glucose	0.609	0.588	<0.05	0.587	0.658	<0.05
<i>Streptococcus bovis</i> JB-1	Glucose	1.268	1.231	NS	1.113	1.042	<0.05

Results are from eight replicate cultures of each strain grown in microtiter plates.
ns = not significant at *P* = 0.05.

that inhibit common species of bacteria known to be responsible for a wide array of fermentative activities in the rumen. Therefore, it appears unlikely that aversion-inducing compounds in LS are seriously inhibiting normal forage digestion in the rumen of cattle that could, thus, cause them to learn to avoid consuming additional LS. These results are consistent with those of Thomas et al. (1994) who concluded that leafy spurge had no negative effects on rumen digestion in sheep when it made up 50% of their diet.

In summary, fermentation of LS with bovine ruminal digesta generally increased the toxicity of extracted compounds to brine shrimp, a general model system for studies of animal toxicity. This indicates that interactions between LS toxins and rumen microbes are may be increasing the toxicity of these compounds to cattle. Introduction of a gram-negative antibiotic into the LS and cattle rumen digesta mixture did not increase or decrease toxicities of this mixture regardless of fermentation length. Levels of toxic ingenol that we observed in LS before or after exposure to bovine ruminal digesta were consistent with levels of ingenols others have reported for LS. Finally, an aversion-inducing extract of LS had little negative effect on several common species of rumen bacteria comprising a broad array of ruminal metabolic activities. This indicates that the mechanism of action of aversion-inducing compounds in LS is not inhibition of forage digestion by rumen microbes but rather is direct toxicity to cattle.

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CONSTITUTIVE AND JASMONATE-INDUCIBLE TRAITS OF *Datura wrightii*

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Abstract—Plants in the family Solanaceae possess numerous traits that are induced from damage from herbivores. Many of these also can be induced by exposing plants to the plant hormone jasmonic acid or its volatile ester methyl jasmonate. *Datura wrightii* (Solanaceae) is dimorphic for leaf trichome morphology in most southern California populations. Trichome phenotype is governed by a single gene, and the glandular trichome condition is dominant and under developmental control. This study addressed two major objectives. The first was to determine if mature plants with glandular or nonglandular trichomes responded differentially to methyl jasmonate. The second objective was to determine if exposure of seedlings to methyl jasmonate during the period of trichome differentiation altered either the phenotype or the density of trichomes that mature plants expressed. Methyl jasmonate induced from 200 to 800 µg/ml of proteinase inhibitor activity and increased the activity of polyphenol oxidase by more than threefold depending on the experiment. These increases did not differ significantly between plants expressing glandular or nonglandular trichomes. Methyl jasmonate exposure did not increase the activity of peroxidase or the concentration of scopolamine or hyoscyamine, the two major alkaloids of *Datura*. Exposure to methyl jasmonate during trichome differentiation did not affect either the final trichome phenotype or the density of either type of trichome, but did increase the production of acylsugars in glandular trichomes by 44%. Because trichome phenotype was not inducible, and because both trichome phenotypes showed similar increases in proteinase inhibitors and polyphenol oxidase activity, the methyl-jasmonate-inducible responses of *D. wrightii* are independent of trichome phenotype in *D. wrightii*.

Key Words—Acylsugars, alkaloids, chemical defense, *Datura wrightii*, methyl jasmonate, inducible traits, polyphenol oxidase, proteinase inhibitors, trichomes.

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INTRODUCTION

Plants possess numerous traits that reduce damage by herbivores and subsequent losses in fitness caused by herbivory. These defensive traits may be expressed constitutively or induced following herbivory. Responses induced after damage by herbivores are becoming more widely documented and include changes in plant nutritional quality, the induction of defensive proteins and other allelochemicals, and the release of complex blends of volatile compounds (reviewed by Karban and Baldwin, 1997; Walling, 2000). Many induced responses are mediated by the jasmonic acid (JA) signaling pathway, and, in many cases, responses can be induced simply by exposing plants to JA or its methyl ester (MeJA) in the absence of damage by herbivores (Baldwin, 1996; Thaler et al., 1996). Induced responses have been extensively studied in solanaceous crops, such as tomato, potato, and tobacco, and often include the production of proteinase inhibitors (Pins), polyphenol oxidase (PPO), and sometimes alkaloids (Farmer and Ryan, 1990; Baldwin, 1996; Thaler et al., 1996; Constabel and Ryan, 1998).

In crucifers, one of the responses induced by herbivory can be an increase in leaf trichome density (Agrawal, 1999; Traw and Dawson, 2002), and trichome density in *Arabidopsis thaliana* can also be induced by exposure to MeJA (Traw and Bergelson, 2003). More recently, a JA-insensitive (*jai1*) mutant of tomato (Li et al., 2001, 2004), which is deficient in the accumulation of PPO and Pins compared to wild type when exposed to MeJA, was found that exhibits altered trichome development on fruit and leaves (Li et al., 2001). Whereas wild-type tomato produces two types of glandular trichomes on fruit prior to ripening, the developing fruits of *jai1* plants are devoid of glandular trichomes (Li et al., 2004). Moreover, the densities of four-lobed glandular trichomes on *jai1* leaves are only 25–35% that of wild-type plants. In another study, exposing young tomato plants to MeJA increased the densities of four-lobed glandular trichomes by up to ninefold (Boughton et al., 2005). Collectively, these data suggest that increases in trichome density can be mediated by exposure to MeJA in some plant species.

In southern California, the native perennial species *Datura wrightii* Regel (Solanaceae) expresses two distinct trichome phenotypes in most populations (van Dam et al., 1999; Hare and Elle, 2001). Some plants are densely covered with short, nonglandular trichomes, whereas others are less-densely covered with two types of uniseriate, multicellular glandular trichomes. These trichome types correspond to the type V nonglandular trichomes and types I and IV glandular trichomes of tomato described by Luckwill (1943). Both phenotypes also produce a small number of four-lobed glandular (type VI) trichomes as well (van Dam et al., 1999). Glandular trichomes secrete glucose esterified with several aliphatic acids (van Dam and Hare, 1998a), which makes

the plants feel sticky. Plants with nonglandular trichomes are velvety to the touch (van Dam et al., 1999). Trichome phenotype is controlled by a single Mendelian gene, and the sticky condition is dominant (van Dam et al., 1999). All seedlings produce glandular trichomes when young, but homozygous recessive plants produce leaves with a progressively greater ratio of nonglandular to glandular trichomes over a period of approximately 10 wk, after which homozygous recessive plants produce leaves that are essentially covered by nonglandular trichomes. Heterozygous and homozygous dominant plants continue to produce leaves with nearly 100% glandular trichomes throughout their lives (van Dam et al., 1999). At least 10 insect species attack *D. wrightii*, and they are differentially affected by glandular trichomes (van Dam and Hare, 1998a,b; Elle et al., 1999; Elle and Hare, 2000; Hare and Elle, 2002). A group of herbivores including flea beetles, hornworms, leafhoppers, and whiteflies preferentially attack velvety plants, whereas a mirid bug, *Tupiocoris notatus*, preferentially attacks sticky plants. The chrysomelid beetle *Lema daturaphila*, however, attacks both plants (Elle et al., 1999; Hare and Elle, 2002).

Although *Datura* spp. are notorious for their high constitutive levels of the alkaloids, scopolamine, and hyoscyamine (e.g., Parr et al., 1990), relatively little is known about the inducible responses of *Datura*. In the annual species *Datura stramonium*, proteinase inhibitor 1 (Pin1) activity was induced in detached leaves (Gurusiddaiah et al., 1972), but alkaloids were not induced in intact plants by damage by flea beetles (Shonle and Bergelson, 2000). Undamaged sticky and velvety phenotypes of *D. wrightii* do not differ in concentrations of water, total nitrogen, soluble sugars, total phenolics, or total alkaloids (van Dam and Hare, 1998a). There is no information available, however, about which plant traits may be inducible in *D. wrightii*, or if the two trichome phenotypes might be differentially inducible.

Here, the nature and magnitude of inducible traits in *D. wrightii* was investigated. First, we determined if short-term exposure to MeJA induced the accumulation of defensive proteins and alkaloids, and if defensive proteins or alkaloids were differentially induced in the two adult trichome phenotypes. This helps us understand the breadth of induced responses of *D. wrightii* relative to other solanaceous species, and whether or not induced responses are more important to one trichome phenotype or the other. Additionally, we determined if long-term exposure to MeJA during the period of trichome differentiation altered the ultimate adult trichome phenotype, the densities of either glandular or nonglandular trichomes, or the concentration of acylsugars that are produced by glandular trichomes. These results help us understand to what extent adult trichome type or density are themselves induced or modified by herbivore damage that occurs during the seedling or juvenile plant stages.

METHODS AND MATERIALS

Experimental Plants. “Velvety” plants possess the homozygous recessive genotype and express the nonglandular trichome phenotype, whereas “sticky” plants possess the heterozygous genotype and express the glandular trichome phenotype. Seedlings from 7 randomly selected lines originating from crosses between a randomly selected velvety pollen donor and a heterozygous sticky pollen acceptor from 4 local populations were used. These lines were backcrossed to the velvety pollen donor for two generations. Because the sticky phenotype is inherited as a dominant Mendelian character, the expected ratio of adult heterozygous sticky to homozygous recessive velvety offspring from these backcrosses is 1:1.

Batches of seeds were germinated in the fall of 2004 and winter of 2005 in a heated greenhouse equipped with four 1000-W high-pressure sodium lamps. Greenhouse temperatures ranged between 15 and 35°C, and the lamps were used to extend the photoperiod to 16 hr. The greenhouse also was equipped with a “pad and fan” cooling system that permitted a constant cross-flow of air entering the greenhouse on one side and exiting through an exhaust fan on the opposite side; the exhaust fan was constantly in operation during all experiments. Plants were placed onto the two center benches of the greenhouse with control plants placed 1 m upwind of treated plants to avoid inadvertent contamination with MeJA vapors.

Methyl Jasmonate Application. There are several ways to treat plants with MeJA or JA ranging from dipping leaves in dilute MeJA solutions (Spoel et al., 2003), spotting MeJA on leaf surfaces (Penninckx et al., 1998), enclosing plants in chambers and allowing MeJA to be taken up through the transpiration stream (Gu et al., 1996) to applying MeJA in lanolin to leaf surfaces (Kessler and Baldwin, 2001). We chose the lanolin–MeJA treatments that were previously used in field experiments with *Nicotiana* sp. (Kessler and Baldwin, 2001). This technique has proven its effectiveness, easily allows for renewed applications, and does not require detachment of leaves or the use of chambers for the long-term studies that we anticipated (see below). A 20-ml quantity of lanolin (Sigma, St. Louis, MO, USA) was heated to 60°C. The liquefied lanolin was poured into a wide-mouth jar, 200 μ l of MeJA (Bedoukian Research, Inc.) were added, and the mixture was rapidly stirred. The jar was sealed, and the mixture was allowed to solidify. Approximately 20 μ l of this mixture were applied to the stem or abaxial surface of the midrib of a leaf of treated plants by using a dissecting needle; a similar quantity of lanolin was applied to control plants. Only one application was made to plants during the short-term experiments, whereas applications were made three times per wk in the long-term treatments.

Experimental Designs. We carried out a preliminary experiment with 3 groups of 5 seedlings randomized over families that had produced four true

leaves. These seedlings were approximately 3-wk-old and of both genotypes, and all were still producing glandular trichomes at the time of treatment. The treated group of 5 plants was placed on the downwind side of the center aisle of the greenhouse and treated with MeJA in lanolin. One control group of 5 was placed 1 m away on the upwind side of the center aisle of the greenhouse and treated with lanolin only. A second control group of 5 plants was moved to a separate greenhouse with similar growing conditions and treated with lanolin only. Plants were maintained in these greenhouses for 10 d, and treatments were renewed every other day. All plants were assayed for Pin activity on day 11 to determine if the MeJA/lanolin treatments could induce Pins, and if control plants, when housed 1 m upwind of treated plants in the same greenhouse as treated plants, remained uninduced.

We carried out two short-term experiments using plants approximately 11-wk-old that had just completed their trichome differentiation. This was done to determine if the conditions that induced a direct gene product, such as Pins, might also increase the concentration of two other defensive proteins, PPO and peroxidase (POD), in one experiment or alkaloids in the other. Eight plants of each trichome phenotype, matched for size and randomized over families, were randomly assigned to “control” and “treated” treatments. Control plants were placed on the upwind side of the greenhouse, and treated plants on the downwind side, 1 m away. A 20- μ l quantity of either lanolin or MeJA/lanolin was applied to the midrib of one leaf per plant as above. After 72 hr, we assessed Pin, PPO, and POD activities or Pin activity and alkaloid content. Assays were performed on the treated leaf and the next youngest leaf for each of the 16 plants by using methods described below.

We completed a long-term experiment by using a total of 99 seedlings from the seven backcross families to determine whether long-term exposure to MeJA affected trichome phenotype or final density. The transition from glandular to nonglandular trichomes occurs over approximately 10 wk (van Dam et al., 1999), and it is uncertain when the developmental transition is initiated. Accordingly, we chose a long-term persistent exposure to MeJA to ensure that the experimental treatment encompassed the developmental transition, and we treated plants with MeJA/lanolin or lanolin alone throughout the period of trichome differentiation. There were no additional observable effects of these long-term MeJA treatments on plant growth or development other than those described below.

Seedlings from different families were randomly assigned to treatments. Fifty-one seedlings were assigned to the “control” treatment and placed on the upwind side of the greenhouse, and 48 were assigned to the “treated” treatment and placed on the downwind side 1 m away. Beginning on January 21, 2005, plants were treated with lanolin or MeJA/lanolin by applying a 20- μ l quantity to the main stem below the growing tip. Plants were approximately 3-wk-old at

this time, and all were still producing glandular trichomes. Treatments were renewed three times a wk for a total of 9 wk. Trichome phenotype was assessed every 2 wk by examining the newest leaf with a hand lens. Five randomly selected plants from treated and control groups also were assayed for Pin production at this time to verify the efficacy of MeJA treatments relative to control plants. The experiment was terminated on March 28, when glandular to nonglandular differentiation of control plants was complete (see Results). At this time, an additional 20 randomly selected plants, five velvety and five sticky, each from the treated and control groups, were assayed for Pin, PPO, and POD activities and total alkaloid content. All chemical assays were performed on tissue from the same leaf per plant.

Defensive Proteins. Pin activity was assayed semiquantitatively by using a radial diffusion assay (Ryan, 1967). Freshly harvested leaf tissue (100 mg) was ground in 100 μ l 10 mM phosphate buffer (pH 7.2) in microcentrifuge tubes with a pestle. Debris was removed by two consecutive centrifugations at 18,000 $\times g$ for 5 min each, and the soluble proteins were recovered. Agar plates with Pin1 antiserum (provided by Dr. Clarence Ryan, Washington State University) were prepared, and leaf soluble protein samples and Pin1 standards (0.5, 1.0, and 2.0 μ g, also provided by Dr. C. Ryan) were added to wells and allowed to absorb into the agar; plates were inverted and incubated at room temperature for 20 hr. Plates were flooded with 7.5% acetic acid for 10 min to visualize immunoprecipitin rings and subsequently rinsed in water. The diameters of the rings were measured and used to determine Pin1 quantities. Pin1 quantities are proportional to ring diameters and are reported in units of μ g/ml of leaf extract as per Green and Ryan (1972).

PPO and POD were analyzed using methods based on those of Stout et al. (1998) and Thaler et al. (1996). Chemicals were purchased from Sigma. Two or three 1.0-cm diam. leaf disks were punched from a leaf with a cork borer, excluding the midrib, and weighed to provide leaf tissue weighing between 100 and 150 mg (fresh wt.). The leaf tissue was placed into 1.0 ml 0.1 M potassium phosphate buffer (pH 7.0; 4°C) with 7% (w/v) insoluble polyvinylpyrrolidone. The tissue was homogenized in a motorized ground-glass tissue homogenizer. The slurry was transferred to a 1.5-ml microcentrifuge tube, 0.3 ml of 10% Triton X-100 was added, and the mixture was vortexed. The extracts were centrifuged for 5 min at 5000 $\times g$ at 10°C, and the supernatant was removed and used immediately for enzyme assays without further purification. PPO activity was assessed by determining the rate at which the extract would polymerize caffeic acid, and the rate of polymerization was monitored at 470 nm for 30 sec. POD activity was assessed similarly at 470 nm by monitoring the rate that guaiacol was oxidized by H₂O₂ in the presence of POD. The activities of both enzymes are reported as the change in absorbance per minute per gram (fresh wt.) of leaf tissue (Thaler et al., 1996).

Alkaloids. The quantities of scopolamine and hyoscyamine in samples of lyophilized leaf tissue were determined by high-performance liquid chromatography by using methods described by Fliniaux et al. (1993) as implemented by van Dam and Hare (1998a) with anisodine hydrobromide (Sigma) as an internal standard. Calibration curves were made by using commercially available scopolamine and hyoscyamine (Sigma). Only total alkaloids (mg/g dry wt) are reported to simplify data presentation. The ratio of scopolamine/hyoscyamine averaged 16:1 and never differed between MeJA treatments or trichome types (data not shown).

Trichomes and Acylsugars. The densities of all trichomes for all surviving plants in the long-term experiment were determined microscopically with techniques described previously (van Dam et al., 1999). Briefly, the density of glandular (types I and IV combined), nonglandular (type V), and four-lobed (type VI) trichomes were counted in a 3-mm² area along the abaxial midrib at 25× magnification. In previous studies, midrib densities predicted trichome densities on the leaf surface (van Dam et al., 1999), but trichomes are more easily counted on the midrib. To avoid losing any acylsugars during counting of glandular trichomes, a piece of aluminum foil was placed below the leaf on the microscope stage. After trichomes were counted, the leaf was wrapped in foil and placed into a refrigerator (4°C). For leaves with nonglandular trichomes, leaf area was determined with a leaf area meter (Li-Cor 3000, Li-Cor Corp., Lincoln, NE, USA), and the leaf was discarded. Leaves with glandular trichomes were extracted with CHCl₃ to remove the acylsugars (see below) before leaf area was determined.

The quantities of acylsugars produced by leaves with glandular trichomes were determined by extraction of both surfaces of the leaves, saponifying the acylsugars to glucose and aliphatic acids, then quantifying the liberated glucose spectrophotometrically (Hare, 2005). Aluminum foil that was used to wrap and store the leaf also was extracted to recover any acylsugars that might have been transferred to the foil during trichome counting and leaf storage. The millimoles of glucose liberated equals the millimoles of acylsugars extracted because each acylsugar molecule in *D. wrightii* is composed of a glucose molecule esterified with one or more C₆–C₉ acids (van Dam and Hare, 1998a). Acylsugars are reported as micromoles per square centimeter of leaf surface.

Statistical Analyses. For the short-term experiments, the data were analyzed by a two-factor analysis of variance (ANOVA) using PROC GLM of SAS/STAT[®], version 8.0 (SAS Institute, 2000), when sample sizes were equal among treatments and PROC MIXED of SAS when sample sizes were unequal. The effects of treatment, trichome phenotype, and treatment × phenotype interaction were tested over the variation among plants within

treatment \times phenotype combinations. Alkaloid concentration was transformed by using the square root transformation to ensure that the residuals were normally distributed, but Pin, PPO, and POD activities required no transformation.

In the long-term experiment, the frequencies of sticky and velvety plants in the treated and control groups were compared by using a replicated goodness-of-fit G test (Sokal and Rohlf, 1995) to compare the frequencies of the two groups and to compare the observed frequencies of each group to the expected frequency of a 1:1 ratio of sticky to velvety plants in these backcross progenies. The chemical concentrations of leaves and densities of four-lobed trichomes were compared by using a multiway mixed model ANOVA using PROC MIXED. Treatment and type were fixed effects, and the effect of family and all interactions that included the effect of family were random effects. Random effects that were not statistically significant were sequentially deleted, and the analysis was repeated to yield the final reduced model. The densities of glandular and nonglandular trichomes were analyzed separately by trichome phenotype. The MeJA treatment was a fixed effect, whereas the family and the family \times treatment interaction were random effects. Leaf area was also included as a potential covariate in these analyses. Non-significant effects were sequentially deleted from the full model to yield the final reduced model.

RESULTS

Preliminary Experiment. Treatment with MeJA/lanolin induced *D. wrightii* to produce a mean of 375 μg (± 85 SE, $N = 5$) of Pin activity per milliliter of leaf extract. None of the control plants treated only with lanolin, either in the same or separate greenhouse, produced Pins. On the basis of these results, we concluded that applying MeJA/lanolin to the leaves of plants was an effective way to induce plant defense responses, and that plants 1 m upwind of treated plants in the same greenhouse were not contaminated by exposure to MeJA. Therefore, we used the induction of Pins as a marker for the efficacy of the induction treatment and tested for MeJA-induced changes in the other traits in all subsequent experiments only in plants where MeJA also induced Pin activity.

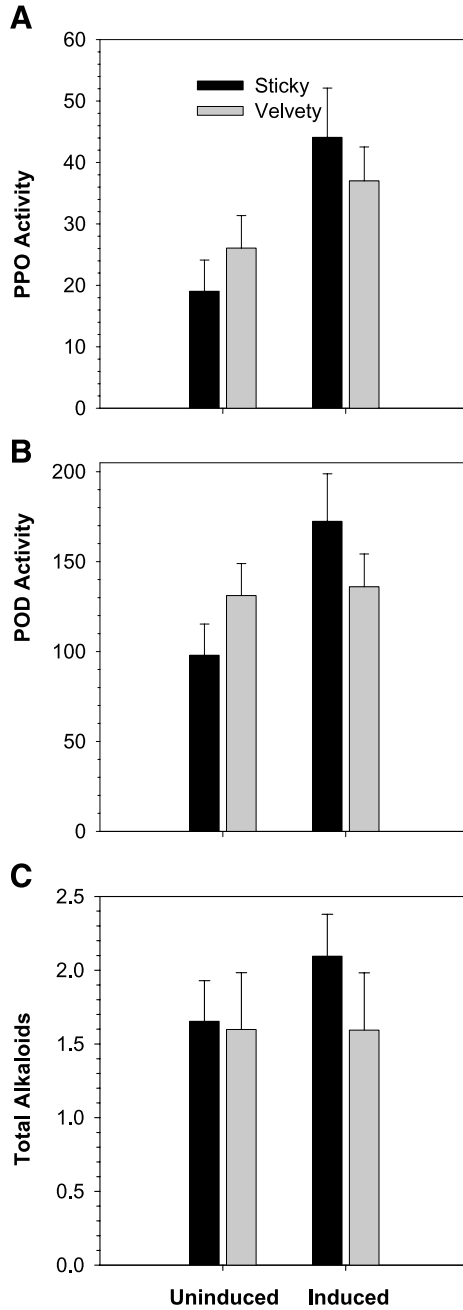
Defensive Proteins. There was some variability in the induction of Pins in the short-term experiments. In the experiment to investigate the induction of PPO and POD, three of the eight plants exposed to MeJA failed to produce Pins. Leaves from sticky plants in which Pins were induced, produced an average of 791.6 ± 240.4 μg of Pin activity per milliliter of leaf extract (least-squares mean \pm SE, $df = 5$), whereas induced velvety leaves pro-

duced an average of 560.8 ± 169.5 $\mu\text{g/ml}$ of Pin activity (least-squares mean \pm SE, $df = 5$). There was no significant difference in Pin activity between sticky and velvety leaves of induced plants ($F_{1,5} = 0.83$, $P = 0.40$). PPO activity increased overall by nearly twofold in leaves induced to produce Pins ($F_{1,10} = 8.73$, $P = 0.014$; Figure 1A) but did not differ between sticky and velvety leaves ($F_{1,10} = 0.00$, $P = 0.99$), nor because of the induction \times leaf-type interaction ($F_{1,10} = 1.35$, $P = 0.27$). Unlike PPO activity, POD activity was independent of the induction of Pin activity ($F_{1,10} = 3.81$, $P = 0.08$; Figure 1B). POD activity also did not vary between sticky and velvety leaves ($F_{1,10} = 0.01$, $P = 0.94$), nor because of the induction \times leaf-type interaction ($F_{1,10} = 2.95$, $P = 0.12$).

At the end of the long-term experiment, Pin activity was consistently expressed in all sampled MeJA-treated plants, but was never expressed in the controls. Pin activity averaged 250 ± 88 $\mu\text{g/ml}$ in induced sticky plants (least-squares mean \pm SE, $df = 5$) and 225 ± 83 $\mu\text{g/ml}$ in induced velvety plants (least-squares mean \pm SE, $df = 5$), and Pin activity did not differ between leaf phenotypes ($F_{1,8} = 0.04$, $P = 0.84$). Exposure to MeJA increased PPO activity more than threefold in the long-term study ($F_{1,16} = 145.18$, $P < 0.001$; Figure 2A), but MeJA did not increase POD activity ($F_{1,16} = 0.16$, $P = 0.70$; Figure 2B). In neither case did PPO or POD activities differ between leaf phenotypes ($P \geq 0.17$) nor because of the treatment \times type interaction ($P \geq 0.60$) in the long-term experiment.

Alkaloids. There was also some variability in the induction of Pins by MeJA in this short-term experiment as in the first. One treated plant of eight failed to express Pins, but a low level of Pin activity was detected in three of the eight control plants. Leaves from sticky plants in which Pins were induced produced an average of 243.28 ± 129.8 $\mu\text{g/ml}$ of Pin activity (least-squares mean \pm SE, $df = 10$), and leaves from induced velvety plants produced 372.8 ± 117.5 $\mu\text{g/ml}$ of activity (least-squares mean \pm SE, $df = 10$). Pin activity also did not differ between leaf phenotypes in this experiment ($F_{1,10} = 1.44$, $P = 0.26$). Alkaloid concentration did not vary significantly between Pin-induced and -uninduced plants ($F_{1,10} = 0.00$, $P = 0.95$), between leaf types ($F_{1,10} = 1.99$, $P = 0.19$), nor because of the treatment \times leaf-type interaction ($F_{1,10} = 0.87$, $P = 0.37$; Figure 1C). MeJA also did not increase leaf alkaloid concentration in the long-term experiment ($F_{1,15} = 2.78$, $P = 0.12$; Figure 2C). In addition, as seen in the short-term studies, alkaloid concentrations did not differ between leaf phenotypes of differentiated plants ($F_{1,15} = 0.18$, $P = 0.68$) nor because of the treatment \times leaf-type interaction ($F_{1,15} = 0.05$, $P = 0.82$) after long-term exposure to MeJA.

Trichomes and Acylsugars. MeJA-treated plants expressed Pin activities averaging between 100 and 400 $\mu\text{g/ml}$ of leaf extract during trichome differentiation, depending on the sampling period (Figure 3A). None of



the control plants showed Pin activity except for one plant in the March 14 assay. Despite the effectiveness of thrice-weekly MeJA applications to induce Pin activity, MeJA had no effect on timing of trichome differentiation or final leaf phenotype. Trichome differentiation occurred over a period of 9 wk in this experiment, as is typically the case for *D. wrightii* (van Dam et al., 1999). After trichome differentiation completed, 55% of the control plants and 46% of the treated plants expressed the velvety phenotype (Figure 3B). These frequencies did not differ from each other ($G_1 = 0.815$, $P = 0.36$), nor did either differ from the expected value of 50% ($G_1 \leq 0.486$, $P \geq 0.49$).

The density of glandular trichomes on sticky plants did not differ between control and MeJA-treated plants ($F_{1,52} = 1.25$, $P = 0.27$; Figure 4A). Similarly, the densities of nonglandular trichomes on velvety plants did not differ between control and treated plants ($F_{1,47} = 0.23$, $P = 0.63$). Type VI trichomes comprised only a few percent of total trichomes (Figure 4B), and the densities of these trichomes also were similar in control and treated plants ($F_{1,99} = 0.02$, $P = 0.89$). In no case did the densities of any of the three types of trichomes differ among families or due to any interactions involving families (all $P \geq 0.65$), and in no case did the densities of any trichome type covary significantly with leaf area (all $P \geq 0.14$).

Although MeJA did not alter the densities of glandular trichomes nor prevent the differentiation of nonglandular trichomes, exposure to MeJA caused the glandular trichomes of sticky plants to produce about 44% more acylsugars per square centimeter of leaf area than control plants ($F_{1,46} = 4.43$, $P = 0.041$; Figure 4C). Acylsugar production did not differ, however, among families or due to the treatment \times family interaction (all $P \geq 0.95$).

DISCUSSION

The putative herbivore defenses of solanaceous species range from completely inducible to completely constitutive. The relative importance of constitutive and inducible traits may vary among solanaceous species and populations within species. The two best-studied solanaceous genera are *Lycopersicon*, including the cultivated tomato, and *Nicotiana*, including cultivated and wild

FIG. 1. Mean (+SE) polyphenol oxidase (PPO) and peroxidase (POD) activity [$\Delta\text{OD}_{470} \text{ g (fresh wt.)}^{-1} \text{ min}^{-1}$, A and B], and mean (+SE) alkaloid concentration (mg/g, dry wt., C) from induced or uninduced leaves after 72-hr exposure to MeJA.

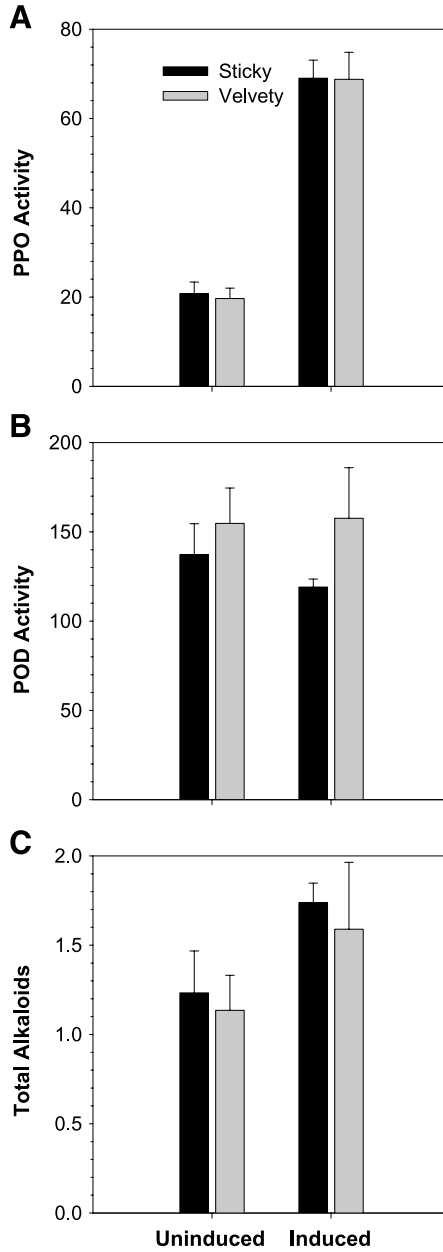


FIG. 2. Mean (+SE) PPO and POD activity [$\Delta\text{OD}_{470} \text{ g (fresh wt.)}^{-1} \text{ min}^{-1}$, A and B] and mean (+SE) alkaloid concentration (mg/g, dry wt., C) of sticky and velvety plans after 9 wk of exposure to MeJA.

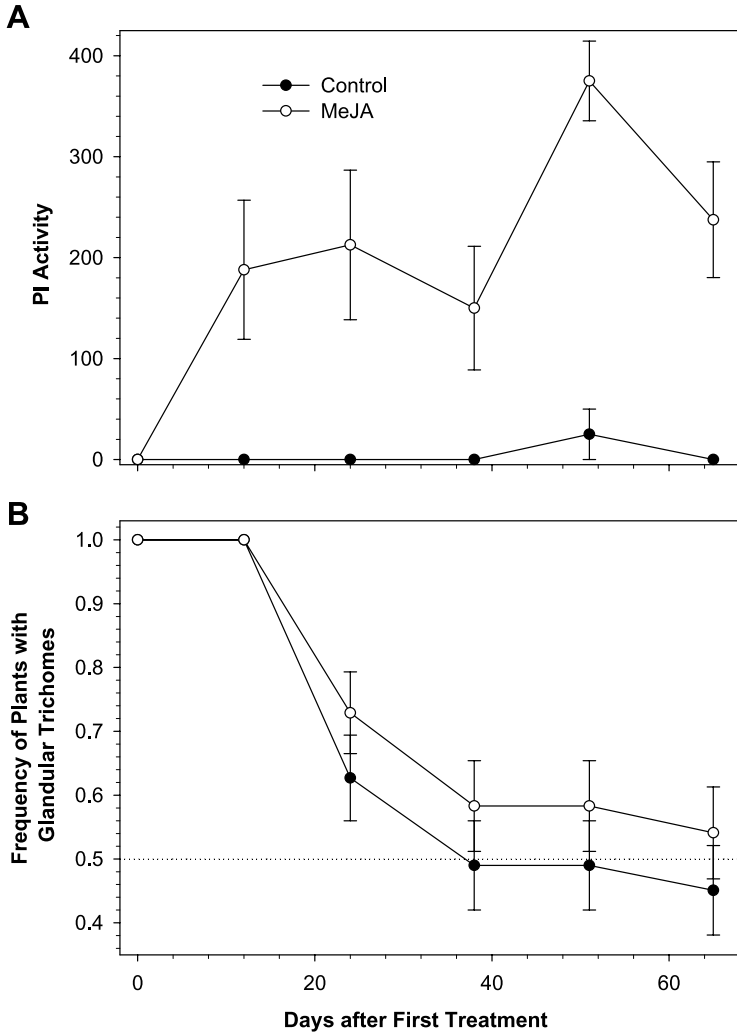
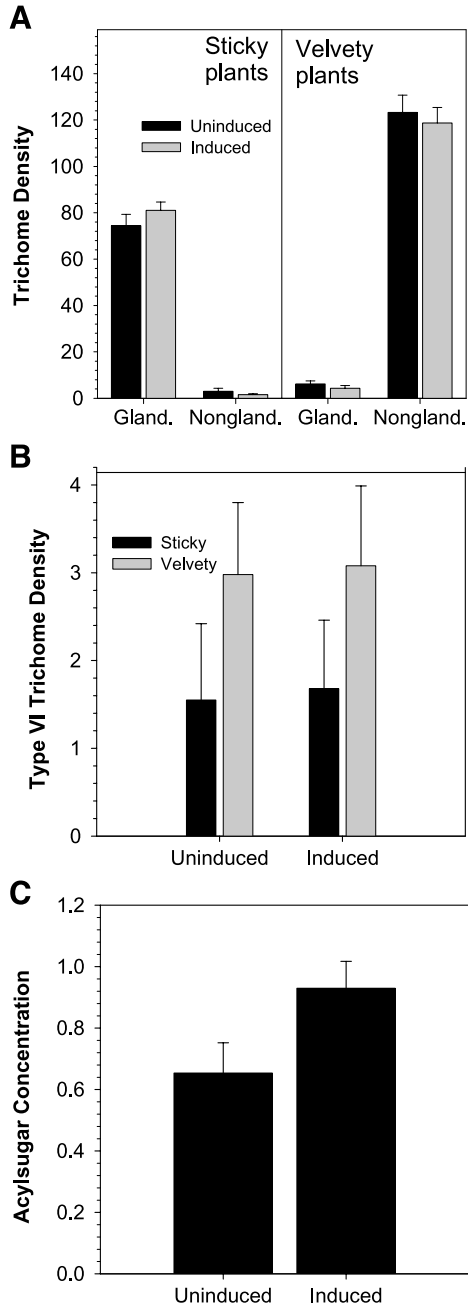


FIG. 3. Proteinase activity and trichome differentiation during 9 wk of exposure to MeJA. Mean (\pm SE) Pin activity ($\mu\text{g/ml}$ leaf extract) in treated and control plants (A) and frequency (\pm SE) of plants producing glandular trichomes (B). The dotted line in B shows the expected 1:1 frequency of sticky/velvety plants after trichome differentiation is completed.

tobacco species. In these genera, Pins are often expressed constitutively at low levels in leaves, but can be induced many fold by MeJA (Thaler et al., 1996; van Dam et al., 2001). PPO activity also is commonly expressed constitutively in many solanaceous species, but can be induced to higher levels by wounding



or exposure to MeJA. It is difficult to compare across studies because of the variation in magnitude of induced responses with variation in plant ontogeny, growing conditions, and experimental methodology (Constabel and Ryan, 1998; van Dam et al., 2001), but the observed Pin and PPO activities induced by MeJA in *D. wrightii* seem to compare favorably with those of other solanaceous species. The Pin activity of induced *D. wrightii* is similar to, or higher than, previous studies of tomato (202 $\mu\text{g/ml}$) and potato (336 $\mu\text{g/ml}$) in which the same radial diffusion assay was used (Green and Ryan, 1972). Similarly, although PPO activity of induced *D. wrightii* was lower than in tomato (Thaler et al., 1996), it was higher than in *Nicotiana tabacum*, *Solanum tuberosum*, *S. nigrum*, or *Capsicum annuum* (Constabel and Ryan, 1998). Thus, *D. wrightii* appears to share with other better-studied solanaceous species the ability to produce substantial quantities of Pins and PPO after exposure to MeJA.

Although the differences in POD levels approached statistical significance in the short-term experiments, neither the magnitude nor the pattern of difference was repeated in the long-term experiment. Others have noted inconsistency of induction of POD activity in response to MeJA in tomato and have suggested that POD induction may be mediated by signaling pathways other than those involving MeJA (e.g., Thaler et al., 1996). POD activity also was independent of MeJA exposure in *D. wrightii*.

The results of the short-term induction experiments were variable in that a single MeJA application failed to induce Pins in all plants, whereas multiple MeJA applications consistently induced Pins in the other two experiments. Multiple MeJA applications also induced a higher level of PPO activity than a single application. The variability in responsiveness to MeJA in the short-term experiments was observed in both genotypes and may be the result of differences in the number of MeJA applications or differences in age or developmental stage of plants at the initiation of each experiment (e.g., van Dam et al., 2001). A complete understanding of the causes of variable responses to MeJA observed in our short-term experiments would require a more extensive series of experiments.

In contrast to Pins and PPO, the concentrations of alkaloids in *D. wrightii* were not inducible. For this trait, *D. wrightii* differs from *Nicotiana* sp., in which alkaloid concentration is inducible (e.g., Baldwin, 1996). Our result for *D. wrightii* is similar to that of Shonle and Bergelson (2000), who found no

FIG. 4. Trichome densities of uninduced and MeJA-induced velvety and sticky plants, and acylsugar concentration of sticky plants. MeJA-induced plants were treated three times per week for 9 wk. (A) Mean (+SE) densities (no./mm²) of glandular (types I and IV) and nonglandular (type V) trichomes. (B) Mean (+SE) densities (no./mm²) of type VI lobed trichomes. (C) Mean (+SE) acylsugar concentration ($\mu\text{mol/cm}^2$).

increase in alkaloid concentration in response to feeding damage by flea beetles in either field- or greenhouse-grown *D. stramonium*. The little information available suggests that alkaloid profile and quantities may be a more constitutive characteristic of *Datura* spp. than *Nicotiana* spp.

Trichome phenotype and density also are more constitutive traits of *D. wrightii* genotypes than some tomato genotypes and cruciferous species, as long-term exposure to MeJA did not influence either trichome differentiation or final density. These results are in accord with previous qualitative observations. Neither the density nor the type of trichomes produced by *D. wrightii* on newer leaves distally on stems was visibly altered after insect damage of more proximal leaves on the same stem (Hare and Elle, 2002). In contrast to *D. wrightii*, tomato showed a transient increase in the density of type VI trichomes 7–21 d after exposure to MeJA (Boughton et al., 2005). Several annual crucifers also show a transient increase in trichome density on leaves initiated after older leaves on the same stem are damaged (e.g., Agrawal, 1999; Traw and Dawson, 2002). The different types of glandular trichomes in tomato and potato are apparently controlled by different genes (Lemke and Mutschler, 1984; Vallejo et al., 1994), and the developmental pathways leading to different types of trichomes also may vary in their sensitivity to MeJA. The absence of any trichome density response in *D. wrightii* was not because of inadequate MeJA treatments, as at least two MeJA-responsive gene products, notably Pin and PPO, accumulated substantially in the long-term MeJA studies.

This is, to our knowledge, the first example of acylsugar production being induced by MeJA. In previous studies with *D. wrightii*, the concentration of acylsugars on leaves of sticky plants not only varied seasonally, but also was reduced on leaves from irrigated compared to unirrigated plants (Hare, 2005). Acylsugar quantities also were independent of trichome density in *D. wrightii* (Forkner and Hare, 2000) and were only weakly correlated with trichome density in tomato (Blauth et al., 1998). Moreover, quantitative trait locus (QTL) analyses of acylsugar production and trichome densities in both potato and tomato indicate that although some QTLs are associated with both trichome density and acylsugar accumulation, other QTLs are associated with each independently (Bonierbale et al., 1994; Blauth et al., 1998). Although circumstantial, these independent lines of evidence suggest that type IV glandular trichomes do not simply secrete acylsugars passively, but that acylsugar production and secretion may be actively regulated within these trichomes.

It would not be surprising if different herbivores induced different traits and activated different groups of genes from those that are induced by MeJA alone (Stout et al., 1994; van de Ven et al., 2000; Traw and Dawson, 2002; Heidel and Baldwin, 2004). The present study provides a baseline to compare

and evaluate plant responses to damage by different herbivore species. Because of the known differences in susceptibility of Lepidoptera and Coleoptera to Pins and PPO (Murdock et al., 1987; Felton et al., 1992), the value of induced Pins and PPO may be higher when the herbivore community is dominated by lepidopterans than by coleopterans. In addition, because the structures of herbivore communities vary in space and time on *D. wrightii* (Elle and Hare, 2000; Hare and Elle, 2002), the realized benefit of these traits also is likely to vary spatially and temporally among and within *D. wrightii* populations.

Finally, because the expression of adult trichome phenotype by *D. wrightii* genotypes was not altered by exposure to MeJA, and because Pins and PPO were induced similarly in the two trichome genotypes, the induced responses that we examined probably have similar defensive roles within genotypes. Because of the similarities in responses across genotypes, induced responses probably contribute little to the variation in the frequencies of trichome genotypes among plant populations.

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THE EFFECTS OF NECTAR–NICOTINE ON COLONY FITNESS OF CAGED HONEYBEES

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Abstract—Nectar of many bee flowers contains secondary compounds, which are considered toxic for honeybees on repeated exposure. Although many anecdotal reports indicate the toxicity of secondary compounds to bees, only a few studies have tested the extent of toxicity at different honeybee ages, especially at the larval stages. Honeybees encounter nicotine at trace concentrations (between 0.1 and 5 ppm) in floral nectar of a few *Nicotiana* spp. and in *Tilia cordata*. Adult honeybee workers tolerate these nicotine concentrations. In controlled nonchoice feeding experiments with caged bees, we investigated the effect of nicotine on hatching success and larval and forager survival. Naturally occurring concentrations of nectar–nicotine did not affect hatching success of larvae or their survival, but the latter was negatively affected by higher concentrations of nicotine (50 ppm). Concentrations of nicotine in fresh honey samples from the hives were 90% lower than the concentrations in the offered experimental sucrose solutions. Our results indicate that honeybees can cope with naturally occurring concentrations of nicotine, without notable mortality, even when consumed in large quantities for more than 3 weeks.

Key Words—Nectar, secondary compounds, nicotine, *Nicotiana* spp. honeybees, toxicity.

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INTRODUCTION

Nectar of several plants contains secondary compounds (hereafter SC), which are suspected to be toxic to bees and other pollinators (Baker and Baker, 1975). The prevalence of SC in bee nectars has given rise to the hypothesis that bees are less susceptible to SC than are flower-inconstant butterflies (Baker and Baker, 1975). Conceivably, bees developed counteradaptations to SC (Rhoades and Bergdahl, 1981). Still, it is unclear whether bees consume SC deliberately or only for lack of alternative food sources. The actual concentrations of SC in nectar are known for only a few plants, yet, they are frequently reported to be toxic to honeybees (Adler, 2000). Detzel and Wink (1993) tested a wide array of nectar SC for their toxic (LD_{50}) effects on adult honeybees and found that most SC (particularly alkaloids) are highly toxic to bees across a wide range of concentrations.

Most reports on toxicity of SC to honeybees have not addressed the extent of toxicity in relation to the age of the bees. Toxic nectar may not affect forager bees but may still be toxic to brood or young nursing bees (Sharma et al., 1986). Conversely, anecdotal accounts indicate that SC may be fatal for foraging bees (Adler, 2000). Accordingly, it is important to test the age-related toxicity of SC on bees as they undergo clear-cut transitions in tasks and physiological plasticity with age (Smirle and Winston, 1988). The shift from the protected hive environment to the open field exposes honeybees to numerous challenges, such as pesticides and SC in nectar. The bees' ability to withstand exposure to such toxicants may be a critical factor determining colony foraging performance (Smirle and Winston, 1988). Plant SC and pesticides have been shown to induce detoxifying enzymes in honeybees (Terriere and Yu, 1974).

Nicotine is encountered by foraging honeybees in floral nectar of some *Nicotiana* species at concentrations between 0.1 and 5 ppm (Detzel and Wink, 1993; Tadmor-Melamed et al., 2004). Although birds and Lepidoptera are the typical pollinators of most *Nicotiana* species, honeybees also consume nectar of *N. tabacum* (Bhuiyan et al., 2002; Oddo et al., 2004) and possibly of *N. sylvestris* (Jakobsen et al., 1995) and *N. suaveolens* (Loughrin et al., 1991, 1993). Honeybees also actively collect nectar containing nicotine from *Tilia cordata* (Naef et al., 2004; the precise concentration is not known).

Detzel and Wink (1993) assessed the toxicity of nicotine in sucrose solutions for caged bees after an exposure of 48 hr. They found that nicotine was toxic to adult bees at LD_{50} concentration of 2000 ppm, a dosage that foragers probably never experience in nectar. Lower concentrations (<300 ppm) were tolerated for over a week without increased mortality (Detzel, 1990). Free-flying honeybees tolerated and even prefer nicotinated sucrose solutions at their naturally occurring concentration in nectar (Singaravelan et al., in press). Forager bees unload the collected (toxic) nectar to young workers in the hive,

who process and store it as honey, but they also feed it directly to young developing larvae. An effect can, therefore, be expected even in hive-bound adult bees and the developing brood. Developing larvae were more sensitive to such toxic constituents in the food than adult workers (Sharma et al., 1986; Miranda et al., 2003; Amir and Peveling, 2004), perhaps because of the low level of induced physiological specialization they have to manage. A remarkable increase was found in the activities of detoxification enzymes (glutathione *S*-transferases and the mixed-function oxidases) in forager bees. The detoxifying power proved positively correlated with workers' age (Smirle and Winston, 1988). Hence, because toxic nectar is also consumed by the developing brood (larvae), they should be more susceptible to it than adult worker bees.

Here, we investigated the effect of natural concentrations of nectar–nicotine on the hatching success of bee larvae and on survival of larvae and forager bees (*Apis mellifera ligustica*). Knowing the effect of nicotine at the colony level is essential for understanding the interactions between honeybees and nicotine-containing plants.

METHODS AND MATERIALS

Maintenance of Caged Hives. Minibeehives (400–2000 bees) of four-frame strength were obtained from an apicultural farm (Noga Reuven, Manoot, Israel). In the fall, experimental hives were randomly assigned to four nylon-meshed outdoor cages (3 × 3 m, allowing natural environments) erected at the Oranim Campus of the University of Haifa, Israel. Each beehive was introduced into a cage, and the bees were offered sucrose solution (20%) and pollen on Petri dish plates. In addition, pollen substitute (Bee-Pro^R) was provided *ad libitum* on plastic bowls placed inside the hives. To prevent ants from gaining access to test solutions, we applied tangle foot at the base of the table on which the solutions were provided. We routinely removed mites (*Varrora* spp.) and caterpillars of lesser wax moth (*Achroia grisella*) from the experimental colonies.

Experimental Procedure. The work schedule is shown in Table 1. To investigate the direct effect of nicotine, after 1 d of adjustment, the bees in each cage were fed exclusively with one of the following solutions: control (20% sucrose) or treated 20% sucrose laced with three concentrations of nicotine—0.5, 5, and 50 ppm. To trace the fate of individual eggs, after 10 d of bees' exposure to the test solutions, we mapped all frame cells on drawn sheets. We marked the cells of 15–30 new eggs randomly on two or three frames each day for 3 d using color dyes. We marked the cells of 75 (± 10 SD) new eggs in each colony. The developmental stage of each individual was classified as follows: “egg 1” to “egg 3” for 3 d in the egg stage; “L1d” through “L5d” for a 5-d larval period; and “CL” for capped larvae. We monitored the marked cells until

TABLE 1. WORK SCHEDULE WITH CAGED HIVES

Days	Tasks ^a
Day 1	Acclimatizing bees to the cage environment
Days 2–10	Exposure session
Days 11–14	Mapping, marking cells of new eggs
Days 15–17	Monitoring the cells for egg hatching
Days 18–26	Monitoring the cells for larval survival (capping)

^aNote that during the entire session, the hives were exposed to a single source of nectar.

the egg became a capped larva (usually about 9–10 d after the “egg 1” stage). Disappearances of eggs and larval death were noted regularly. To test the effect of nicotine concentrations on survival of forager bees, we numbered a minimum of 20–25 foragers in each colony in all replicates, and these were monitored for about 15 d. When our experiments were completed, we returned the hives to the apicultural farm. This setup was repeated four times (between August and November 2004), each time with new beehives that were randomly placed in the cages. In two cases, we terminated the experiment early, as the bees remained outside the hive. Therefore, the 5- and 50-ppm nicotine treatments had only three replicates.

Nicotine Concentration in Fresh Honey Samples. To determine the concentrations of nicotine in stored honey, we collected fresh honey samples from the combs with a blunt-tipped syringe. The samples were dried by a speed-vac (VR-Maxi, Heto, Allerød, Denmark) and kept at -20°C . Methanol (150 or 250 μl) was added to each of the dried samples, and after vortexing, the samples were centrifuged at 13,000 rpm for 5 min. Some 50 μl of the supernatant was derivatized, and the following solutions were sequentially added: 25 μl 4 M acetate buffer (pH 4.7); 10 μl 1.5 M potassium cyanide in water; 10 μl 0.4 M chloramine-T in water; 50 μl 50 mmol l^{-1} thiobarbituric acid in water/acetone (50:50, v/v). The contents were mixed and incubated for 5 min; the reaction was stopped by the addition of 10 μl 0.1 M sodium metabisulfite in water.

High-performance liquid chromatography (HPLC) analysis was performed exactly 3 min after the reaction had stopped. The HPLC configuration (HPLC, Beckmann system gold, Beckmann, Fullerton, CA, USA) for determination of nicotine consisted of an HPLC pump (Beckmann 125P) connected to a photodiode array detector (Beckmann 168; wavelength, 505 nm). The mobile phase—linear gradient was water/acetonitrile from 0 to 100% acetonitrile in 15 min (flow rate, 1 ml min^{-1}). The column used was Merck LiChroCART RP-18 (5 μm , 250 \times 4 mm; Merck, Darmstadt, Germany). The injection volume was 20 μl . The column was equilibrated for 3 min prior to each injection. Concentrations of nicotine were determined by calibration curves by using standards at concentrations between 0.3 and 50 $\text{ng } \mu\text{l}^{-1}$.

Data Analysis. The differences in the percentages of larval hatching and larval survival between the control and the different treatment groups and the differences in percent mortality among larval instars exposed to 50-ppm nicotine were analyzed with one-way analysis of variance on arcsine square-root-transformed data, followed by Tukey's multiple comparison mean separation test.

RESULTS

Foraging Activities and Survival of Forager Bees. Bees collected the test solutions until the end of the experiment except in the colonies exposed to 50-ppm nicotine; there, the bees reduced their usual flight activities as well as their food uptake after 15 d. We monitored the survival of foragers with minimal sample size (as it is already known that foragers tolerate nicotine up to 300 ppm without increased mortality; Detzel, 1990) for about 15 d and found that more than 80% of the bees survived (Table 2).

Hatching Success. Larvae hatched from about 70% of the eggs in all colonies. Exposures to nicotinated sucrose solutions did not affect the hatching success ($F_{3,10} = 2.858$, $P > 0.05$; Figure 1A). A slight nonsignificant reduction in hatching was found in the 50-ppm treatment (Figure 1A).

Larval Survival. Naturally occurring concentrations of nicotine (0.5 and 5 ppm) in nectar did not affect larval survival. About 70% of larvae survived. However, 50-ppm nicotine reduced larval survival 30% more than other concentrations ($F_{3,10} = 28.23$, $P < 0.001$; Figure 1B). Mortality rates differed significantly among larval instars ($F_{4,10} = 20.95$, $P < 0.001$) exposed to 50-ppm nicotine. High mortality occurred mainly in 3-d-old larvae (Tukey's multiple comparison, $P < 0.05$; Figure 2).

Concentrations of Nicotine in Fresh Honey Samples. On average, there was more than a 90% reduction in concentrations of nicotine in honey samples compared with the concentrations of the test solutions offered. The average concentration of nicotine traced in honey samples of colonies treated with 50-ppm nicotine was 3.23 ± 0.41 ppm (in wet mass; mean \pm SE; $N = 15$), whereas

TABLE 2. PERCENT SURVIVAL OF NUMBERED FORAGERS MONITORED IN DIFFERENT COLONIES (POOLED DATA)

Colonies	Foragers monitored	Foragers survived	Percentage
Control	79	68	86.1
0.5 ppm	84	72	85.7
5 ppm	63	51	80.9
50 ppm	58	49	84.5

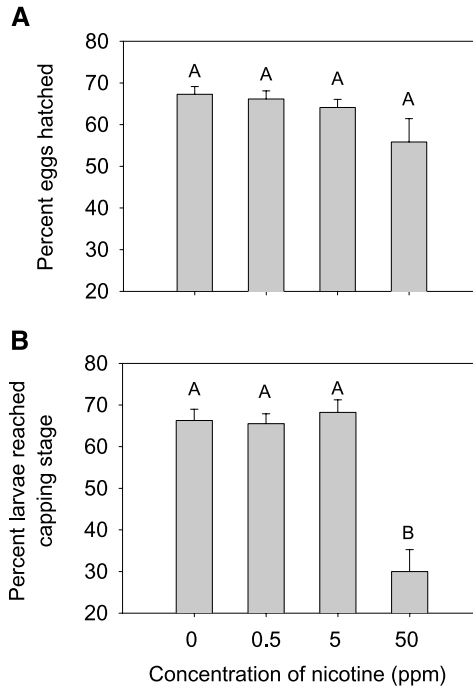


FIG. 1. Effect of nicotine consumption on honeybees. (A) Hatching success; (B) larval survival ($P < 0.05$, Tukey's multiple comparison). $N = 4$ hives with 0- and 0.5-ppm nicotine and $N = 3$ hives with 5- and 50-ppm nicotine. Values given as mean \pm SE. Bars followed by different letters are statistically significant.

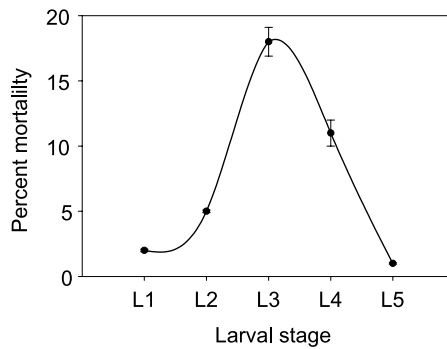


FIG. 2. Percent mortality (mean \pm SE) of different larval instars in the colonies exposed to 50 ppm of nicotine.

concentrations in honey samples of colonies treated with 0.5- and 5-ppm nicotine were hardly detectable.

DISCUSSION

The results indicate that bees' consumption of nicotine in its naturally occurring concentrations in floral nectar does not adversely affect hatching success and larval survival, even in the absence of alternative nectar sources for more than 3 wk. However, the effect of nicotine was dose-dependent, and a higher concentration (50 ppm) significantly reduced larval survival (Figure 1). Adult forager honeybees proved unaffected at any of the tested nicotine concentrations. Previously, we found that free-flying bees tolerated and were even stimulated by naturally occurring concentrations of nicotine in artificial nectar (Singaravelan et al., 2005). Adult forager honeybees may escape the acute toxic effects of nicotine because they unload the collected nectar almost completely to receiver bees immediately upon returning to the hive (von Frisch, 1965). By contrast, nectarivorous sunbirds that consume naturally occurring concentrations of nicotine were adversely affected (Tadmor-Melamed et al., 2004). Unlike honeybees, birds immediately digest the consumed nectar (Tadmor-Melamed et al., 2004) and, hence, are more liable to suffer negative effects.

Baker and Baker (1975) suggested that the level of tolerance to SC in nectar by pollinators is related to their pollination efficiency. In our experiments, we used the European race of bees (*A. mellifera ligustica*), which may encounter nicotine naturally in nectar of *Nicotiana tabacum* (Detzel and Wink, 1993) and *T. cordata* (Naef et al., 2004). Although these plants are native to tropical America and Europe, respectively, they have been introduced and are cultivated in many areas, including Israel. General pollinators usually serve invasive plants in similar strategies that are used in their natural habitats (Richardson et al., 2000). Accordingly, interpreting the reciprocal adaptations of different flower visitors to tolerate nectar–nicotine in respective host plants is more complex and entails several lines of considerations. For just a few, this can be illustrated by the following questions: Is a particular pollinating species more tolerant or better adapted to nectar–nicotine concentrations in areas where it has coevolved with the host plant than in areas where it has not? If the host plant has an array of pollinating species in a given area, how does the ability of the different species to tolerate the nectar–nicotine level vary? Most nectar SC have proved toxic for honeybees across a wide range of concentrations (Detzel and Wink, 1993). Nicotine is toxic for adult workers at LD₅₀ concentration of 2000 ppm (Detzel and Wink, 1993). Detzel and Wink (1993) chose a high concentration range probably because bees also encounter the SC in pollen, in which their concentrations are many times higher than in nectar (Detzel

and Wink, 1993; Kretschmar and Baumann, 1999; London-Shafir et al., 2003). Because high concentrations of nicotine and other SC cause severe toxicity in forager bees, it has been suggested that they are not especially adapted to cope with them (Detzel and Wink, 1993). However, if the bees encounter SC only in nectar, it is reasonable to consider the toxic nature of SC only within the concentration range actually found in nectar. We focused on nicotine concentrations actually found in floral nectar of *Nicotiana* spp. because it is unknown whether the bees collect and consume the pollen of these plants. If they collect both nectar and pollen of a particular plant that contains SC, one has to consider the concentration ranges of SC of both. Pollen is vital for plant reproduction, so the higher concentrations of SC in pollen may reflect a differential allocation providing high protection from being consumed.

What factors may enable larvae to tolerate naturally occurring concentrations of nicotine? In nature, bees collect nectar from various plants, which may be mixed in the hive to reduce the concentrations of SC. In our experiment, they had no alternative nectar source, but only the experimental test solutions. We found a drastic reduction (>90%) in the concentration of nicotine in honey as compared with the consumed experimental solutions. This is consistent with previous observations on reduction in concentrations of SC, such as amygdalin (50% reduction) and caffeine (90% reduction) in honey (including fresh honey samples) compared with the nectar (Kretschmar and Baumann, 1999; London-Shafir et al., 2003). The actual mechanisms whereby bees reduce SC concentrations in honey are still unknown. Larvae are fed with honey containing reduced SC concentrations. Moreover, induction of detoxifying enzymes known in larval bees (Nielsen et al., 2000) should help to cope with SC toxicity.

In summary, our study demonstrates that naturally occurring concentrations of nicotine in nectar do not affect colony fitness of honeybees, even when such nectar has been consumed for nearly 3 wk and no alternative nectar was offered. However, higher concentrations of nicotine cause high larval mortality. Further studies are essential for clearer insights into the effect of SC in nectar and pollen on both adult honeybees and developing brood regarding their differential susceptibility and/or physiological resistance. In the future, it will be important to study what concentration spectra of different SC the bees encounter in both nectar and pollen and what concentration spectra of these SC they can handle at all developmental stages.

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POLLINATION BY SEXUAL MIMICRY IN *Mormolyca ringens*:
A FLORAL CHEMISTRY THAT REMARKABLY MATCHES
THE PHEROMONES OF VIRGIN QUEENS OF *Scaptotrigona* sp.

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Abstract—The chemical composition of some volatile (2-heptanol) and nonvolatile constituents (a homologous 9-alkene/alkane series) of *Mormolyca ringens* flowers and *Scaptotrigona* sp. queen waxes (homologous 9-alkene/alkane series) and cephalic extracts (homologous series of 2-alkanols, including 2-heptanol) involved with the pseudocopulation or sexual mimicry in Orchidaceae pollination is compared. The similarity in chemical composition of flowers and insects is assigned to the chemically induced copulatory activity in *Scaptotrigona* males.

Key Words—*Mormolyca ringens*, chemical constituents, Orchidaceae, queens, *Scaptotrigona postica*, sexual mimicry, waxes.

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INTRODUCTION

Pseudocopulation or sexual mimicry is a remarkable and puzzling pollination strategy within the Orchidaceae. Flowers pollinated through pseudocopulation offer no floral reward to their pollinators and display sets of characters that prompt visitation and pollination by sexually excited male insects (mostly Hymenoptera; Kerr and Lopes, 1962; Van der Pijl and Dodson, 1966; Dressler, 1993; Ayasse et al., 2000). These flowers emit fragrances that mimic the sexual pheromones of virgin insect females (Borg-Karlson, 1990; Borg-Karlson and Tengö, 1986). Visual clues, such as coloration and floral indument of the labellum (median petal), reinforce the insect-like appearance of these flowers (Kullenberg, 1961; Van der Pijl and Dodson, 1966). In this pollination strategy, sexually excited male insects arrive at flowers and attempt copulation (normally with the median petal). As in most orchids, the pollen is packed into discrete units called pollinia. Additional flower secretions or structures hold the pollinia together. The pollinia and these secretions or structures form a complex pollen-dispersal unit or pollinarium (plural: pollinaria) (Singer and Koehler, 2004). During attempts by male insects to copulate with the flower, the pollinaria stick to the insects' body surface (Kullenberg, 1961; Van der Pijl and Dodson, 1966; Dressler, 1993). Insects laden with pollinaria then promote pollination during successive visits to other flowers by depositing the pollinia (or parts of them) on the surface of the floral stigma (Kullenberg, 1961; Van der Pijl and Dodson, 1966; Dressler, 1993).

Pollination through pseudocopulation has been particularly well documented in terrestrial orchids of the subfamily Orchidoideae from Europe, Africa, and Oceania (Kullenberg, 1961; Van der Pijl, 1995, 2001; Borg-Karlson, 1990; Borg-Karlson and Tengö, 1986; Schiestl et al., 2000; Ayasse et al., 2000, 2003). The pollination strategies of these taxa are closely associated with the biology of seasonal insects (Van der Cingel, 1995, 2001). The insects involved in pollination by pseudocopulation in orchids in the Old World are solitary bees and wasps. As a rule, these plants flower only when male insects are around, and, consequently, the males mistake the flowers for their respective female partners and promote pollination (Kullenberg, 1961; Van der Pijl and Dodson, 1966). When females finally emerge, males may be able to recognize "true females" and avoid the flowers (Van der Pijl and Dodson, 1966; but, see Ayasse et al., 2003). Consequently, pollination in these orchids is usually low (Neiland and Wilcock, 1998) and limited to a brief period of time prior to female emergence (Kullenberg, 1961; Van der Pijl and Dodson, 1966). Although a number of Neotropical orchid genera have been suggested as being pollinated by pseudocopulation (Van der Pijl and Dodson, 1966), these reports are preliminary and lack the details of literature on Afro-European or Australian taxa (Kullenberg, 1961; Borg-Karlson, 1990; Borg-Karlson and Tengö, 1986;



FIG. 1. (A) Flower of *Mormolyca ringens* (Orchidaceae: Maxillariinae) in frontal view. (B) *Scaptotrigona* drone attempting copulation with a flower of *M. ringens*.

Borg-Karlson, 1990; Van der Cingel, 1995; Ayasse et al., 2000, 2003; Schiestl et al., 2000; Van der Cingel, 2001). Recently, pollination through pseudocopulation has been reported for *Trigonidium obtusum* Lindl. and *Mormolyca ringens* (Lindl.) Schltr. (Figure 1A and B; both belonging to the subtribe Maxillariinae, sensu Whitten et al., 2000; Kerr and Lopes, 1962; Singer and Koehler, 2004; Singer, 2002; Singer et al., 2004). Interestingly, sexually excited drones of Meliponini pollinate both orchid species. In contrast to cases documented in the Old World, these bees are eusocial (i.e., there are castes, and there is division of labor in the hives). The hives are perennial and produce fertile individuals several times a year. In agreement, plants of *T. obtusum* and *M. ringens* produce flowers throughout most of the year (Singer, 2002; Singer et al., 2004). Gas chromatography–mass spectrometry (GC-MS) analysis of *T. obtusum* floral fragrance reveals that pentadecane is the main constituent (over 95%; Flach et al., 2004). In contrast, the fragrance of *M. ringens* contains 31 main components (Singer et al., 2004) and is visited and pollinated by *Scaptotrigona* and *Nannotrigona testaceicornis* (Apidae: Meliponini).

As part of our multidisciplinary studies on Maxillariinae orchids pollinated by pseudocopulation, we investigated the floral chemistry of *Mormolyca* and compared it with that of virgin queen bees, assuming that similar chemical components should be present in both organisms. Specifically, we wished to determine and compare the floral chemistry of *M. ringens* and the sex pheromone of virgin queens of *Scaptotrigona* sp. (Meliponini). *Scaptotrigona* is one of the two Meliponini genera recently reported to pollinate this orchid

(Singer et al., 2004; Figure 1A and B). There are around eight species of *Scaptotrigona* in Brazil (Silveira et al., 2002), but they vary in morphology and behavior and represent a taxonomically difficult group. Readers interested in the biology, taxonomic details, and phylogenetic affinities of *Scaptotrigona* bees are referred to Silveira et al. (2002).

METHODS AND MATERIALS

Plant Material. Fresh flowers were obtained from plants grown in the orchidarium of the Escola Superior de Agronomia Luiz de Queiroz, Universidade de São Paulo, Piracicaba, São Paulo State, Brazil. Voucher specimens (ESA 7247, ESA 16802, and ESA 1648) were deposited in the ESA and UEC herbaria. Insect voucher specimens were deposited in the Laboratório de Abelhas (Instituto de Biociências, Universidade de São Paulo, São Paulo State, Brazil).

Flower Extract. Pollinated ($N = 5$) and unpollinated ($N = 4$) flowers were extracted by immersion in bidistilled hexane (500 μ l) for 24 hr. The hexane was evaporated to 50 μ l under a nitrogen stream, and 1 μ l was analyzed by GC-MS.

Rearing Meliponini Queens. Young larvae were transferred to artificial cells (6.6-mm diam, 8.8 mm high), where the amount of food was controlled. To obtain complete success (100% of female larvae developing into queens), the amount of food necessary to develop a worker (35.4 mg) was tripled (106 mg).

Artificial cells and their caps were made from molded *Apis mellifera* L. wax. Food for larvae was harvested from 100 natural cells from a *Scaptotrigona* colony. Artificial cells were incubated at 28°C, with ca. 75% RH that was sustained by using a saturated KCl solution. Pupae were transferred to new cells to avoid fungal infection. Ten queens were reared and manually fed every 2 hr with a syrup of honey, pollen, and water. Only queens were reared. In addition, bees were kept in captivity until they were sacrificed and excludes the possibility that these bees were fertilized.

Extraction of Cuticular Waxes from Virgin Queens. The waxes of 10 virgin queens of *Scaptotrigona* sp. were extracted by washing their abdomens with bidistilled hexane (500 μ l). The solvent was reduced to 50 μ l under a nitrogen stream, and 1 μ l was analyzed by GC-MS.

Virgin Queens Cephalic Extract. The heads of 10 virgin queens of *Scaptotrigona* sp. were crushed and extracted with 200 μ l of bidistilled ethyl acetate. After 10 min, the solution was filtrated, and the solvent was evaporated to 50 μ l under a nitrogen stream, and 1 μ l was analyzed by GC-MS.

GC-MS Analysis. GC analysis was carried out using a Hewlett Packard 6890 apparatus fitted with an HP-5 fused silica capillary column (30 m \times 0.25 mm \times

0.25 μm). A sample volume of 1 μl was injected, and pressure programming was used to maintain a constant flow (1 ml/min) of the helium carrier gas. A Hewlett Packard 5973 mass spectrometer was used in the EI mode (ionization energy of 70 eV) and set to scan the mass range of 50–700 u at a rate of 2.94 scans/sec. The interface temperature was maintained at 280°C. The resulting data were processed using the Hewlett Packard Chemstation Software package. The temperature program was from 50 to 310°C at 4°C/min, plus 40 min at the final temperature. The injector temperature was 240°C.

Identification of Alkanes. Alkanes identification was confirmed by comparing mass spectra and retention times with those of standard alkanes and by using a Wiley275 database.

Derivatization of Alkenes. Extracts (0.3–0.5 mg) were solubilized in 50 μl of dimethyldisulfide and 10 μl of a 10% solution of iodine in ethyl ether. The reaction mixture was kept at 50°C for 12 hr, and the reaction was quenched with aqueous $\text{Na}_2\text{S}_2\text{O}_3$. The organic phase was extracted with hexane (0.5 ml) and evaporated to 50 μl under a nitrogen stream, and 1 μl of the solution was analyzed by GC-MS. The dimethyldisulfide adducts were identified, and the positions of the methylsulfide substituents were deduced from the fragmentation pattern.

Trans-Cinnamic Acid Methyl Ester. Cinnamic acid (1 mg) was treated with 200 μl of a diazomethane solution in ethyl ether at room temperature for 15 min. The solvent was evaporated and the product was analyzed by GC-MS and ^1H nuclear magnetic resonance (NMR). EIMS (70 eV): m/z (%) = 162 (M^+ , 50%), 147 (2%), 131 (100%), 103 (74%), 77 (36%), 51 (12%). ^1H NMR (300.067 MHz, CDCl_3): δ = 3.81 (s, 3H), 6.45 (d, 1H, J = 15.9 Hz), 7.38 (m, 3H), 7.53 (dd, 2H), 7.70 (d, 1H, J = 15.9 Hz).

Trans-Cinnamic Acid Ethyl Ester. Cinnamic acid (2 mg) in ethanol (200 μl) and sulfuric acid (10 μl) were heated to reflux for 12 hr. The reaction mixture was extracted with dichloromethane (2×1 ml) and washed with aqueous NaHCO_3 , after which the solvent was evaporated and the product was analyzed by GC-MS. EIMS (70 eV): m/z (%) = 176 (M^+ , 34%), 147 (20%), 131 (100%), 103 (44%), 77 (25%), 51 (10%). ^1H NMR (300.067 MHz, CDCl_3): δ = 1.34 (t, 3H), 4.26 (q, 2H), 6.44 (d, 1H, J = 16.2 Hz), 7.38 (m, 3H), 7.52 (dd, 2H), 7.69 (d, 1H, J = 16.1 Hz).

Electrophysiology. *N. testaceicornis* male antennae ($N = 5$) were excised (pulled from the head) with forceps, and a few segments were cut off at the base and the tip (Bjostad, 1998). Each antenna was then fixed between two stainless-steel electrodes by pushing the base and tip into droplets of an electrically conductive gel (Spectra 360[®] electrode gel, Parker, Orange, NJ, USA) applied onto the metal electrodes.

Electroantennogram (EAG) recordings were carried out by using a Syntech EAG system (Hilversum, The Netherlands). An antenna was stimulated by

subjecting it to puffs (0.3 sec) of purified and humidified air (1.2 l/min) delivered through a Pasteur pipette, containing a filter paper strip (ca. 0.8 cm), impregnated with 5 μ l of test solution. Hexane and dichloromethane were used as control stimulus: crude floral extract and volatiles were captured by dynamic *headsapce*. Control stimulation was made before and after each test compound of each EAG experiment. Test compounds were applied randomly at intervals of 90 sec. The Syntech EAG software calculated the normalized values automatically. The mean normalized responses of the different compounds were submitted to ANOVA for statistical analysis and were compared by using Tukey's test ($P < 0.05$).

RESULTS AND DISCUSSION

Monitoring Mormolyca Floral Chemistry. While studying *M. ringens* floral chemistry, we observed that pollinated flowers attracted neither *Scaptotrigona* (Singer et al., 2004) nor *Nannotrigona*, the alternative pollinator. We assigned this phenomenon to floral chemistry changes in pollinated and unpollinated flowers.

Monitoring (Table 1, Figure 2) of the hexane extracts of unpollinated (Figure 2A) and pollinated (Figure 2B) flowers by GC-MS showed that the floral chemistry changed as soon as pollination occurred. The total relative abundance of alkene/alkane ratio for unpollinated flowers is 0.8, and in pollinated flowers, the ratio is lower (0.3). Additionally, the 9,17-octadecadienal and C-25 and C-27 alkenes were less abundant in pollinated flowers (1.60, 2.02, and 1.15%, respectively; Table 1) than in unpollinated flowers (7.88, 10.20, and 9.7% respectively; Table 1), and the males were no longer attracted to the pollinated flowers (Singer et al., 2004).

This analysis was repeated five times with different flowers, and the same results were always obtained. To determine the position of unsaturation, we derivatized the floral extract with dimethyldisulfide and iodine, as described by Buser et al. (1983). The fragmentation patterns of all the *bis*-methylsulfide derivatives in the total ion chromatogram were similar with major fragments at m/z 173 and at m/z 243 + n14, which was consistent with a homologous series of 9-alkenes ranging from C-23 to C-31, all of them possessing odd carbon numbers. The remaining constituents (peaks 1–6) were identified by coinjection with standards. Schiestl and Ayasse (2002) analyzed flower extracts of *Ophrys fusca* and *O. bilunulata* and detected alkenes ranging from C-25 to C-29, but with unsaturations at C-7, C-9, C-11, or C-12. In *M. ringens*, however, the double bond was always found at C-9.

TABLE 1. COMPOUNDS IDENTIFIED IN HEXANE EXTRACTS OF UNPOLLINATED AND POLLINATED FLOWERS OF *MORMOLYCA RINGENS* AND CUTICULAR WAXES FROM VIRGIN QUEENS OF *SCAPTOTRIGONA* SP

Peak no. in Figure 1	Compound	Unpollinated flower (%)	Pollinated flower (%)	Virgin queen bees (%)	Method of identification
1	Cinnamic acid methyl ester	1.35	1.00	—	MS, standard
2	Cinnamic acid	2.23	0.01	—	MS, standard
3	Cinnamic acid ethyl ester	1.20	0.80	—	MS, standard
4	Palmitic acid	4.62	3.20	—	MS, standard
5	Henicosane	0.30	—	0.99	RI, MS, standard
6	Linoleic acid	3.50	2.61	—	MS, standard
7	9,17-Octadecadienal	7.88	1.60	—	MS, Wiley275 database
8	9-Tricosene	1.05	1.40	3.13	MS, DMDS derivative
9	Tricosane	4.50	3.10	24.34	RI, MS, standard
10	Tetracosane	1.36	1.11	0.68	RI, MS, standard
11	9-Pentacosene	10.20	2.02	1.34	MS, DMDS derivative
12	Pentacosane	10.40	13.50	26.15	RI, MS, standard
13	Hexacosane	1.21	1.60	0.66	RI, MS, standard
14	9-Heptacosene	9.70	1.15	7.99	EM, DMDS derivative
15	Heptacosane	14.20	28.00	21.80	RI, MS, standard
16	Octacosane	1.25	2.02	—	RI, MS, standard
17	9-Nonacosene	10.10	16.20	8.38	MS, DMDS derivative
18	Nonacosane	11.40	20.10	2.65	RI, MS, standard
19	9-Hentriacontene	3.45	0.28	0.69	MS, DMDS derivative
20	Hentriacontane	0.10	0.30	1.20	RI, MS, standard
Total		100.0	100.0	100.0	

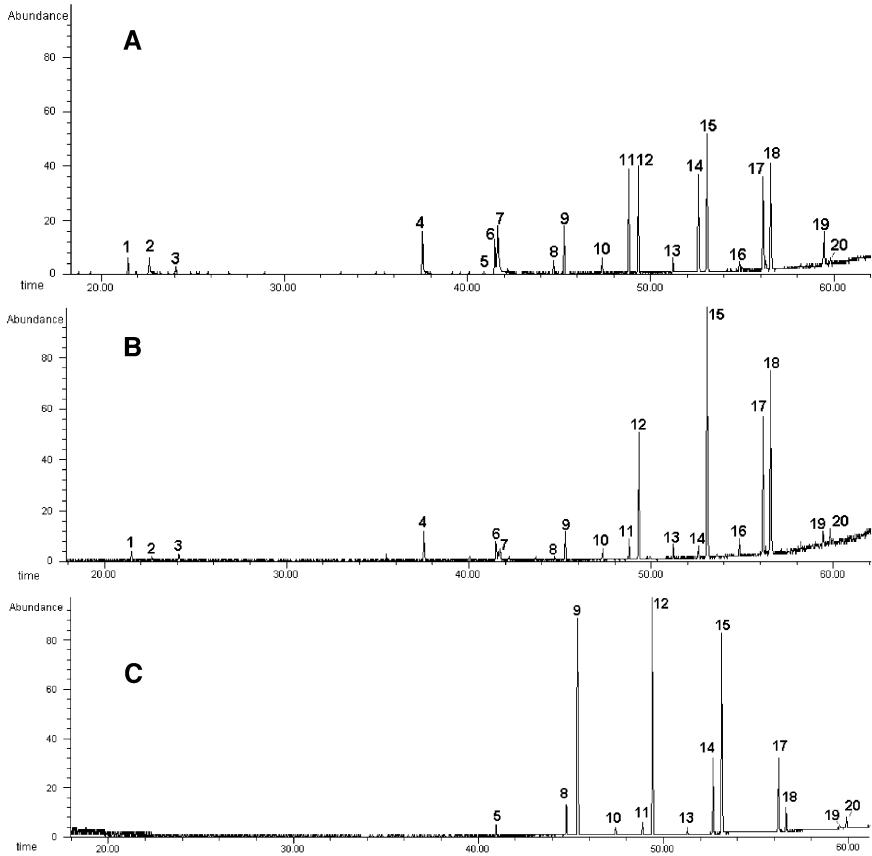


FIG. 2. Total ion chromatogram of hexane extracts from unpollinated (A) and pollinated (B) flowers of *M. ringens* and cuticular waxes from virgin queens of *Scaptotrigona* sp. (C) Identification of numbered compounds in Table 1.

Monitoring Scaptotrigona sp. Virgin Queens (bee) and M. ringens (Orchidaceae) Chemistry. The attraction of *Scaptotrigona* sp. and *Nannotrigona* males to *M. ringens* flowers and the induction of pseudocopulation suggested that the flowers had chemical compositions similar to virgin queen bees that were cultivated for investigation. This was based on the previous knowledge that in all Meliponini genera (except for *Melipona*), workers and queen castes are differentiated by a quantitative feeding system (Kerr, 1948). Thus, larvae ingesting moderate amounts of food will develop into workers, while larvae ingesting large amounts of food will develop into queens (Camargo, 1972b). Therefore, we used the technique of Camargo (1972a) and Menezes et al. (2004) to

TABLE 2. 2-ALKANOLS IDENTIFIED IN ACETATE CEPHALIC EXTRACTS OF VIRGIN QUEENS OF *Scaptotrigona* SP

Compound	Relative abundance (%)
Heptan-2-ol	28.3
Nonan-2-ol	25.6
Undecan-2-ol	19.1
Tridecan-2-ol	27.0
Total	100.0

produce queens “in vitro.” We were more successful in rearing *Scaptotrigona* sp. than *N. testaceicornis*, thus, the chemistry from different *Scaptotrigona* sp. virgin queen’s (all originated from the same hive) bodies and heads ($N = 10$ individuals) was investigated. GC-MS analysis of the cephalic acetate extracts revealed the presence of a mixture of 2-alkanols (Table 2). A similar mixture of 2-alkanols was reported by Engels et al. (1997) in the volatile fraction of the cephalic secretions of receptive virgins of *S. postica*. These authors also demonstrated that the 2-alkanols acted as long-range attraction signals (Engels et al., 1997). Because we also detected the same 2-alkanol mixture, we concluded that the virgin queen bees we were dealing with were receptive to males.

In addition to an attractive, long-range “perfume,” virgin queen bees also require additional short-range chemical signals to prompt male sexual behavior (Schiestl et al., 2000). However, this additional short-range precopulatory signal has not yet been identified for *Scaptotrigona*. We hypothesized that the short-range attractive components were located on the abdominal surface. To examine this possibility, female bees ($N = 10$) were washed with hexane, and the extract was analyzed by GC-MS. This analysis revealed a homologous series of alkanes and alkenes. The latter showed an odd series C-23 to C-31 with unsaturation at position 9, identical to that seen in the *M. ringens* hexane extract, but in a slightly different alkane/alkene ratio.

Working with *Ophrys* (Orchidaceae) and *Andrena* bees, Schiestl and Ayasse (2002) have demonstrated that these two organisms have similar alkane/alkene constituents, not identical in abundance; the alkane/alkene mixture (present in both virgin bees and orchid labella) was demonstrated to be responsible for the pseudocopulatory behavior in the *Andrena* bees–*Ophrys* system (Schiestl et al., 2000). Extrapolating these results to the *Mormolyca* × *Scaptotrigona* system, we suggest that the homologous 9-alkane/alkene series present in the flowers and virgin queen bees may be responsible for the chemically induced copulatory activity in males.

Changes in Floral Chemistry. As indicated above, floral chemistry undergoes a compositional change following pollination (a decrease in the relative alkene/alkane abundance, assumed to be the short-range floral attractant), so that

the flower no longer attracts *Scaptotrigona* males. Although the relative floral alkene/alkane composition and abundance were similar in *M. ringens* and in *Scaptotrigona* sp. virgin queen bees, we were intrigued by the lack of similarity in the long-range attraction chemicals detected in the cephalic extracts of *Scaptotrigona* sp. (homologous series of 2-alkanols). A detailed examination of the floral hexane extract revealed no 2-alkanol. Because we considered that this negative result might reflect an inappropriate sampling method, we trapped the emitted volatiles of an unpollinated flower by using dynamic headspace methodology (Singer et al., 2004). At first, 2-alkanol was not detected, but a closer analysis of the total ion chromatogram for specific ions revealed the presence of 2-heptanol. We concluded that male attraction does not require large quantities of the active substance, and that, in this case, 2-heptanol may have been the active principle responsible for attracting *S. postica* males.

Based on these results, and on a similar phenomenon (Ayasse et al., 2000), we suggest that the 2-alkanols (specifically, 2-heptanol) act as long-range chemical signals, whereas homologous odd 9-alkene/alkane series act as short-range signals and induce copulation in *Scaptotrigona* sp. To confirm, *in vivo*, the pollinators' response to floral chemistry, we collected as many male bees as possible, all flying around the *M. ringens* flowers. However, many did not survive long enough to reach the analytical laboratory, and only the remaining specimens, i.e., *N. testaceicornis* drones, were investigated by electroantennography (EAG). Mean depolarization achieved by *N. testaceicornis* male antennae in response to the crude floral extracts and volatile components of *M. ringens* is

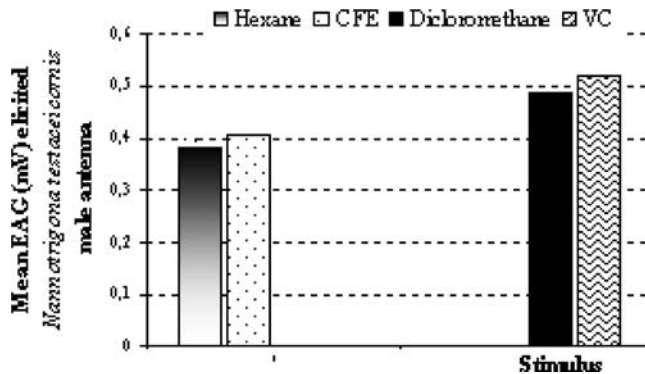


FIG. 3. Mean values (\pm SD, in mV) of electroantennography (EAG) responses of *Nannotrigona testaceicornis* male antenna to crude floral extracts (CFE) and volatile components (VC) of *M. ringens*. Mean values marked with the same letter are not significantly different at $P < 0.05$ based on Tukey's test ($N = 5$). Hexane, 100%; CFE, 1 mg/ml; dichloromethane, 100%; VC, not determined.

shown in Figure 3. Both extracts elicited EAG responses statistically different from controls. These results indicated that *N. testaceicornis* recognized the chemical components present in both extracts via antennae chemoreceptors and suggested that this bee uses *M. ringens* volatiles to find these specific flowers. EAG provided conclusive data on the selective antennae depolarization by the orchid floral constituents compared with the control (solvents).

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SPECIES-SPECIFIC ANTENNAL RESPONSES TO TIBIAL FRAGRANCES BY MALE ORCHID BEES

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Abstract—Male neotropical orchid bees (Euglossini) collect odoriferous substances from orchids and other sources and store them in tibial pouches, accumulating complex and species-specific bouquets. These fragrances are later exposed at display sites, presumably to attract females or conspecific males or both. We hypothesized that the necessity to detect and recognize specific fragrance bouquets has led to peripheral chemosensory specializations in different species of orchid bees. To test this, excised male antennae of four species of *Euglossa* were stimulated with complete tibial extracts of the same four species in a crosswise experiment. In the majority of the tested extracts, the amplitude of the electroantennogram (EAG) response was significantly different between species and always maximal in males of the extracted species. This effect did not appear to result from a given species' increased sensitivity toward certain attractive components: gas chromatography with electroantennographic detection (GC-EAD) of one extract of *Euglossa tridentata* evoked similar and generalized response patterns in all four species, encompassing a total of 34 peaks that elicited antennal responses. Therefore, the species effect in EAG responses to complete extracts likely resulted from species-specific interactions of compounds at the receptor level. Antennal specialization to conspecific bouquets adds additional strength to the argument that specificity is an important evolutionary aspect of euglossine tibial fragrances.

Key Words—Sensory specialization, olfaction, EAG, GC-EAD, odor bouquet, Euglossini.

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INTRODUCTION

Male orchid bees are uniquely adapted for collecting and storing volatile chemicals, which they seek at flowering orchids, aroids, palms, and other plants, as well as at nonfloral sources such as rotting wood and feces (Vogel, 1966; Dressler, 1982). The fragrances are dissolved in lipoid labial gland secretions and are transferred to spacious cavities in the bees' hind tibiae. Here, complex mixtures of terpenoids and aromatics accumulate over weeks or months (Whitten et al., 1989; Eltz et al., 1999). Mounting evidence suggests that males release the substances during bouts of courtship display, for which they defend small territories around perch trees in the forest understory (Bembé 2004; Eltz et al., 2005a). On rare occasions, females visit these territories to mate, but it is unknown whether their attraction is mediated by the fragrances or indeed by any other chemical stimulus (Kimsey, 1980; Eltz et al., 2003).

Chemically, the tibial contents seem to be species-specific in a rather broad sense: males of *Euglossa* vary substantially in qualitative and quantitative aspects of their bouquets, but much less so within than between species. Notably, each species has a characteristic set of fragrance compounds (~6–15) that is shared with high probability even by individuals from distant and ecologically divergent localities (Eltz et al., 1999, 2005b). Field experiments suggest that this chemical profile is sufficient to promote specific long-range attraction. When hexane extracts of *Eulaema meriana* and *Eulaema bombiformis* hind legs were applied to filter paper and exposed at known male display sites in the forest understory, these extracts quickly and reliably attracted other males of the correct species (Zimmermann, Roubik and Eltz, submitted). Thus, it seems that fragrance perception is important to male euglossines in two rather different contexts. First, males must find remote fragrance sources, which emit mostly either single components (e.g., some nonfloral odors (Whitten et al., 1989, 1993; Eltz et al., 1999) or simple blends of 2 to 10 components (most euglossophilous orchids; Williams and Whitten, 1983; Gerlach and Schill, 1991). Second, fragrance perception is important in the context of territorial behavior, where males encounter the much more complex blends previously accumulated by their peers. In either context, one might expect strong selection pressures leading to sensory specialization in favor of certain odor qualities and divergent sensory tuning in different species of bees.

Here, we used electroantennography (EAG) to quantify antennal responses to conspecific and heterospecific tibial extracts in four species of *Euglossa*. EAG records the sums of receptor potentials from the entirety of olfactory neurons located in the antenna, and the amplitude of the recorded signal is assumed to reflect the strength of stimulation evoked by an odor. The method has previously been used to investigate odor detection in a wide variety of insects (Roelofs, 1984; Schiestl and Marion-Poll, 2002), including orchid bees (Schiestl and Roubik, 2003). In addition to recording EAG responses to

complete extracts, we used gas chromatography coupled with electroantennographic detection (GC-EAD) to screen tibial extract components for their ability to elicit responses from antennae of male bees.

METHODS AND MATERIALS

Bees. Males of *Euglossa imperialis* (imp), *E. cognata* (cog), *E. mixta* (mix), and *E. tridentata* (trid) were captured during the first 2 wk of March 2005 in forests of the Barro Colorado National Monument, Panama. Individuals were captured upon arrival at screened 1,8-cineole (imp, cog, trid), methyl salicylate (imp, cog, mix), *p*-dimethoxybenzene (cog, trid, mix), and skatole (mix) baits, and were later transferred to Düsseldorf, Germany, in individual vials. The bees were introduced into 50 × 50 × 60 cm mesh cages placed in a greenhouse (25–30°C, 70–90% relative humidity) where they learned to drink honey-water from artificial flowers. Individuals were subjected to EAG (Düsseldorf) and GC-EAD (Ulm) over the course of 4 wk.

Test Extracts. Hexane extracts were prepared from males of all four species collected at the same time at the same Panamanian locality (see above). Individual heads and pairs of hind legs were extracted separately in 0.5 ml of p.a. grade hexane (Merck). Hind-leg extracts were screened by GC-MS for fragrance content, and three rich extracts of each species were selected as EAG stimuli. This screening for tibial content was necessary because many individual male *Euglossa* contain only tiny amounts of fragrances (Eltz et al., 1999). The selected extracts were likely derived from relatively old males that had accumulated substantial quantities. The hind-leg extracts contained a large quantity and variety of lower molecular weight (<300 Da) terpenoids and aromatics [the exogenous fragrances; see Eltz et al., (1999)], a range of long chain saturated and unsaturated hydrocarbons, alcohols, acids, acetates, diacetates, and esters (“lipids,” mostly products of the bees’ labial glands, added in the process of fragrance collection; Williams and Whitten, 1983), and a few high molecular weight compounds presumably derived from plant surfaces (e.g., triterpenoids) (see Appendix; Available online at www.springerlink.com; Search for DOI: 10.1007/s10886-006-9352-0). To test whether labial gland lipids alone stimulated antennae, we prepared and tested one head extract of each of the species. Head extracts contained roughly similar amounts of labial gland lipids as did the hind-leg extracts, but lacked fragrance compounds (see Appendix; Available online at www.springerlink.com; Search for DOI: 10.1007/s10886-006-9352-0).

EAG with Complete Extracts. Single antennae cut at the tip and at the third antennal segment were mounted between two glass pipettes filled with insect Ringer solution and connected to silver electrodes. All extracts were tested once on each antenna. During the test series, one stimulus was applied every 2 min, with the

individual extracts arranged in blocks of four (one of each species per block, randomized within-block sequence), interspersed by solvent blanks. For every stimulus, 5 μ l of the test solution were pipetted onto a fresh 2 \times 10 mm strip of filter paper. The solvent was allowed to evaporate before the strip was placed in a clean pipette tip. For stimulation, 200 μ l of air were puffed over the filter paper and injected into a purified and moistened air stream blowing over the antenna. EAG responses were amplified and recorded (in mV) using Syntech (Hilversum, The Netherlands) electrode holders, IDAC-232 acquisition controller, and EAG recording software. The amplitude of the negative baseline deflection was used as a measure of response. To analyze whether a species' response to an extract was greater than that elicited by the averaged hexane blanks, we calculated rank-based Wilcoxon matched-pairs tests for all extracts, separately in each species. We then standardized responses to extracts across antennae to control for variations in the size of bee and the quality of preparation. Single-factor ANOVA was calculated to test for the overall effect of the factor *species* on the response to a given extract.

GC-EAD Screening. The tibial extract *trid 3* was selected to screen for the electrophysiological effect of its single components. Antennal preparations were identical to those described above. GC-EAD was done with an HP 6890 gas chromatograph fitted with a DB-5 column (J&W Scientific, Folsom California, 30 m \times 0.25 mm I.D.). A variable outlet splitter (SGE) was used to divide the effluent 3:1, and the larger fraction was directed onto the bee antennae. Nitrogen was used as a makeup gas. Flame ionization detector and EAD signals were recorded with Syntech EAD recording software. For each run, 1 μ l of the extract was injected splitless, testing antennae of males of all four species. The GC oven was programmed from 50 to 300°C at 10°C/min. A peak was classified as "GC-EAD active" when it coincided with a negative EAD baseline deflection in all analyzed runs. Each antenna was tested only once, and all scored antennae originated from separate individuals.

RESULTS

EAG Responses to Complete Extracts. All tested hind-leg extracts elicited substantially larger antennal responses than did solvent blanks in each of the species [Wilcoxon matched-pairs tests: $P < 0.01$ in all 48 comparisons (12 extracts \times 4 species); Figure 1]. Irrespective of the perceiving species, the response level varied between extracts, with *E. tridentata* extracts eliciting relatively large and *E. imperialis* extracts relatively small responses (Figure 1). This variation corresponded to the overall concentration of fragrances in the different extracts (see Appendix; Available online at www.springerlink.com; Search for DOI: 10.1007/s10886-006-9352-0). In 9 of the 12 hind-leg extracts, there was a significant effect of the perceiving species on the magnitude of the

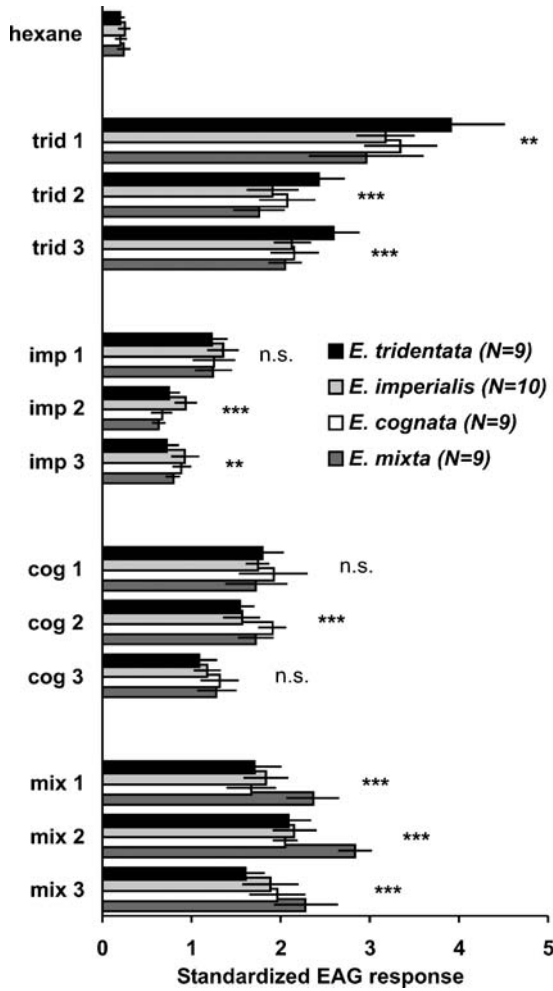


FIG. 1. Standardized electroantennogram response (mean and standard deviation) of males of four species of *Euglossa* to hind-leg extracts taken from males of the same four species captured in Central Panama. Three extracts of each species were tested (trid = *E. tridentata*, mix = *E. mixta*, imp = *E. imperialis*, cog = *E. cognata*). Significance levels of the factor “perceiving species” on response amplitude are given (single-factor analysis of variance: $N = 37$; ** $P < 0.01$; *** $P < 0.001$).

response (see Figure 1), with the correct (extracted) species showing the largest response in all cases. The species effect was particularly pronounced in the case of *E. tridentata* and *E. mixta*. Here, the extracted species showed a response that was roughly 20% larger than that of the other species.

In contrast to hind-leg extracts, the head extracts elicited only weak responses that were not significantly different from averaged solvent blanks in most comparisons (data not shown here). The following responses were marginally significant (Wilcoxon matched-pairs tests; $P < 0.05$): *E. cognata* to *E. tridentata*, *E. tridentata* to *E. tridentata*, and *E. imperialis* to *E. cognata*, *E. imperialis*, and *E. tridentata*.

GC-EAD Screening. The tibial extract *trid* 3 was selected to screen for physiologically active components. We analyzed a total of 17 GC-EAD runs: 5 with *E. tridentata*, 6 with *E. cognata*, 3 with *E. imperialis*, and 3 with *E. mixta* antennae. A total of 33 GC peaks elicited reproducible negative baseline deflections in all species, and 1 additional peak, an uncharacterized trace compound (28 in Figure 2), did so only in *E. tridentata*. Most of the dominant fragrance components elicited EAD responses, frequently causing strong negative deflections. Reproducible deflections also were caused by several minor fragrance components and by eicos-9-enyl-1,20-diacetate (34), the major component of *Euglossa* labial gland secretions. A few peaks were not active in spite of their relatively large size. These included the moderate peaks of α -pinene and β -pinene and the massive peak of (*E*)-nerolidol. The overall response pattern was similar between species, although some late eluting fragrance compounds seemed to cause somewhat larger responses in *E. tridentata* (29 and 30 in Figure 2).

DISCUSSION

Tibial fragrance bouquets of male *Euglossa* bees elicited larger EAG responses from conspecific than heterospecific antennae. This suggests that peripheral olfactory specialization has occurred in the different species of orchid bees, presumably because of different olfactory cues being important in their respective life cycles.

The various species of euglossine bees differ in the type of fragrances they prefer to collect. This is evident in the range of synthetic chemicals that can be used to lure males of different species during baiting assays (Ackerman, 1989). In a previous study, we tested a broad range of these pure compounds, but did not find a species-specific difference in the EAG responses, although the tested species had quite distinct chemical preferences on the behavioral level (Eltz and Lunau, 2005). These findings are broadly confirmed by the GC-EAD screening of extract components in the present investigation: response patterns to bait chemicals such as 1,8-cineole, eugenol, *p*-dimethoxybenzene, 2,3-epoxygeranyl acetate, or benzyl benzoate were surprisingly similar across species.

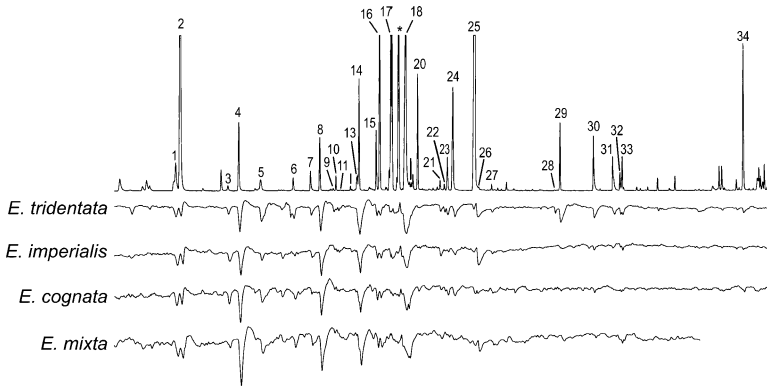


FIG. 2. Antennal responses of four male *Euglossa* spp. to components of a hind-leg extract of *E. tridentata* (*trid* 3 in Figure 1) eluting from the gas chromatograph. The flame ionization detector chromatogram is shown along with one representative electroantennographic detection (EAD) signal for each of the study species. Numbered peaks correspond to reproducible negative deflections of the baseline in all four species, except peak (28), which was active in *E. tridentata* only. Peaks (12) and (19) are marked but not numbered on the chromatogram because of limited space. (1) Limonene + 1,8-cineole; (2) (*E*)- β -ocimene; (3) ipsdienol; (4) *p*-dimethoxybenzene; (5) ?; (6) trace; (7) 2,3-epoxygeraniol; (8) eugenol; (9) (*E*)-methyl cinnamate; (10) β -elemene, ?; (11) trace; (12) (*E*)-caryophyllene; (13) (*E*)-isoeugenol; (14) 2,3-epoxygeranyl acetate; (15) (*E,E*)- α -farnesene; (16) β -bisabolene; (17) sesquiterpene alcohol; (18) *p*-methoxycinnamic alcohol; (19) methoxyeugenol; (20) (*E*)-farnesene epoxide; (21) (*E*)-methyl *p*-methoxycinnamate; (22) hedycaryol; (23) similar methoxyeugenol; (24) (*E*)-*p*-methoxycinnamyl acetate; (25) benzyl benzoate; (26) trace; (27) hexahydrofarnesyl acetone; (28) trace; (29) ?; (30) 3,7,11,15-tetramethyl-hexadeca-2,6,10,14-tetraen-1-ol, ?; (31) diterpene alcohol; (32) + (33) long-chain unsaturated acids; (34) eicos-9-enyl-1,20-diacetate; (35, not shown) long-chain acetate. *Peak of (*E*)-nerolidol, the only major component that did not elicit an EAD response. See text for further specifications.

Why should complete tibial bouquets elicit species-specifically different EAG responses if critical components do not? Natural fragrance cues encountered by male bees are in fact mixtures, either the tibial fragrances themselves (as exposed by displaying males) or the more simple blends of the floral sources from which they are derived. Detecting and discriminating among mixtures of odorants is certainly important to the bees and may be a challenging task given that more than 30 species of euglossines may coexist in neotropical forests. Antennal specializations to certain odorant combinations might facilitate fragrance resource partitioning among competing males of different species as well as benefit fragrance-based chemical communication within species (e.g., facilitate species recognition).

On the mechanistic level, species-specific differences in EAG responses to complete extracts could have accumulated from small species-specific differences in sensitivity to certain components. Each of these differences may have been too small to be detected when components were tested individually (as in Eltz and Lunau, 2005), but when combined in complex blends, they might produce a significant overall effect. Furthermore, with mixtures of odorants, antennal responses may also be influenced by how different components interact on the level of olfactory receptor neurons (Akers and Getz, 1993; Cromarty and Derby, 1997). Recent work on *Drosophila* suggests that individual receptor proteins can confer either excitatory or inhibitory effects on an olfactory neuron, depending on precisely which odorant is binding to them (Hallem et al., 2004). In the case of complex mixtures, a number of different odorants could bind simultaneously to any given neuron, with relative concentrations determining the outcome of the neuron-level response. If tested as mixtures, inhibitory effects of certain components may have substantial effects on the overall antennal response. For orchid bees, Schiestl and Roubik (2003) have argued that inhibitory interaction of compounds at the receptor level may be responsible for decreased EAG responses to certain synthetic fragrance mixtures. We hypothesize that species-specific inhibitory effects were responsible or at least contributed to differential responses to tibial bouquets. This view has interesting implications for the orchid–euglossine bee coevolution. Normally, a given euglossophilous orchid attracts only one or a few species of euglossine bees (Roubik and Hanson, 2004), although its scent may be dominated by a broadly attractive component. In such a case, selectivity appears to be achieved by minor modifying compounds that render a given bouquet unattractive to all but the pollinating species (Williams and Dodson, 1972). The inhibitory effects of these modifiers may already be operating at the antennal level. Future studies should attempt to imitate naturally occurring orchid fragrances by mixing synthetic components in the correct blends. Using stringent comparative EAG testing with pollinating and nonpollinating bees might help to elucidate the sensory basis of specialized orchid–bee interactions.

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CHEMICAL CONSTITUENTS OF THE AQUATIC PLANT
Schoenoplectus lacustris: EVALUATION OF
PHYTOTOXIC EFFECTS ON THE GREEN ALGA
Selenastrum capricornutum

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Abstract—Forty-nine secondary metabolites were isolated from aqueous and alcoholic extracts of the aquatic plant *Schoenoplectus lacustris*. All compounds were characterized based on spectroscopic data. Eleven free and glycosylated low-molecular polyphenols, 17 cinnamic acid and dihydrocinnamic acid derivatives, 11 flavonoids, and 10 C₁₃ *nor*-isoprenoids were identified. The structure of the new compound, 1-benzoyl-glycerol-2- α -L-arabinopyranoside, was elucidated by 2D NMR experiments (COSY, HSQC, HMBC, NOESY). To evaluate potential phytotoxic effects, all compounds were tested on the green alga *Selenastrum capricornutum*, a unicellular organism commonly used in tests of toxicity as a bioindicator of eutrophic sites. The most active compound was (–)-catechin, showing an inhibition similar to that of the algaecide CuSO₄.

Key Words—*Schoenoplectus lacustris*, polyphenols, cinnamic acids, dihydrocinnamic acids, flavonoids, C₁₃ *nor*-isoprenoids, (–)-catechin, phytotoxicity, antialgal, *Selenastrum capricornutum*.

INTRODUCTION

In aquatic systems, plants can release allelochemicals into the water, and complex interactions between phytoplankton and vascular plants have been in-

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vestigated. A number of plants possess inhibitory effects on populations of microalgae by releasing phytotoxic substances into the environment (Gross et al., 2003).

Early studies on interactions between aquatic plants and microalgae were conducted by Schreiter (1928), Hasler and Jones (1949), and Fitzgerald (1969). Later, experiments were designed to identify interactions between hydrophytes and microalgae (Kogan and Chinnova, 1972; Brammer, 1979; Godmaire and Planas, 1983).

Allelochemicals have been isolated from aquatic plants, and the ability of some natural products to inhibit *in vitro* development of microalgae has been reported by our research group in recent years (Cangiano et al., 2001; DellaGreca et al., 2001, 2003a, b).

In this research, we report on a phytochemical study of *Shoenoplectus lacustris* L. (Cyperaceae) and its effects on *Selenastrum capricornutum*, an algal species selected as a bioindicator of eutrophic sites for studies in aquatic environments (OECD, 1984; ISO, 1982). The effects of 49 low-molecular weight secondary metabolites on microalga *S. capricornutum* are reported.

METHODS AND MATERIALS

General Experimental Procedures. NMR spectra were recorded at 500 MHz for ^1H and 125 MHz for ^{13}C on a Varian spectrometer (Varian Inc., Palo Alto, CA, USA) in CDCl_3 or CD_3OD solutions at 25°C . Optical rotations were measured on a Perkin-Elmer 343 Polarimeter (Norwalk, CT, USA). CD spectra were obtained in MeOH solutions on a Jasco J-715 Spectrophotometer Polarimeter (Jasco, Great Dunmow, UK). Electronic impact mass spectra (EI-MS) were obtained with an HP 6890 instrument equipped with an MS 5973 N detector (SIS Instruments, Ringoes, NJ, USA). The HPLC apparatus (Shimadzu, Kyoto, Japan) consisted of a pump (Shimadzu LC-10AD), a refractive index detector (Shimadzu RID-10A), and a Shimadzu Chromatopac C-R6A recorder. Preparative HPLC was made by using a 250×10 mm i.d., $10 \mu\text{m}$, RP-18 Luna column (Phenomenex, Torrance, CA, USA) and SiO_2 (MAXSIL, 250×10 mm i.d., Phenomenex) columns. Analytical TLC was conducted on Merck Kieselgel 60 F_{254} or RP-8 F_{254} plates with 0.2-mm layer thickness (Merck, Darmstadt, Germany). Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates, with 0.5 or 1 mm film thickness. Flash column chromatography (FCC) was carried out with Merck Kieselgel 60 (230–400 mesh) at medium pressure. Column chromatography (CC) was performed on Merck Kieselgel 60 (70–240 mesh), Baker Bond Phase C18 (0.040–0.063 mm), Fluka (Fluka, Buchs, Switzerland), Reversed phase silica gel 100 C8 (0.040–0.063 mm) or on Sephadex LH-20[®] (Pharmacia).

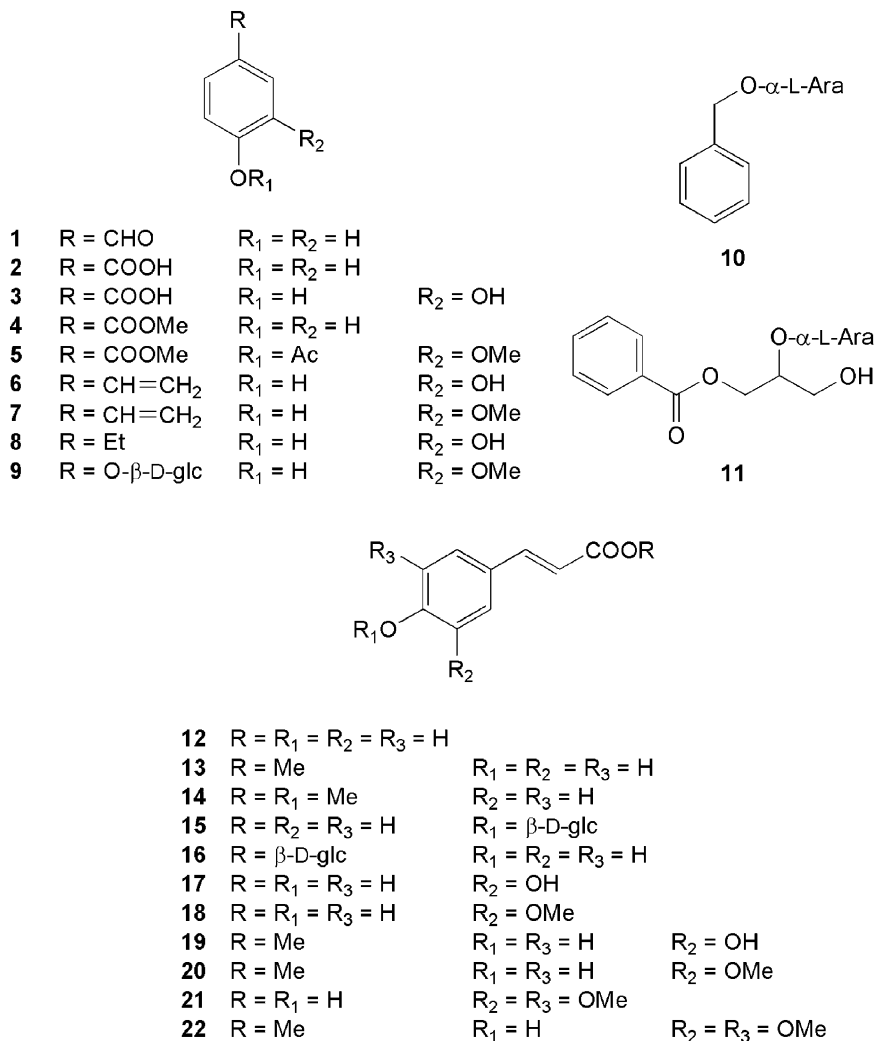
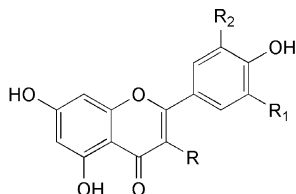
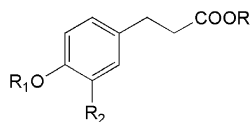


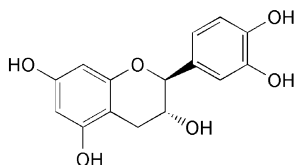
FIG. 1. Chemical constituents of *Schoenoplectus lacustris*.

Plant Material. Plants of *S. lacustris*, collected in the summer of 2002 from the Volturno River (Campania, Italy), were identified by Professor Assunta Esposito at the Second University of Naples (SUN). Voucher specimens (CE 31) were deposited at the Herbarium of the Dipartimento di Scienze della Vita of the SUN.

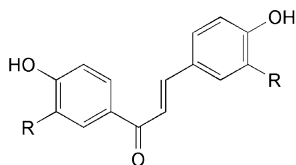
Extraction and Isolation. Fresh plants (12 kg) were extracted in 10% MeOH-H₂O over 3 d, in the dark at 4°C. After removal of the solution, plants



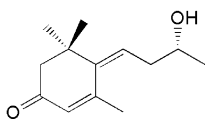
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| 24 $\text{R} = \text{R}_1 = \text{Me}$ $\text{R}_2 = \text{H}$ | 30 $\text{R} = \text{H}$ | $\text{R}_1 = \text{R}_2 = \text{OMe}$ |
| 25 $\text{R} = \text{Me}$ $\text{R}_1 = \text{R}_2 = \text{H}$ | 31 $\text{R} = \text{H}$ | $\text{R}_1 = \text{R}_2 = \text{OH}$ |
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| 28 $\text{R} = \text{Me}$ $\text{R}_1 = \text{H}$ $\text{R}_2 = \text{OH}$ | 34 $\text{R} = \text{O}-\beta\text{-D-glc}$ | $\text{R}_1 = \text{OH}$ $\text{R}_2 = \text{H}$ |
| | 35 $\text{R} = \text{O}-\alpha\text{-L-rha}(1\text{-}6\text{-})\beta\text{-D-glc}$ | $\text{R}_1 = \text{R}_2 = \text{H}$ |
| | 36 $\text{R} = \text{O}-\alpha\text{-L-rha}(1\text{-}6\text{-})\beta\text{-D-glc}$ | $\text{R}_1 = \text{OH}$ $\text{R}_2 = \text{H}$ |



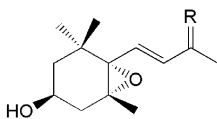
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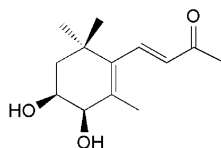
38 $\text{R} = \text{H}$
39 $\text{R} = \text{OMe}$



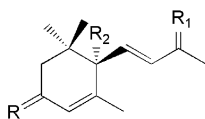
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41 $\text{R} = \text{O}$
42 $\text{R} = \alpha\text{OH}, \beta\text{H}$



43



- | |
|---|
| 44 $\text{R} = \text{O}$ $\text{R}_1 = \alpha\text{OH}, \beta\text{H}$ $\text{R}_2 = \text{H}$ |
| 45 $\text{R} = \text{OH}, \text{H}$ $\text{R}_1 = \alpha\text{OH}, \beta\text{H}$ $\text{R}_2 = \text{H}$ |
| 46 $\text{R} = \text{O}$ $\text{R}_1 = \alpha\text{OH}, \beta\text{H}$ $\text{R}_2 = \text{OH}$ |
| 47 $\text{R} = \text{OH}, \text{H}$ $\text{R}_1 = \alpha\text{OH}, \beta\text{H}$ $\text{R}_2 = \text{OH}$ |
| 48 $\text{R} = \text{OH}, \text{H}$ $\text{R}_1 = \alpha\text{OH}, \beta\text{H}$ $\text{R}_2 = \text{O}-\beta\text{-D-glc}, \text{H}$ |
| 49 $\text{R} = \text{O}$ $\text{R}_1 = \text{O}$ $\text{R}_2 = \text{OH}$ |

FIG. 1. Continued.

were extracted with MeOH for 5 d. After removal of methanol, the hydroalcoholic fraction was extracted first with methylene chloride and then with EtOAc. The two fractions were evaporated under reduced pressure to obtain crude residues (Scheme 1). Structures are provided in Figure 1. CH₂Cl₂ extract (13.3 g) was chromatographed on SiO₂ eluting with petroleum ether and EtOAc solutions to give seven fractions I–VII: Fraction I, eluted with petroleum ether, furnished compounds **6**, **7**, and **40**. Fraction II, eluted with petroleum ether–EtOAc (19:1), gave seven compounds: **5**, **13**, **14**, **4**, **8**, **23**, and **24**. Fraction III, eluted with petroleum ether–EtOAc (9:1), furnished three compounds: **1**, **17**, and **25**. Fraction IV, eluted with petroleum ether–EtOAc (4:1), gave six compounds: **2**, **20**, **22**, **27**, **28**, and **31**. Fraction V, eluted with petroleum ether–EtOAc (3:1), led to the isolation of **26**, **44**, **49**, and **45**. Fraction VI, eluted with petroleum ether–EtOAc (3:2), furnished compounds **38**, **41**, and **42**. Fraction VII, eluted with petroleum ether–EtOAc (1:1), gave isoprenoid **43**.

EtOAc extract (16.8 g) was chromatographed on SiO₂ eluting with CHCl₃ and EtOAc solutions to give six fractions VIII–XIII. Fraction VIII, eluted with CHCl₃, led to the isolation of **34** and **35**. Fraction IX, eluted with CHCl₃–EtOAc (19:1), gave flavone **36**. Fraction X, eluted with CHCl₃–EtOAc (9:1), furnished compound **32**. Fraction XI, eluted with CHCl₃–EtOAc (4:1), gave compounds **37** and **47**. Fraction XII, eluted with CHCl₃–EtOAc (3:2), led to the isolation of **21**, while fraction XIII, eluted with CHCl₃–EtOAc (1:4), gave compounds **42** and **46**.

MeOH extract (50.8 g) was dissolved in water and beaten with EtOAc to obtain an organic fraction and an aqueous one (Scheme 2). Structures are shown in Figure 1. The EtOAc fraction (30.4 g) was chromatographed by CC on Sephadex LH-20, eluting with hexane–CHCl₃–MeOH (1:1:1) to give five fractions, A–F. Fraction A furnished metabolites **12**, **21**, **29**, and **30**. Fraction B contained quercetin **33**. Fraction C consisted of compound **37**. Fraction D gave compounds **2** and **18**. Fraction E led to the isolation of **39** and **19**, whereas fraction F gave **3** and **35**.

The H₂O fraction (20.4 g) was chromatographed on Amberlite XAD-4 and eluted first with H₂O to eliminate sugars and proteins from the extract, then with MeOH–H₂O (1:1) and finally with MeOH. The eluate obtained with MeOH–H₂O (1:1) gave a fraction (G) that furnished compounds **15** and **16**. The eluate obtained with MeOH was chromatographed with H₂O and MeOH solutions to give five fractions H–L. Fraction H, eluted with H₂O–MeOH (17:3), gave compounds **10** and **48**. Fraction I, eluted with H₂O–MeOH (4:1), yielded glucoside **9**. Fraction J, eluted with H₂O–MeOH (4:1), gave compounds **11** and **36**. Fraction K, eluted with H₂O–MeOH (1:1), led to the isolation of glucoside **34**, whereas fraction L, eluted with H₂O–MeOH (1:3), gave rutinoid **35**.

Bioassay. The effects of the extracts and the isolated compounds were assessed on *S. capricornutum*, more recently renamed *Pseudokirchneriella*

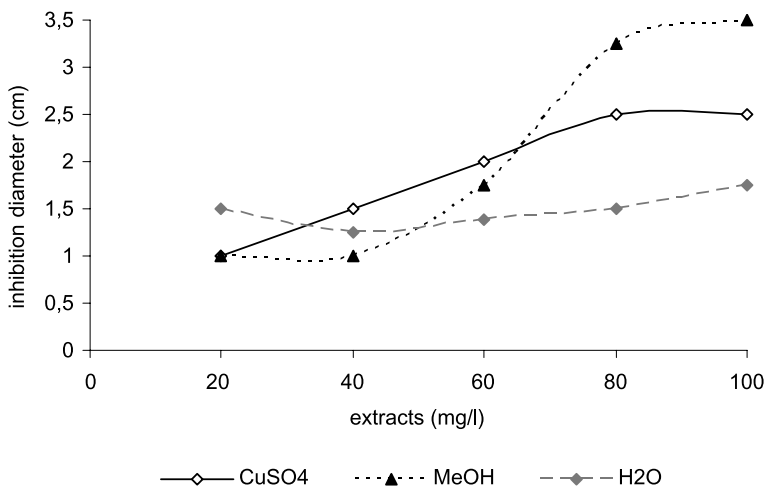


FIG. 2. Antialgal activity of the extracts of *Schoenoplectus lacustris*.

subcapitata. The alga was provided in immobilized algal beads by MicroBio Test (Nazareth, Belgium). The antialgal test was performed by deimmobilizing algae from the matrix with ethylene-bis(oxyethylenenitrilo)tetracetic acid (EGTA). *S. capricornutum* was cultivated on Bold basal medium (BBM) (Nichols, 1973). Cell density was measured with an electronic particle dual threshold counter (Coulter Counter Z2, 100 μm capillary; Instrumentation Laboratory, Miami, FL, USA). When the culture reached exponential growth phase, 1 ml (1.0×10^4 cells/ml) was poured onto a Petri dish with 20 ml of BBM containing 1.5% agar. The plates were incubated in a growth chamber at 25°C under continuous illumination (8000 lx). After 24 hr, a green layer of algae was evident on the agar, and subsequently a known quantity (0.5, 1.0, 2.0, and 3.0 μmol) of each chemical, dissolved in acetone or ethanol, was poured onto paper disks (sterile banks, Difco Bacto Concentration Disks, 3 mm). Each treatment was repeated with four replicates. After evaporation of the solvent, disks were placed on each Petri dish inoculated 24 hr before. Positive controls, for tests on single compounds were prepared by inoculating 0.5, 1.0, 2.0, and 3.0 μmol of algaecide CuSO_4 in 10 μl of Milli-Q water on the disk. A solvent control using acetone or ethanol on each disk was also prepared and placed on plates after evaporation (2 hr). The same procedure, with acetone or ethanol as solvent, was used for the extracts, and three independent experiments were performed in four replicates. The plates were incubated in a growth chamber for 96 hr at 25°C under continuous illumination (8000 lx). Chemical inhibition was

calculated by measuring the diameter of the no-growth zone excluding the paper disk (3 mm). The values obtained represent the average of three experiments. Phytotoxicity was compared with that of algaecide CuSO_4 .

Data Analysis. The statistical significance of differences between groups was determined by Student's *t*-test, calculating mean values for growth inhibition. The significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

Aliquots of hydroalcoholic and methanolic extracts of *S. lacustris* were dried under reduced pressure, and the residues were tested on *S. capricornutum* to evaluate the anti-algal effects. A common algaecide, CuSO_4 , was used as positive control. This compound shows a median effective concentration (EC_{50}) in standard algal growth inhibition test (ISO 8692) equal to 0.045 mg/l with confidence interval at 95% ranging from 0.032 to 0.070. In this study, high concentrations of CuSO_4 were used because no information existed on the activity of the investigated extracts. Our results, reported in Figure 2, showed that the MeOH extract had a no-growth zone 66% greater than that of algaecide CuSO_4 at the maximum concentration tested, but became inactive below 40 mg/l. The aqueous extract was less active than the positive control at the highest concentrations but remained active up to 20 mg/l.

The hydroalcoholic fraction was extracted first with methylene chloride and then with EtOAc to obtain crude residues CH_2Cl_2 and EtOAc. The MeOH extract was dissolved in water and shaken with EtOAc to obtain an organic fraction and an aqueous one. Chromatographic processes of the fractions (Schemes 1 and 2) led to the identification of 49 compounds (Figure 1).

Compounds 1–4 were identified as 4-hydroxybenzaldehyde, benzoic acid, protocatechuic acid, and methyl benzoate, respectively. Compound 5 was identified as methyl 4-acetyloxy-3-methoxybenzoate. Its EI-MS spectrum showed the molecular peak at $m/z = 224$. The ^1H NMR spectrum showed three aromatic protons of a 1,2,4-trisubstituted benzene, two methoxyls at $\delta = 3.87$ and 3.84 and a methyl at $\delta = 2.20$. These data, together with NOE observed between this latter methyl and the aromatic doublet, allowed an acetoxy group to be localized at the C-4 carbon and a methoxyl at C-3 carbon with a methyl benzoate structure. In compounds 6 and 7, ^1H NMR evidenced the ABX spin system of the vinyl group. The differences in their collected spectral data could be justified by the 3,4-dihydroxystyrene structure for 6 and 4-hydroxy-3-methoxystyrene for 7. Compound 8 was identified as 1-(3,4-dihydroxyphenyl)ethane, and compound 9 as 4-hydroxy-3-methoxyphenyl-1- O - β -D-glucopyranoside. ^1H NMR showed three aromatic protons as two doublets at $\delta = 6.82$ ($J = 2.1$ Hz) and 6.79 ($J = 8.2$ Hz)

and a double doublet at $\delta = 6.58$. The spectrum also indicated a methyl singlet at $\delta = 3.82$, a doublet at $\delta = 4.75$, and six protons ranging from 3.75 to 3.30 ppm. GC analysis of the compounds, after hydrolysis, reduction, and acetylation, showed the sugar moiety to be glucose. The coupling constant (7.4 Hz) of the anomeric proton indicated a β configuration at C-1'. NOE, between the H-1' proton at $\delta = 4.75$ and the H-2 and H-6 protons localized the sugar at the C-1 carbon of the aromatic ring. Compound **10** was identified as benzyl-1-*O*- α -L-arabinopyranoside. EI-MS spectrum showed the molecular ion at $m/z = 240$, and ^1H NMR revealed signals of five aromatic protons as well as two diastereotopic protons as two doublets ($J = 11.4$ Hz) at $\delta = 4.25$ and 4.67. NMR data of the glycidic moiety revealed the presence of a pentose, identified as arabinose by GC analysis of the alditol acetate derivative. Compound **11** showed a molecular formula $\text{C}_{15}\text{H}_{20}\text{O}_8$, according to EI-MS spectrum, indicating a molecular ion peak at $m/z = 328$. The ^1H NMR spectrum revealed three aromatic signals as a doublet integrated for two protons at $\delta = 8.02$, a triplet at $\delta = 7.59$, and a triplet integrated for two protons at $\delta = 7.48$. These data suggested the presence of a benzoyl unit in the molecule. In the aliphatic region of the spectrum, there were also a doublet at $\delta = 4.92$, two double doublets at $\delta = 4.34$ and 4.44, as well as eight protons in the range 4.20–3.40 ppm. ^{13}C NMR showed a carboxyl carbon at $\delta = 167.1$ and four signals in the aromatic range, two of them due to the homotopic C-2'/C-6' and C-3'/C5' carbons of the benzoyl group. Eight carbons, five methines, and three methylenes were also evident in the spectrum. In the HMBC experiment, carboxyl carbon showed correlations with the H-2'/H-6' and H-3'/H-5' protons. The same carbon was also correlated with the protons at $\delta = 4.34$ and 4.44, which result correlated with the carbon at $\delta = 67.4$ in the HSQC experiment. This carbon was in turn correlated with the proton at $\delta = 4.18$ bonded to the carbon at $\delta = 70.3$, which showed heterocorrelations with the doublet at $\delta = 4.92$, due to an anomeric proton, and with two methylene protons at $\delta = 3.56$ and 3.92 bonded to the carbon at $\delta = 71.2$. The remaining ^1H and ^{13}C NMR signals were consistent with the presence of a pentose in the molecule. GC analysis of alditol derivative allowed the sugar moiety to be identified as L-arabinose.

Compounds **12–22** were identified as *p*-coumaric acid (**12**), caffeic acid (**17**), ferulic acid (**18**), and synapic acid (**21**), their corresponding methyl esters **13**, **19**, **20**, and **22**, 4-methoxycinnamic acid (**14**), and glucoside *p*-coumaryl-4-*O*- β -D-glucopyranoside (**15**) and glucoside *p*-coumaryl-1-*O*- β -D-glucopyranoside (**16**). Compounds **12**, **17**, **18**, and **21** were reported as allelochemicals by Li et al. (1993). These phenols inhibited lettuce seedling growth and seed germination. Compounds **19** and **20** were isolated from *Cestrum parqui* (D'Abrosca et al., 2004a), and their phytotoxicity assayed on the seeds of lettuce, tomato, and onion. The glucoside of *p*-coumaric acid (compounds **15** and **16**) were isolated from Riesling wine and play an important role as antioxidant components of this white wine (Baderschneider and Winterhalter, 2001).

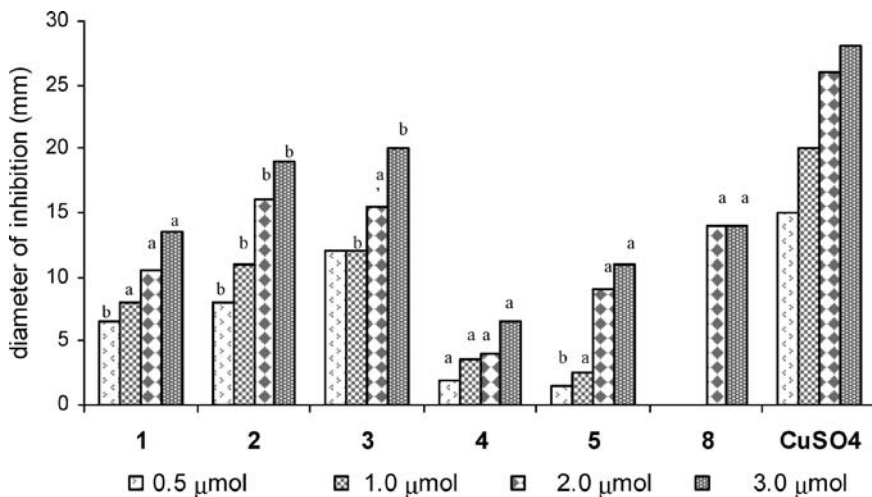


FIG. 3. Antialgal activity of phenols on *Selenastrum capricornutum*. Values are presented as diameter of no-growth zone (mm) for Student's *t*-test. (a) $P < 0.01$, (b) $0.01 < P < 0.05$.

Compounds **23–28** were identified as phenyl propionic acid derivatives. 3-(4-Hydroxyphenyl)propionic acid (**23**) and 3-(4-hydroxy-3-methoxyphenyl)propionic acid (**26**) were reported from *C. parqui* (D'Abrosca et al., 2004a). Compounds **24**, **25**, **27**, and **28** were characterized as methyl 4-methoxydihydrocinnamate, methyl 4-hydroxydihydrocinnamate, methyl dihydroferulate, and methyl dihydrocaffeate, respectively.

Compounds **29–39** were identified as flavonoids. Compound **29** was identified as chrysoeriol, the aglycone of compounds isolated from *Potamogeton* spp. (Boutard et al., 1973; Roberts and Haynes, 1986; Les and Sheridan, 1990). Compound **30** was identified as tricetin, an allelopathic flavone released from rice seedlings (Kong et al., 2004). Compounds **31–33** were identified as luteolin, kaempferol, and quercetin, respectively. Glycosides **34–36** were isolated from *Sambucus nigra* (D'Abrosca et al., 2001). Compound **37** showed spectral data corresponding to catechin (Nahrstedt et al., 1987). It showed an $[\alpha]_D$ value (c, 0.16) -14.8 and a positive Cotton effect $\Delta\epsilon_{280\text{ nm}} +3.2$ nm, in accordance with the presence of (–)-catechin (Bais et al., 2003a). Calcones **38** and **39** were identified on the basis of their spectral data. $^1\text{H NMR}$ of **38** showed two protons as doublet of a *trans* double bond at $\delta = 6.27$ and 7.59 , an α,β unsaturated carbonyl group and four doublets of two aromatic system at $\delta = 7.44$, 7.16 , 6.80 , and 6.73 . EI-MS spectrum confirmed the proposed structure showing the molecular ion at $m/z = 240$ and fragments at $m/z = 147$ $[\text{CO}-\text{CH}-\text{CH}-\text{C}_6\text{H}_4\text{OH}]^+$, 119 $[\text{CH}-\text{CH}-\text{C}_6\text{H}_4\text{OH}]^+$, 121 $[\text{CO}-\text{C}_6\text{H}_4\text{OH}]^+$, and 93

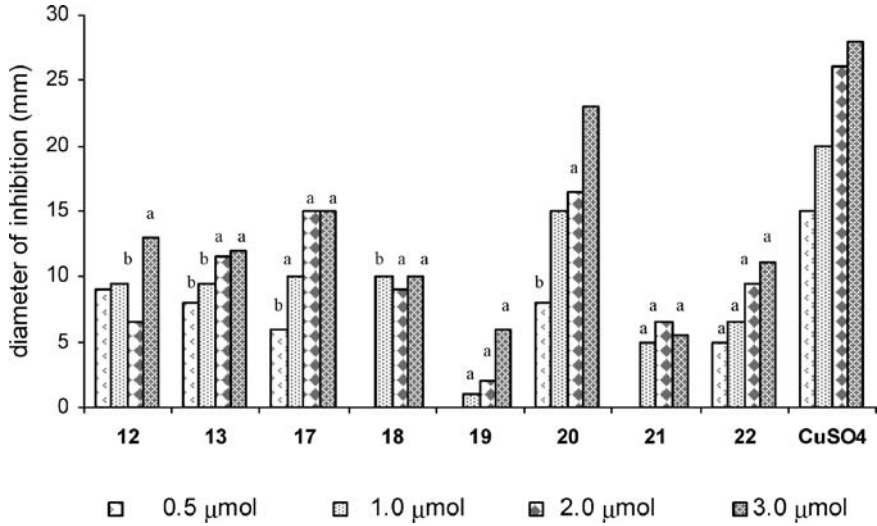


FIG. 4. Antialgal activity of cinnamic acids derivatives on *S. capricornutum*. Values are presented as diameter of no-growth zone (mm) for Student's *t*-test. (a) $P < 0.01$, (b) $0.01 < P < 0.05$.

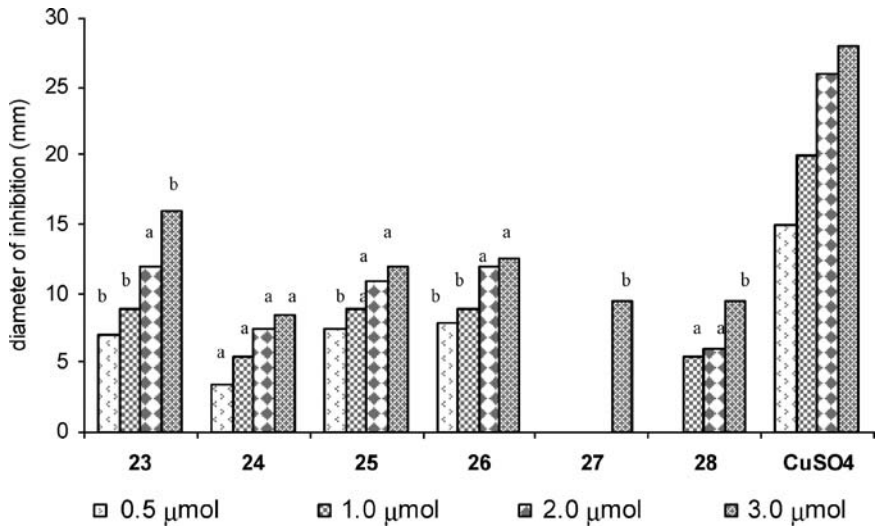


FIG. 5. Antialgal activity of dihydrocinnamic acids derivatives on *S. capricornutum*. Values are presented as diameter of no-growth zone (mm) for Student's *t*-test. (a) $P < 0.01$, (b) $0.01 < P < 0.05$.

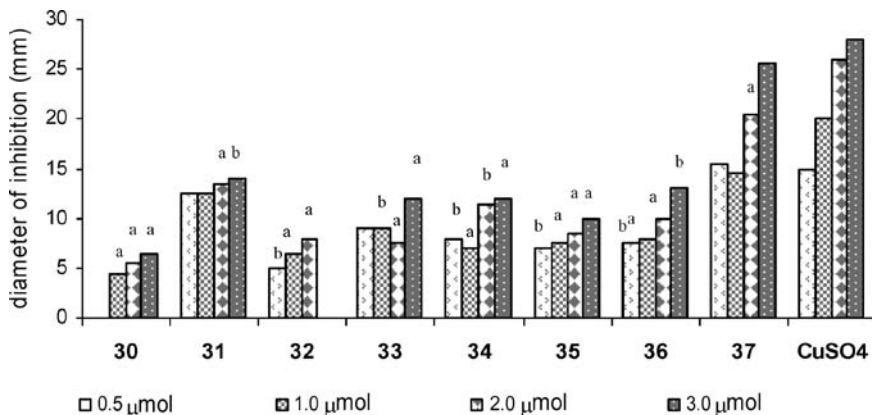


FIG. 6. Antialgal activity of flavonoids on *S. capricornutum*. Values are presented as diameter of no-growth zone (mm) for Student's *t*-test. (a) $P < 0.01$, (b) $0.01 < P < 0.05$.

$[\text{C}_6\text{H}_4\text{OH}]^+$. ^1H NMR of compound **39** showed two doublets of the *trans* double bond at $\delta = 7.32$ and 6.33 , two methoxyl groups as a singlet at $\delta = 3.88$, and two 1,2,4-trisubstituted aromatic rings at $\delta = 7.58$ (d, $J = 2.0$ Hz), 7.48 (dd, $J = 8.5$ and 2.0 Hz), 7.11 (d, $J = 2.0$ Hz), 6.97 (dd, $J = 8.5$ and 2.0 Hz), 6.76 (d, $J = 8.5$ Hz), and 6.74 (d, $J = 8.5$ Hz). EI-MS spectrum showed the molecular ion at $m/z = 300$ and fragments at $m/z = 177$ $[\text{CO}-\text{CH}-\text{CH}-\text{C}_6\text{H}_3(\text{OCH}_3)\text{OH}]^+$, 149 $[\text{CH}-\text{CH}-\text{C}_6\text{H}_3(\text{OCH}_3)\text{OH}]^+$, 151 $[\text{CO}-\text{C}_6\text{H}_3(\text{OCH}_3)\text{OH}]^+$, and 123 $[\text{C}_6\text{H}_3(\text{OCH}_3)\text{OH}]^+$.

Compounds **40–49** were identified as C_{13} *nor*-isoprenoids. Compounds **40–42** and **44–45** were isolated and characterized from *C. parqui* (D'Abrosca et al., 2004b), whereas compounds **46**, **47**, and **49** were isolated from *Chenopodium album* (DellaGreca et al., 2004). Compound **43** was identified as the aglycone of some glucoside isolated from *Acanthus ebracteatus* (Kanchanapoom et al., 2001).

All compounds were tested on the green alga *S. capricornutum* to assay their antialgal effect. This organism is commonly present in aquatic systems and has been selected as bioindicator of the eutrophic sites. The test was conducted on agar using a modified antibiotic assay method. The phytotoxicity results are summarized in Figures 3–6, where only the active compounds are reported.

The results of the active low-molecular phenols **1–11**, which are reported in Figure 2, show a correspondence between dose and activity. The antialgal effect for compounds **1–3** increased with their polarity. The most active was protocatechuic acid **3**. The activity should be correlated with the hydrophilicity and/or acidity: the antialgal effect of the methyl ester of **1** (compound **4**) was

about 50% of the activity of the corresponding acid. Compound **5**, which has all its hydroxyl groups derivatized, was less phytotoxic in respect to the more polar compounds **1–3**. Compound **8** only showed a moderate antialgal effect for the highest doses used (2 and 3 μmol).

Figure 3 reports the results of the cinnamic acid derivatives. The most active compound was methyl ferulate **20**. For ferulic and synapic acids, esterification of the carboxyl group increased activity (**18** vs. **20** and **21** vs. **22**). *p*-Coumaric acid and its methyl derivative **13** showed a similar toxicity, whereas methyl caffeate was over 50% less active than caffeic acid (**19** vs. **17**). The most active of the dihydrocinnamic acid derivatives (Figure 4) was 3-(4-hydroxyphenyl)propionic acid (**23**); the introduction of another hydroxylated function in the molecule reduced activity (cf. **26** and **28**). Methylation also made the molecule less active with the carboxymethyl group in compound **25**, and with the carboxy and methoxy groups in compound **24**. Hydrogenation of the double bond of cinnamic acid increased correspondence between dose and activity and had a slow effect on toxicity (**12** vs. **23** and **18** vs. **26**). Hydrogenation of methyl ester **20** reduced the activity considerably, whereas methyl dihydroferulate **27** was less phytotoxic in respect to methyl ferulate **19**.

Results of bioassay with the flavonoids are reported in Figure 5. The presence of the catechol ring made the compound especially active (**31**, **33**, **34**, **36**, **37**). Glycosylation had little effect on activity, and the presence of the hydrophobic groups (methoxyls) decreased activity. The most active compound was (–)-catechin **37**, showing a no-growth zone similar to that of algacide CuSO_4 . C_{13} *nor*-isoprenoids were inactive at all quantities tested. Only **42**, **44**, and **45** metabolites showed slight no-growth zones of 3.0-, 4.2-, and 2.5-mm diameters, respectively, with 3.0 μmol of chemicals.

Among the constituents of *S. lacustris*, (–)-catechin **37** was the most active compound. The no-growth zone showed diameters similar to those of CuSO_4 , with an anomalous brown coloration probably as result of a photooxidation reaction that caused blocking of photosystem activity. The production of the reactive species (i.e., singlet oxygen) and the absence of photoprotection could cause the degradation of the photosynthetic pigments. The toxicity of (–)-catechin seemed to inhibit the photorespiration phenomenon. These results were in accordance with Bais et al. (2003b), showing that allelopathic effects of the weed *Centaurea maculosa* are attributable to a racemic mixture of (\pm)-catechin. They demonstrated that phytotoxic activity was due to (–)-catechin, whereas (+)-catechin had an antibiotic activity (Bais et al., 2002).

The natural abundance of *S. lacustris* along our rivers could optimize its use. In fact, the chemical characterization of the algal inhibitory component of the plant could provide a reliable method for controlling algal growth in eutrophic sites. Among the isolated metabolites, low-molecular phenolic compounds are toxic to algae and show a superior inhibitory activity on algal growth.

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ACTIVATED CHEMICAL DEFENSE IN *Aplysina* SPONGES REVISITED

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Abstract—Sponges of the genus *Aplysina* accumulate brominated isoxazoline alkaloids in concentrations that sometimes exceed 10% of their dry weight. We previously reported a decrease in concentrations of these compounds and a concomitant increase in concentrations of the monocyclic nitrogenous compounds aeroplysinin-1 and dienone in *Aplysina aerophoba* following injury of the sponge tissue. Further investigations indicated a wound-induced enzymatic cleavage of the former compounds into the latter, and demonstrated that these reactions also occur in other *Aplysina* sponges. A recent study on Caribbean *Aplysina* species, however, introduced doubt regarding the presence of a wound-induced bioconversion in sponges of this genus. This discrepancy motivated us to reinvestigate carefully the fate of brominated alkaloids in *A. aerophoba* and in other *Aplysina* sponges following mechanical injury. As a result of this study we conclude that (1) tissue damage induces a bioconversion of isoxazoline alkaloids into aeroplysinin-1 and dienone in *Aplysina* sponges, (2) this reaction is likely catalyzed by enzymes, and (3) it may be ecologically relevant as the bioconversion products possibly protect the wounded sponge tissue from invasion of bacterial pathogens.

Key Words—Wound-induced bioconversion, chemical defense, biotransformation, brominated alkaloids, enzymatic cleavage, marine sponge.

INTRODUCTION

Sessile organisms are dependent on protective mechanisms other than flight or active defense. Depending on the predictability of the influencing stress factors

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they are exposed to, they follow different strategies. In cases of predictable or constant stress factors, constitutive defense mechanisms (e.g., shells and stings or the constitutive accumulation of protective metabolites) have usually evolved (Hay and Fenical, 1988). However, if the disturbing impacts display a high spatial or temporal variability, facultative defense mechanisms have often developed instead (Adler and Harvell, 1990; Harvell, 1990). The latter can be divided into two different forms: induced defenses and activated defenses.

“Induced defenses” such as induced defense metabolite biosynthesis (Havel, 1986; Roda and Baldwin, 2003) or induced morphological changes (Steneck and Adey, 1976; Lewis et al., 1987; Lurling, 2003) are slow. Inducing factors include predation or attack by harmful microorganisms. After the initial attack, the priorities of resource allocation in the attacked organism may be shifted from growth to defense. Sometimes it takes weeks until a protective effect occurs (Hammerstrom et al., 1998; Taylor et al., 2002). Thus, this type of mechanism can not be expected to provide immediate protection, but rather prepares the harmed organism for future attacks.

A faster response to stress factors is the attack-induced transformation of inactive precursor compounds stored in the tissue of the attacked organism, thereby yielding transformation products that exhibit a pronounced defensive activity. Paul and van Alstyne (1992) described this phenomenon as “activated defense” to distinguish it from the slower “induced defense.” Such mechanisms are fast (often occurring within seconds after wounding) in order to facilitate immediate protection against the affecting organism. These reactions are usually catalyzed by enzymes that convert inactive storage compounds into defensive products (Paul and van Alstyne, 1992; Hickel et al., 1996).

In the terrestrial environment, activated defense reactions involving enzymatic transformations of inactive precursors are frequently found in plants. Examples include cyanogenic glycosides that are transformed into highly toxic HCN (Conn, 1979; Wajant and Effenberger, 1996; Gleadow and Woodrow, 2002), or as in Brassicaceae and related plants, glucosinolates that are cleaved to form thiocyanates, isothiocyanate, or isonitriles (Stoewsand, 1995; Fahey et al., 2001). In all reported examples, the respective precursors are physically separated by compartmentalization from the biotransformation enzymes. Upon mechanical damage, the compartments are destroyed, which facilitates the formation of the protective substances as the enzymes and substrates make contact.

In the marine environment, examples of activated chemical defense mechanisms are also found. In green algae, *Halimeda*, tissue damage leads to the transformation of the diterpene halimeditetraacetate to the aldehyde halimedatrial, which is a more potent toxin compared to the precursor (Paul and van Alstyne, 1992). The green alga *Caulerpa taxifolia* accumulates caulerpenyne. After wounding of the algal tissue, it is deacetylated forming a

group of aldehydes, mainly oxytoxins 1 and 2 (Jung and Pohnert, 2001). Presumably, the transformation products are more potent defensive compounds than the precursor. It has been suggested that the rapid biotransformation of caulerpenyne is catalyzed by an esterase (Jung et al., 2002). A third example is the cleavage of dimethylsulfonylpropionate (DMSP) to acrylic acid and dimethylsulfide (DMS) by the enzyme DMSP lyase that was recently observed in macroalgae (van Alstyne et al., 2001; van Alstyne and Houser, 2003). This reaction was previously described in unicellular phytoplankton (Wolfe and Steinke, 1996; Wolfe et al., 1997).

Numerous marine invertebrates face ecological situations that are similar to those in marine or terrestrial plants. Ascidians, sponges, corals, and bryozoans are sessile and unable to protect themselves actively by flight or attack. They have evolved defense mechanisms similar to those of plants. Marine sponges have been particularly well investigated with regard to putative defense metabolites. Within the last 30 yr, over 4000 secondary metabolites have been isolated and described, many of which are biologically active (MarinLit, 2003). In various cases, these metabolites protect invertebrates against predators, competitors for settlement space, or against microbial pathogens (Green, 1977; Bakus et al., 1986; Becerro et al., 1997; McClintock et al., 1997; Thacker et al., 1998; Engel et al., 2002).

Induced chemical defense reactions have also been reported for sponges. Recent examples are *Suberites domuncula* and *Agelas conifera*. In *S. domuncula*, the level of two lyso-PAF (platelet-activating factor) congeners is increased upon exposure to an endotoxin derived from the outer cell wall of gram-negative bacteria (Müller et al. 2004). Lyso-PAF and analogues possess strong antimicrobial activity (Steel et al., 2002) and inhibit the growth of phytopathogenic fungi (Tanaka et al., 1997). In *A. conifera*, the concentrations of the antimicrobial and feeding-deterrent compounds sceptrin and oroidin increase 3- to 4-fold within 0.5–6 d following wounding of the sponge tissue (Richelle-Maurer et al., 2003).

An example for an activated chemical defense mechanism in sponges has been reported for *Aplysina aerophoba*. This sponge, like other *Aplysina* species, accumulates brominated isoxazoline alkaloids in concentrations of up to 10% dry weight (Albrizio et al., 1994; Aiello et al., 1995; Ciminiello et al., 1994a,b, 1995, 1996a,b, 1997, 1999, 2000; Thoms et al., 2003a). X-ray microanalysis revealed that these brominated metabolites are mainly stored in specialized sponge cells called spherulous cells (Turon et al., 2000). Teeyapant and Proksch (1993) reported the cleavage of these isoxazoline alkaloids in tissue of *A. aerophoba* when freeze-dried sponge material was extracted with water or with aqueous MeOH. During this reaction, the monocyclic nitrogenous compounds aeroplysinin-1 (**5**) and dienone (**6**) were formed (Figure 1). By extraction of freeze-dried tissue with 100% MeOH, however, only putative isoxazoline

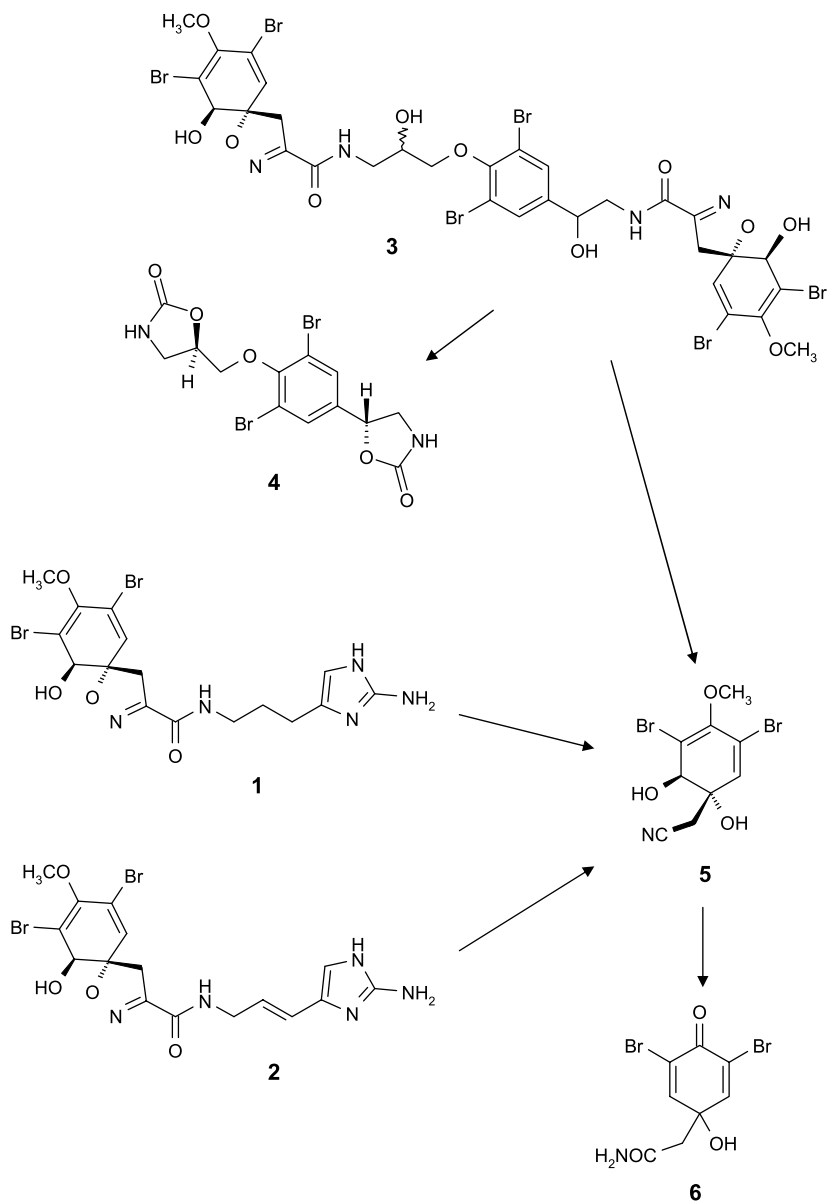


FIG. 1. Wound-induced bioconversion of the brominated isoxazoline alkaloids aerophobin-2 (1), aplysinamisin-1 (2), and isofistularin-3 (3) to aeroplysinin-1 (5) and the dienone (6) in tissue of *Aplysina aerophoba*. When isofistularin-3 (3) is used as a substrate for the reaction, the bisoxazolidinone derivative (4) is recovered as a further product.

precursors, such as isofistularin-3 (**3**) or aerophobin-2 (**1**), were recovered. Subsequently, Ebel et al. (1997) reported that the bioconversion of the isoxazoline precursors into aeroplysinin-1 (**5**) and dienone (**6**) also takes place when fresh sponge tissue is crushed mechanically in the presence of seawater. Additional experiments with cell-free extracts from *A. aerophoba* and from six other *Aplysina* species collected in the Caribbean and in the Mediterranean Sea revealed that all *Aplysina* species had the capability to cleave isoxazoline alkaloids, thereby generating aeroplysinin-1 (**5**) (Ebel et al., 1997). This led to the conclusion that the observed cleavage of the isoxazoline precursors is catalyzed by enzymes present in *Aplysina* sponges. It was concluded that the cleavage reactions consist of at least two different steps: in a first step, degradation of the isoxazoline alkaloids gives rise to the β -hydroxynitrile aeroplysinin-1 (**5**), while in a second, this intermediate is converted into the dienone (**6**) via enolether hydrolysis and partial hydrolysis of the nitrile group (Teeyapant and Proksch, 1993; Goldenstein et al., 2000).

Bioactivity studies revealed that bioconversion of the isoxazoline alkaloids in *Aplysina* sponges is paralleled by an increase in antibiotic and cytotoxic activity (Teeyapant et al., 1993; Weiss et al., 1996). In contrast, feeding deterrent activity of the bioconversion products against the Mediterranean fish species *Blennius sphinx* is significantly weaker than observed for their isoxazoline precursors (Thoms et al., 2004).

Recently, Puyana et al. (2003) investigated the Caribbean sponges *A. insularis* and *A. archeri* with regard to a possible biotransformation of isoxazoline alkaloids following mechanical injury, which was simulated by stabbing the sponges with a scalpel. In this study, the authors came to the conclusion, that a conversion of the isoxazoline alkaloids into aeroplysinin-1 (**5**) and dienone (**6**) as reported for *A. aerophoba* was not apparent in the Caribbean *Aplysina* species, and they assumed that past observations of biotransformation reactions within the genus *Aplysina* were the results of either differential tissue extraction efficiency, hydrolysis from insoluble precursors, or a heterogeneous distribution of metabolites in sponge tissue. These contradictory results motivated us to reinvestigate the fate of brominated alkaloids in *Aplysina* sponges following mechanical injury.

METHODS AND MATERIALS

Sponges of the genus *Aplysina* (Demospongiae, Verongiida, Aplysinidae) frequently occur in the Mediterranean as well as in the Caribbean Sea. From both geographical regions, two representatives each were obtained. Specimens of the sponge *A. aerophoba* were collected by scuba diving and snorkeling at

depths between 3 and 10 m at Banyuls-sur-mer, France, in April 2002 and at Rovinj, Croatia, in August 2003. *A. cavernicola* was collected in May 2001 at Elba, Italy, whereas *A. fistularis* and *A. archeri* were from previous collections in the Bahamas in 1995. The Mediterranean sponge *Crambe crambe* (Demospongiae, Poecilosclerida, Myxillidae) was collected off the coast of Rovinj, Croatia, in August 2003. All sponges were carefully detached from the substratum with a knife to avoid tissue damage.

During sampling, specimens were kept submerged in ziplock bags filled with seawater for transport to the laboratory. All experiments with fresh, living sponge tissue were performed with *A. aerophoba* collected at Rovinj, Croatia. These specimens were directly used. For experiments involving lyophilized tissue, specimens were flash frozen and kept at temperatures below -80°C until freeze-drying and homogenization of the lyophilized tissue.

Wounding Intensity Series. Six tissue slices, each comprising a volume of 8 ml (thickness, 1 cm) were cut from one *A. aerophoba* individual with a scalpel. After the respective treatments, all samples were placed in 15-ml cryo tubes and flash frozen in liquid nitrogen.

Sample “t₀” was flash frozen after it was excised from the living sponge. Sample “b” was repeatedly stabbed with a scalpel over its entire surface for a total of 15 sec. After an additional period of 4 min and 45 sec, during which no further treatment was applied, the sample was flash frozen. Sample “c” was ground in a mortar for 15 sec instead. After an additional 4 min and 45 sec (no further treatment), it was flash frozen. Sample “d” was ground for a total of 5 min and immediately flash frozen. Sample “e” was treated like sample “d” with the exception that 1.5 ml EtOH were immediately added to the fresh tissue prior to grinding.

The same series of treatments was repeated four times, each time with samples that had been cut from a different *A. aerophoba* individual. Additional slices of two *A. aerophoba* individuals (samples “a”) were placed in a mortar without further treatment for 5 min followed by flash freezing.

Prior to HPLC analysis, all samples were lyophilized, ground to a fine powder, and exhaustively extracted with MeOH. Extracts were then injected into an HPLC system coupled to a photodiode array detector (Dionex, Germany). Routine detection was at 254 and 280 nm. The separation column (125 × 4 mm i.d.) was prefilled with Eurosphere C-18 (5 μm) (Knauer, Germany). Compounds were identified by their online UV spectra and by direct comparison with previously isolated standards (Ebel et al., 1997). Identification of compounds in extracts was further verified by LC-MS analysis using a diode array equipped HPLC system (Agilent 1100 Series; Agilent Technologies, Waldbronn, Germany) coupled to an electrospray ionization (ESI) Mass Spectrometer (LC-Q Deca; Finnigan, Bremen, Germany). Separation was achieved using a Knauer Eurospher C-18 column (5 μm, 2 mm diam × 250 mm long).

The amount of each individual compound in the samples was calculated from the detector response in HPLC-UV and the compound-specific molar extinction coefficients. Based on these data, proportions of the respective brominated alkaloids in the samples were expressed as percentage of the total content of brominated alkaloids (set at 100%) present in 1 g lyophilized sponge tissue.

A control experiment was performed with fresh tissue of the sponge *C. crambe* that had been collected at Rovinj. Three samples of the sponge tissue were spiked with one of the *A. aerophoba* metabolites aeroplysinin-1 (**5**), aerophobin-2 (**1**), or isofistularin-3 (**3**), each at concentrations within the range of their natural abundance in *A. aerophoba* (aeroplysinin-1 as found in damaged sponge tissue). To a fourth *C. crambe* tissue piece, no *A. aerophoba* metabolites were added. Samples were ground for 5 min and subsequently flash frozen. After lyophilization, samples were extracted and analyzed as described above.

Time Course Experiment Fresh tissue slices of *A. aerophoba* of the same volume as described in the experiment above (8 ml) were ground for 15 sec in a mortar. Over a total period of 165 sec, measured from the onset of grinding, aliquots were collected from the ground material and immediately frozen in liquid nitrogen. Following lyophilization, they were extracted and analyzed using HPLC-UV and LC-MS under the conditions described above. All samples comprising one time series resulted from one *A. aerophoba* individual. The four parallel experiments were performed with one individual each.

Experiments with Lyophilized Animal Tissue Experiments were performed with tissue of the two Mediterranean sponges, *A. aerophoba* and *A. cavernicola*, and the Caribbean sponges *A. fistularis* and *A. archeri*. For each species, lyophilized tissue from three different individuals was used. Tissue was ground and homogenized. Subsequently, two portions of 100 mg were sampled. One of these was used for exhaustive extraction with MeOH. The other was mixed with 600 μ l seawater, kept therein overnight, and was subsequently lyophilized again. After extraction with MeOH, all samples were analyzed as described above to determine the molar concentrations of the brominated alkaloids.

Additionally, lyophilized and ground tissue of *A. archeri* was spiked with aerophobin-2 (**1**) isolated from *A. aerophoba* in a concentration found naturally in the latter. The mixture was incubated in 600 μ l seawater and subsequently lyophilized, extracted, and analyzed as described above.

Control experiments were performed with lyophilized and ground tissue of the marine opisthobranch *Tyrodina perversa* and the sponge *C. crambe*. Mantle tissue of *T. perversa* naturally contains *Aplysina* alkaloids (Ebel et al., 1999; Thoms et al., 2003b). The opisthobranch tissue was incubated in seawater, lyophilized again, and subsequently extracted and analyzed as described for the lyophilized *Aplysina* sponge samples. Tissue of the sponge *C. crambe* was treated likewise, but prior to incubation in seawater was mixed with aerophobin-2 (**1**) and isofistularin-3 (**3**) in concentrations as found in *A. aerophoba*.

Data Analyses. Data of the wounding intensity series and the time course experiment were analyzed by randomized block analysis of variance, and multiple comparisons were made with the Tukey–Kramer test for unequal sample sizes (Zar, 1999). Percentage data were arcsine-transformed prior to statistical analyses. In case of missing data in the randomized block design, data were estimated iteratively, and the bias in the group sum of squares (group SS) as well as the total degrees of freedom (*df*) was corrected according to Li (1964) and Glen and Kramer (1958).

For analysis of variations in the relative concentration of bioconversion products in lyophilized *Aplysina* sponge tissue upon treatment with seawater, the percentage data were arcsine-transformed and subsequently analyzed using a two-tailed paired *t*-test (Zar, 1999).

RESULTS

Constitutive Alkaloid Patterns in Intact A. aerophoba Individuals Obtained from Croatia and France. The isoxazoline alkaloids aerophobin-2 (**1**) and isofistularin-3 (**3**) were the dominant compounds in individuals collected at Rovinj, Croatia (Table 1A). Aeroplysinin-1 (**5**) was present only at lower concentrations. Other isoxazoline alkaloids that are known for *A. aerophoba* (e.g., purealidines and aerophobin-1) were detected but not quantified, as they occurred only in negligible concentrations. Dienone (**6**) and aplysinamisin-1 (**2**) were not detected. In contrast, in individuals of *A. aerophoba* collected at the French coast at Banyuls, aplysinamisin-1 (**2**) was dominant, followed by aerophobin-2 (**1**) and isofistularin-3 (**3**) (Table 1B). Neither aeroplysinin-1 (**5**) nor dienone (**6**) was detected in these sponges. The sponge zochrome uranidine

TABLE 1A. CONTENTS AND RELATIVE CONCENTRATIONS OF BROMINATED ALKALOIDS IN FRESH TISSUE OF *Aplysina aerophoba* COLLECTED AT THE MEDITERRANEAN COAST OF ROVINJ, CROATIA

Alkaloid	Content in 1 g dry weight (μmol)	Content in 1 ml fresh tissue (μmol)	Alkaloid pattern in percentages (%)
Aerophobin-2 (1)	134.4 \pm 29.6	28.0 \pm 6.2	68.9 \pm 4.4
Isofistularin-3 (3)	48.5 \pm 9.0	10.1 \pm 1.9	25.0 \pm 2.0
Aplysinamisin-1 (2)	n.d.	n.d.	0
Aeroplysinin-1 (5)	11.7 \pm 8.2	2.4 \pm 1.7	6.1 \pm 4.2
Dienone (6)	n.d.	n.d.	0
Total	194.5 \pm 37.9	40.5 \pm 7.9	

N = 10; n.d. = not detected in the sample by HPLC-UV.

TABLE 1B. CONTENTS AND RELATIVE CONCENTRATIONS OF BROMINATED ALKALOIDS IN FRESH TISSUE OF *Aplysina aerophoba* COLLECTED AT THE MEDITERRANEAN COAST OF BANYULS-SUR-MER, FRANCE

Alkaloid	Content in 1 g dry weight (μmol)	Content in 1 ml fresh tissue (μmol)	Alkaloid pattern in percentages (%)
Aerophobin-2 (1)	29.2 \pm 6.6	6.1 \pm 1.4	25.5 \pm 3.3
Isofistularin-3 (3)	18.5 \pm 5.2	3.9 \pm 1.1	16.0 \pm 2.6
Aplysinamisin-1 (2)	67.2 \pm 17.9	14.0 \pm 3.7	58.4 \pm 5.7
Aeropylsinin-1 (5)	n.d.	n.d.	0
Dienone (6)	n.d.	n.d.	0
Total	114.8 \pm 26.7	23.9 \pm 5.6	

N = 3; n.d. = not detected in the sample by HPLC-UV.

was abundantly present in all analyzed sponge individuals, but due to its instability upon contact with air it could not be reliably quantified.

The overall concentration of brominated alkaloids was considerably lower in the *A. aerophoba* samples from France (Table 1B) than in the specimens from Croatia (Table 1A). Consistent with previous studies on other *Aplysina* species (Puyana et al., 2003), the absolute amounts of the various alkaloids detected in individuals of *A. aerophoba* (expressed as micromoles per gram dried tissue or as micromoles per ml volume of sponge tissue) varied considerably, whereas the relative proportions (in % compared to the total amount of alkaloids that was set at 100%) of the individual alkaloids turned out to be remarkably consistent for sponge specimens collected in France as well as in Croatia (Table 1A, B).

Wounding Intensity Series. The intensity of artificial damage to tissue taken from freshly collected *A. aerophoba* individuals was gradually increased by applying different methods of tissue destruction (stabbing with scalpel, grinding in mortar, addition of organic solvent plus grinding). This approach revealed an unequivocal correlation between increase in tissue destruction (wounding intensity), gradual disappearance of isoxazoline alkaloids, and concomitant increase in aeropylsinin-1 (5) content in injured vs. intact *A. aerophoba* tissue (Figure 2 and Table 2).

The possible effect of air exposure (without accessory wounding) on the metabolite pattern in tissue slices was tested (samples "a" in Figure 2 and Table 2). For this purpose, freshly cut slices of *A. aerophoba* were kept exposed to air in a mortar for 5 min before they were flash frozen. The brominated alkaloid composition in this tissue remained largely unchanged by this treatment, when compared to controls ("t₀" samples) that were flash frozen immediately after having been cut from the living sponge.

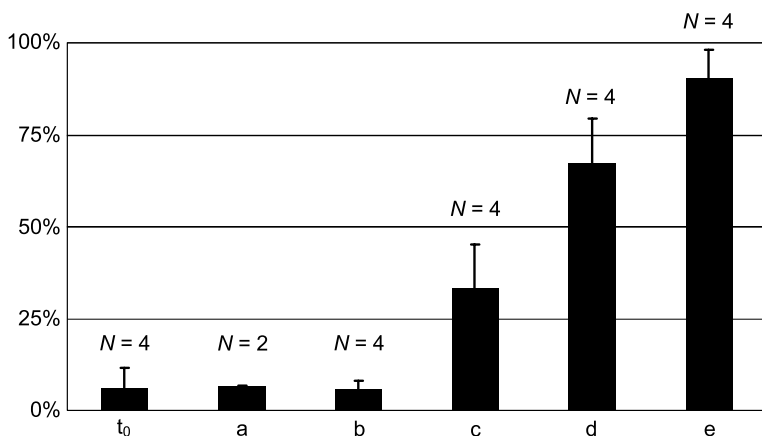


FIG. 2. Variations in the relative concentration of aeroplysinin-1 (**5**) in *A. aerophoba* tissue upon different intensities of wounding. Aeroplysinin-1 proportion is given in percent relative to the total brominated alkaloid content (set at 100%) in the sponge. For explanation of t_0 , a, b, c, d, and e, refer to Table 2. Data were arcsine-transformed prior to statistical analysis. Randomized block analysis of variance: $F = 21.13$, $P < 0.001$. Tukey–Kramer multiple comparisons (significant differences are indicated, only): $d \neq t_0$, $d \neq a$, $d \neq b$, $e \neq t_0$, $e \neq a$, $e \neq b$, $e \neq c$.

Stabbing of the sponge tissue with a scalpel over a period of 15 sec followed by subsequent exposure to air for 4 min 45 sec similarly had no apparent effect on the alkaloid pattern (samples *b*). However, the pronounced increase in tissue destruction caused by grinding the tissue in a mortar for 15 sec substantially changed the metabolite composition (samples *c*). By this treatment, a heterogeneous mixture of different-sized tissue pieces was obtained. As a result, aeroplysinin-1 (**5**) abundance in the sponge tissue increased more than 3-fold when compared to samples “a” or “b” (Table 2), whereas the concentrations of aerophobin-2 (**1**) and isofistularin-3 (**3**) decreased markedly.

When the grinding time of the sponge tissue was extended to 5 min (sample “d”), the tissue was more effectively crushed than observed for sample “c,” yielding a rather homogenous mixture of cellular debris. This resulted in a pronounced increase in the abundance of aeroplysinin-1 (**5**) (compared, for example, to sample “c”), which now accounted for almost two-thirds of all brominated alkaloids detected (Figure 2). In sample “e,” which had been subjected to grinding for 5 min in the presence of 16% EtOH (v/v), aeroplysinin-1 accounted for almost 90% of all brominated metabolites (Figure 2). In samples “c,” “d,” and “e,” the rise in concentration of aeroplysinin-1 was

TABLE 2. VARIATIONS IN THE ABSOLUTE CONCENTRATIONS OF BROMINATED ALKALOIDS IN CRUDE EXTRACTS OF *Aplysina aerophoba* AFTER DIFFERENT INTENSITIES OF WOUNDING

	t ₀ (N = 4)	a (N = 2)	b (N = 4)	c (N = 4)	d (N = 4)	e (N = 4)
Aerophobin-2 (1)	136.0 ± 36.4	105.0 ± 14.3	141.8 ± 33.5	53.3 ± 25.9	15.5 ± 3.5	2.7 ± 1.8
Isofistularin-3 (2)	47.2 ± 13.1	36.2 ± 3.5	48.8 ± 10.2	25.1 ± 12.6	11.5 ± 2.3	6.1 ± 4.1
Aerophysinin-1 (5)	11.8 ± 9.2	10.2 ± 1.1	11.9 ± 5.4	37.8 ± 14.4	62.3 ± 28.8	107.6 ± 54.4
Dienone (6)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	195.0 ± 49.5	151.4 ± 15.4	202.5 ± 46.8	116.1 ± 45.6	89.3 ± 30.2	116.4 ± 51.3

Quantities are expressed in μmol of compound per gram dry weight of sponge tissue ± SD. n.d. = not detected in the sample by HPLC-UV.

t₀ Controls that were immediately flash frozen.

a Controls that were kept exposed to air for 5 min before flash freezing.

b Tissue pieces that were stabbed over 15 sec with a scalpel and were then subsequently left untreated for another 4 min 45 sec before flash freezing.

c Tissue pieces that were ground over 15 sec and were subsequently left untreated for another 4 min 45 sec before flash freezing.

d Tissue pieces that were ground over 5 min.

e Tissue pieces that were ground over 5 min after 16% (v/v) EtOH had been added.

Randomized block analysis of variance: aerophobin-2: $F = 24.68, P < 0.001$; isofistularin-3: $F = 15.21, P < 0.001$; aerophysinin-1: $F = 7.98, P < 0.001$.

Tukey-Kramer multiple comparisons (significant differences are indicated, only): aerophobin-2: c ≠ t₀, c ≠ b, d ≠ t₀, d ≠ a, d ≠ b, e ≠ t₀, e ≠ a, e ≠ b; isofistularin-3: c ≠ t₀, c ≠ b, d ≠ t₀, d ≠ b, e ≠ t₀, e ≠ a, e ≠ b, e ≠ c.

paralleled by an equally impressive decrease in the concentrations of isofistularin-3 (**3**) or of aerophobin-2 (**1**) (Table 2). Dienone (**6**) was not detected in any of the samples.

A control experiment was performed with the Mediterranean sponge *C. crambe* that contains no brominated alkaloids. Aeroplysinin-1 (**5**), aerophobin-2 (**1**), and isofistularin-3 (**3**) isolated from *A. aerophoba* were added to fresh tissue of *C. crambe*. The concentrations remained unchanged (recovery rates of 97–98%) after they were ground with *C. crambe* tissue over 5 min (similar treatment to sample “d” in the experiment with *A. aerophoba*; Table 2) (Figure 3). Neither aeroplysinin-1 (**5**) nor dienone (**6**) was detected in the ground samples that had been mixed with aerophobin-2 (**1**) and isofistularin-3 (**3**). In the ground sample that had been mixed with aeroplysinin-1 (**5**), no dienone (**6**) was detected. The metabolite composition of *C. crambe* also remained unaffected by the treatment.

Time Course Experiment. Grinding of fresh *A. aerophoba* tissue for 15 sec and subsequent collection of samples at set time intervals revealed a gradual and time-dependent increase in the relative proportions of aeroplysinin-1 (**5**) from

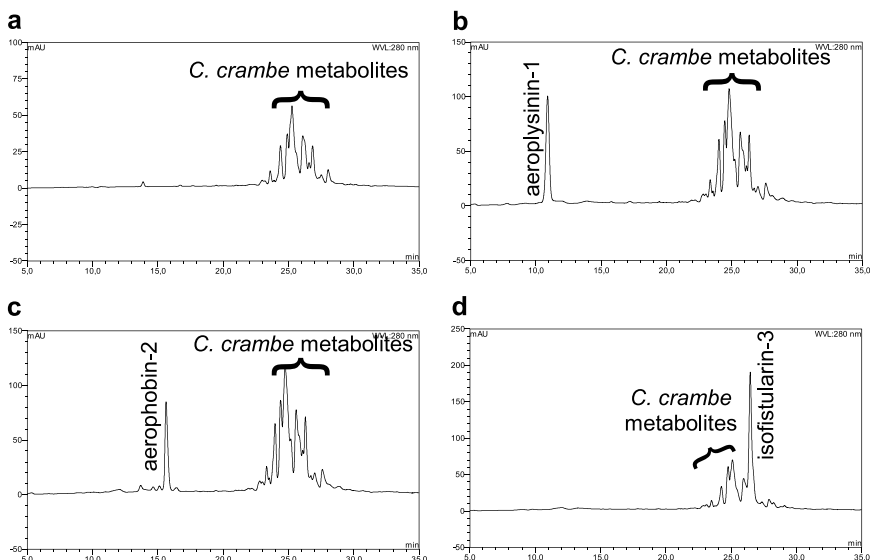


FIG. 3. Comparative HPLC analysis of crude extracts from ground fresh *Crambe crambe* tissue spiked with *A. aerophoba* metabolites (grinding time: 5 min). (a) *C. crambe* tissue without *A. aerophoba* metabolites added. (b) *C. crambe* tissue + aeroplysinin-1 (**5**). (c) *C. crambe* tissue + aerophobin-2 (**1**). (d) *C. crambe* tissue + isofistularin-3 (**3**). Recovery rates for *A. aerophoba* metabolites after grinding: 97–98%.

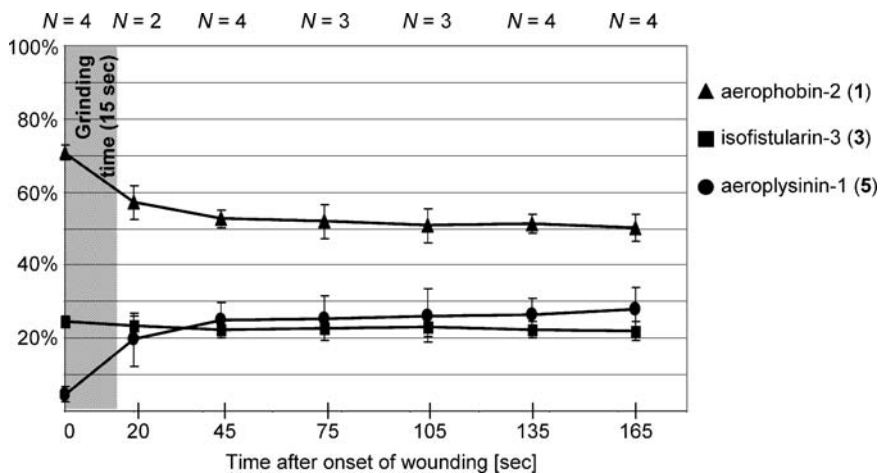


FIG. 4. Variations in the relative concentrations of aerophobin-2 (1), isofistularin-3 (3), and aeropylsinin-1 (5) in *A. aerophoba* tissue after 15 sec of grinding over a 165-sec time course. Proportions of the respective alkaloids are given in percent relative to the total brominated alkaloid content (set at 100%) in the sponge. Data were arcsine-transformed prior to statistical analysis. Randomized block analysis of variance: aeropylsinin-1: $F = 10.75$, $P < 0.001$; aerophobin-2 and isofistularin-3: no statistically significant differences. Tukey–Kramer multiple comparisons for aeropylsinin-1 (significant differences are indicated only): $0 \neq 20$ sec, $0 \neq 45$ sec, $0 \neq 75$ sec, $0 \neq 105$ sec, $0 \neq 135$ sec, $0 \neq 165$ sec.

$4.5 \pm 2.0\%$ in undamaged controls (t_0) to $25.0 \pm 4.8\%$ in samples taken after 45 sec (Figure 4). Thereafter, the aeropylsinin-1 concentrations remained largely unchanged until the observation was terminated after 165 sec.

Experiments with Lyophilized Animal Tissue. In lyophilized tissue of intact *Aplysina* sponges from the Mediterranean Sea (*A. aerophoba* and *A. cavernicola*) and from the Caribbean Sea (*A. fistularis* and *A. archeri*), brominated isoxazoline alkaloids consistently dominated over aeropylsinin-1 (5) or dienone (6) (Tables 1A, B, and 3) even though the alkaloid concentrations showed considerable inter- and intraspecific variation. Following incubation of lyophilized tissue in seawater, the concentrations declined in a pronounced manner, whereas the concentrations of both aeropylsinin-1 (5) and the dienone (6) increased sharply (Table 3, Figure 5). This phenomenon was consistent for all analyzed samples.

A second experiment was performed with lyophilized tissue of *A. archeri*. This sponge contains large amounts of various isoxazoline alkaloids (Table 3). Aerophobin-2 (1), however, which is a major alkaloid in *A. aerophoba*, was not detected in the specimens of *A. archeri* available for this study. Powdered

TABLE 3. CHANGES IN THE BROMINATED ALKALOID CONTENT IN GROUND LYOPHILIZED TISSUE OF DIFFERENT *Aplysina* SPECIES UPON OVERNIGHT INCUBATION IN SEAWATER

Sponge species	Individual no.	Before seawater treatment			After seawater treatment		
		Isoxazol.	Aerophys-1	Dienone	Isoxazol.	Aerophys-1	Dienone
<i>A. aerophoba</i>	1	184.7	26.7	n.d.	43.1	130.4	19.9
	2	232.7	6.4	n.d.	44.3	149.8	18.6
	3	214.9	7.0	0.9	72.8	1.7	43.4
<i>A. cavernicola</i>	1	284.7	12.3	n.d.	22.6	10.8	173.7
	2	223.3	9.4	n.d.	11.9	6.1	122.7
	3	115.1	4.0	6.9	2.4	1.8	76.5
<i>A. fistularis</i>	1	95.3	0.0	0.7	20.4	n.d.	13.2
	2	67.0	0.3	2.2	6.9	0.4	23.4
	3	83.4	0.0	1.4	20.5	n.d.	10.7
<i>A. archeri</i>	1	327.1	0.0	0.8	36.9	2.2	257.1
	2	173.8	11.7	n.d.	90.0	121.8	11.3
	3	196.7	5.7	n.d.	6.9	233.8	71.9

Quantities are expressed in μmol of compound per gram dry weight of sponge tissue.

Isoxazol. = pooled content of all detected isoxazoline alkaloids in the tissue; aerophys-1 = aeropylinin-1 (5) content; n.d. = not detected in the sample by HPLC-UV.

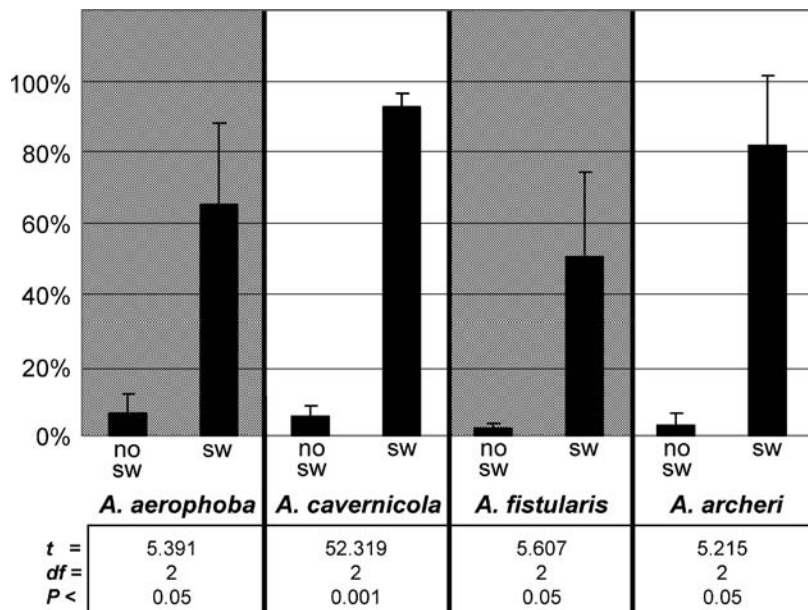


FIG. 5. Variations in the relative concentration of the postulated bioconversion products in ground lyophilized tissue of various *Aplysina* species upon treatment with seawater. The concentrations of bioconversion products aeropylsinin-1 (5) and dienone (6) are pooled. Their proportion is given in percent relative to the total brominated alkaloid content (set at 100%) in the respective sponge. Data were arcsine-transformed prior to statistical analysis. no sw = before treatment with seawater; sw = after treatment with seawater.

lyophilized tissue of *A. archeri* was spiked with aerophobin-2 (1) and subsequently incubated in seawater. Extraction and chromatographic analysis of the sample revealed that the added aerophobin-2, as well as the native isoxazoline alkaloids originally present in *A. archeri*, completely disappeared, whereas dienone (6) showed a considerable increase when compared to a parallel sample that had been lyophilized and extracted without seawater (Figure 6). In contrast, concentrations of the isoxazoline alkaloids aerophobin-2 (1) and isofistularin-3 (3) added to lyophilized and ground tissue of the sponge *C. crambe* remained unchanged, and neither aeropylsinin-1 (5) nor dienone (6) occurred after the tissue had been incubated in seawater [resulting HPLC chromatograms were similar to those shown for the experiments with fresh tissue of *C. crambe* (Figure 3) and, therefore, are not shown separately]. Similarly, in mantle tissue of the marine opisthobranch *T. perversa*, the

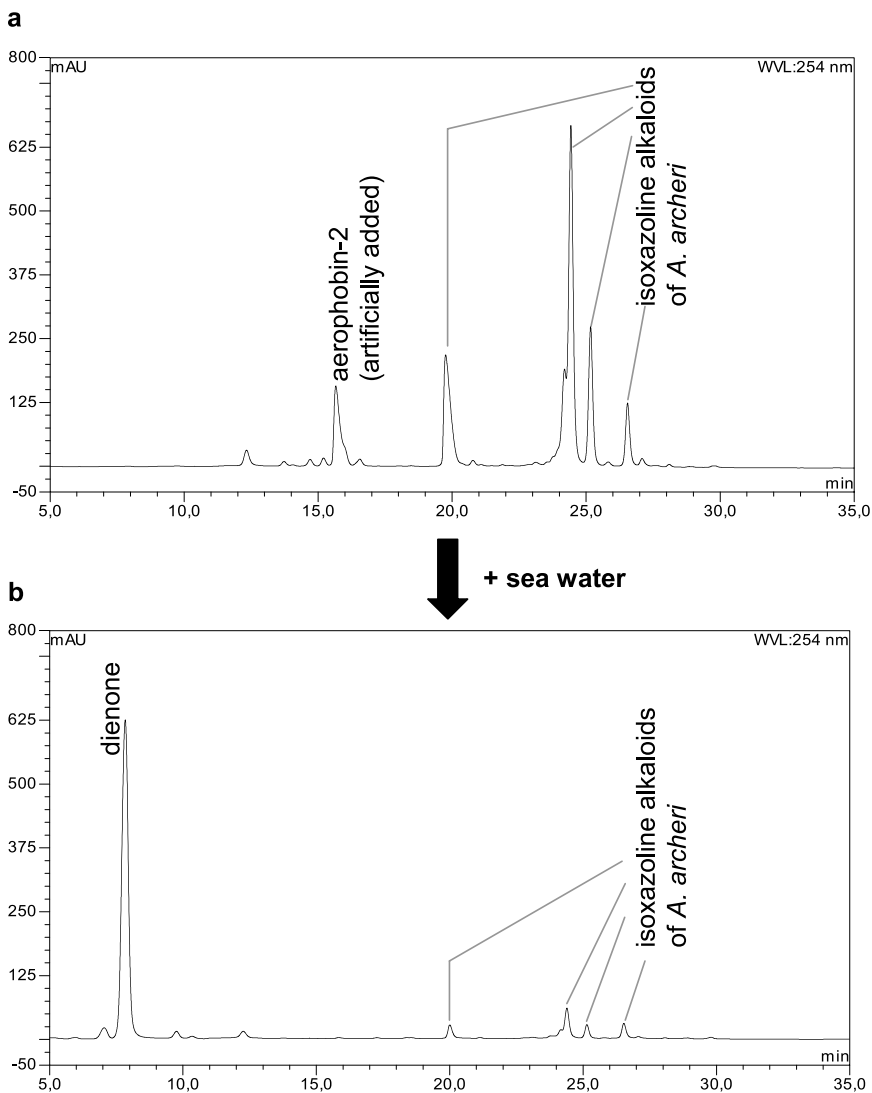


FIG. 6. Comparative HPLC analysis of ground lyophilized tissue of *Aplysina archeri* spiked with aerophobin-2 (**1**) before (a) and after (b) treatment with seawater. The signal intensity axis in both chromatograms is uniformly scaled to facilitate direct comparison.

concentrations of naturally occurring isoxazoline alkaloids remained unchanged, and neither aeroplysinin-1 nor dienone was detected after incubation of the lyophilized and ground material in seawater (data not shown).

DISCUSSION

Comparison of *A. aerophoba* individuals collected at Banyuls-sur-mer with those collected at Rovinj revealed considerable qualitative and quantitative differences in alkaloid patterns (Table 1A, B). The isoxazoline alkaloid aplysinamisin-1 (**2**) that consistently dominated the alkaloid pattern of the sponges from the French Mediterranean coast was not detected in sponges from Croatia. In sponges collected at Rovinj, we detected minor amounts of aeroplysinin-1 (**5**), which, in turn, were not found in individuals from Banyuls (Table 1A, B).

Within 10 specimens of *A. aerophoba* collected along a transect spanning several kilometers at Rovinj, the qualitative composition of the alkaloid patterns turned out to be remarkably similar (Table 1A). However, the absolute contents of brominated alkaloid showed a pronounced variability. Even different tissue samples within a single individual had considerable differences in total brominated alkaloid content. Pronounced variability of secondary metabolite concentrations is a commonly observed phenomenon in Porifera (e.g., Becerro et al., 1995; Betancourt-Lozano et al., 1998; Page et al., 2005), and in regard to the distinct natural variability of the alkaloid pattern in sponges of the genus *Aplysina*, our observations are in accordance with those of Puyana et al. (2003).

This natural variability in *Aplysina* sponges complicates investigations on artificially induced changes in the alkaloid profile, as large standard deviations resulting from replicates with erratic values are likely to obscure possible changes. For our experiments on the injury-induced bioconversion, we, therefore, selected the sponges from Rovinj that showed similarity in relative compositions of their alkaloids. By comparison before and after an experimental treatment (next to the comparison of the absolute values), we circumvented the problem of the vast standard deviations arising when absolute values are compared. We also complimented the experiments involving fresh tissue of *A. aerophoba* with experiments employing lyophilized sponge material. We had determined in our earlier studies (Teeyapant and Proksch, 1993) that the latter has the advantage of being amenable to grinding and homogenization prior to experimental treatment without inducing changes in the alkaloid pattern. Hereby, identical starting material is provided for all samples of an experiment, and even absolute values of the metabolite contents can be compared.

We caused injuries of different intensities to fresh, living tissue of *A. aerophoba* individuals (Table 2, Figure 2). In all samples, the reaction time, i.e.,

the period of time between the onset of the wounding and the flash freezing of the sample, was kept constant at 5 min. Thus, the only variable parameter was the intensity of wounding. In tissue samples that were only damaged to the degree necessary for removal of the sample from the whole sponge (samples "a"), the isoxazoline alkaloid (**1** and **3**) proportion relative to the total brominated alkaloid content amounted to $93.2 \pm 0.1\%$ (Figure 2). Alkaloid proportions in these samples were almost identical to samples that were immediately flash frozen after being cut from the sponge (samples "t₀"). Also, wounding by stabbing with a scalpel (samples "b") did not cause any observable changes in the alkaloid patterns. However, grinding the tissue over 15 sec lowered the isoxazoline alkaloid (**1+3**) proportion to $66.9 \pm 11.9\%$, whereas proportions of aeroplysinin-1 (**5**) increased (samples "c"). By grinding for 5 min, the sponge tissue was more finely homogenized, causing a decrease in the isoxazoline alkaloid proportion to $32.7 \pm 12.0\%$ (samples "d"). Addition of EtOH before 5 min grinding resulted in an isoxazoline alkaloid proportion of merely $9.6 \pm 7.5\%$ (samples "e"). The absolute content of the alkaloids (**1 + 3**) consequently decreased from an original value of $183.3 \pm 49 \mu\text{mol g}^{-1}$ dry weight in the samples "t₀" to $8.8 \pm 5.0 \mu\text{mol g}^{-1}$ in samples "e" (Table 2). At the same time, the aeroplysinin-1 (**5**) content increased from 11.8 ± 9.2 to $107.6 \pm 54.4 \mu\text{mol g}^{-1}$ dry weight sponge tissue. Thus, this experiment revealed a clear relationship between intensity of injury, decline of isoxazoline alkaloids (**1 + 3**), and rise of aeroplysinin-1 (**5**).

A time course experiment conducted for an aliquot of sample "c" that ran over a total of 165 sec (15 sec grinding followed by an additional exposure of the treated tissue for 150 sec) demonstrated the time-dependent increase in aeroplysinin-1 (**5**) (Figure 4). The injury-induced reactions take place within less than 1 min after wounding.

The capability of converting isoxazoline alkaloids to aeroplysinin-1 (**5**) and to the dienone (**6**), which possibly arises from the former by enolether hydrolysis and by partial hydrolysis of the nitrile group thereby giving rise to the amide function, can also be demonstrated for other *Aplysina* sponges such as *A. cavernicola*, *A. fistularis*, and *A. archeri*. From the latter three, only freeze-dried material was available. To obtain comparable results for the different sponges tested, *A. aerophoba* tissue was also lyophilized. Lyophilized and ground material of each *Aplysina* sponge analyzed was divided into two aliquots. One cohort was extracted with MeOH, the other aliquot was incubated in seawater, again lyophilized and subsequently extracted with MeOH. HPLC analysis revealed clear differences in alkaloid profiles between the two treatments (Table 3). Isoxazoline alkaloids dominated in all sponges that had been directly extracted after lyophilization, whereas samples that had been incubated in seawater showed decreased levels and dramatic increases in amounts of aeroplysinin-1 (**5**) and/or dienone (**6**). Exogenously added

aerophobin-2 (**1**), which was not among the brominated isoxazoline alkaloids detected in *A. archeri*, was similarly metabolized by lyophilized powder of this sponge when incubated in seawater (Figure 6). The possibility that the cleavage of the isoxazoline alkaloids is mediated by seawater alone can be excluded as it does not occur in the absence of *Aplysina* sponge tissue (or crude protein extract of these sponges) as demonstrated in our controls with tissue of *C. crambe* and *T. perversa* (Figure 3).

In contrast to compounds such as aerophobin-1 (**1**), alkaloid precursors consisting of two isoxazoline moieties (e.g., isofistularin-3 (**3**) in *A. aerophoba* and aerotionin in *A. cavernicola* and in the Caribbean *Aplysina* species) give rise to two molecules of aeroplysinin-1 upon enzymatic cleavage. This may explain the observation that the total molar concentrations of alkaloids (isoxazoline precursors + generated bioconversion products) in samples treated with seawater in some cases exceed the molar concentrations of precursors in the untreated samples (Table 3). The fact that the dienone (**6**) was also observed for *A. aerophoba* (Table 3) in experiments using lyophilized sponge tissue, whereas it had not been detected for the same sponge in the wounding intensity experiment (Table 2), can probably be explained by the different incubation times used in the experiments (overnight vs. 5 min in case of living tissue).

All experiments unequivocally suggest a wound-induced conversion of brominated isoxazoline alkaloids to aeroplysinin-1 (**5**), which may react further yielding the dienone (**6**). These results are in line with our earlier studies (Teeyapant and Proksch, 1993; Ebel et al., 1997), but are in contrast to the observations and conclusions made by Puyana et al. (2003) in their recent study. Puyana et al. (2003) suggest that our reports on the bioconversion phenomenon were the result of either (1) differential tissue extraction efficiency, (2) formation of aeroplysinin-1 (**5**) and/or dienone (**6**) upon contact of the fresh, wet sponge tissue with organic solvents as artifacts from otherwise insoluble precursors (other than the isoxazoline alkaloids), or (3) heterogeneous distribution of the putative bioconversion products in sponges. Based on these new results and on those from previous studies (Teeyapant and Proksch, 1993; Ebel et al., 1997), we believe that the arguments raised by Puyana et al. (2003) are insufficient to explain the observed phenomenon. We comment on them point by point:

- (1) *Differential tissue extraction efficiency*: Upon artificial wounding of living tissue of *A. aerophoba*, an obvious correlation between wounding intensity, decrease in brominated isoxazoline alkaloids (**1** and **3**), and a concomitant rise of aeroplysinin-1 (**5**) became obvious (Table 2, Figure 2). Different extraction efficiencies as an explanation for this phenomenon are unlikely as all tissue samples had been treated the same way (5 min of exposure followed by flash freezing, lyophilization, and extraction). The only

parameter in which the samples differed was the degree of tissue damage inflicted. While in the aforementioned experiment wounding intensity varied, this parameter was identical for all samples of the time course experiment (Figure 4). Nevertheless, we observed a steady increase the aeroplysinin-1 (5) concentration paralleled by a decrease in isoxazoline alkaloids that was positively correlated with the time between wounding and flash freezing of the damaged tissue.

- (2) *Formation of aeroplysinin-1 (5) and/or dienone (6) upon contact of the fresh, wet sponge tissue with organic solvents as artifacts from otherwise insoluble precursors:* Aeroplysinin-1 (5) is indeed accumulated when living tissue of *A. aerophoba* contacts organic solvents as shown, for example, for sample “e” (Table 2), where living tissue of *A. aerophoba* was treated with diluted EtOH. Except for sample “e,” however, all other samples of living *A. aerophoba* tissue analyzed in this study had been lyophilized prior to exposure to organic solvents. Nevertheless, in all of these samples, aeroplysinin-1 (5) occurred at high concentrations when the respective tissue had been damaged in a substantial manner prior to extraction (Table 2 and Figure 4). At the same time, isoxazoline alkaloid concentrations decreased significantly. Thus, the formation of artifacts upon organic solvent exposition of wet sponge tissue can be ruled out as a plausible explanation for the observed changes in the alkaloid pattern.
- (3) *Heterogeneous distribution of the putative bioconversion products in sponge tissue:* As described in Table 1A and B, alkaloid patterns as well as alkaloid concentrations in different specimens of *A. aerophoba* are subject to intraspecific and even intraindividual variation. Within a given sponge population, these differences are, however, mostly quantitative and not qualitative. For example, all specimens of *A. aerophoba* collected in Rovinj (Croatia) exhibited remarkably homogenous alkaloid profiles and differed only in regard to total alkaloid concentrations (expressed as $\mu\text{mol/g}$ dried tissue or as $\mu\text{mol/ml}$ volume of sponge tissue). The observed changes (reproducible decrease in isoxazoline alkaloids paralleled by a concomitant increase in aeroplysinin-1) we encountered during our experiments, therefore, can not be ascribed to lack of homogeneity of the sponge samples used.

While experiments with lyophilized sponge tissue provide only limited information for processes taking place under *in situ* conditions in living tissue, these experiments have the advantage of providing homogenous material in regard to alkaloid patterns and concentrations. Therefore, differences in alkaloid concentrations of control samples vs. treated samples can not be attributed to nonhomogeneous sponge tissue in experiments using lyophilized material (Table 3, Figure 5 and 6).

The only plausible explanation for the observed changes in the alkaloid patterns of *Aplysina* species is the effect of wounding itself, which results in a breakdown of cellular compartmentalization. We assume that the spherulous cells containing the isoxazoline alkaloids are broken down by mechanical damage. This results in the activation of the bioconversion of isoxazoline alkaloids yielding aeropylsinin-1 (**5**), which may further react to the dienone (**6**). This process would be similar to other known wound-induced bioconversion reactions (e.g., cleavage of cyanogenic glycosides or glucosinolates) in the plant and animal kingdom (Conn, 1979; Paul and van Alstyne, 1992; Wajant and Effenberger, 1996).

The hypothesis that aeropylsinin-1 (**5**) and dienone (**6**) arise from a cleavage of isoxazoline alkaloids is corroborated by a series of further observations: (1) after damaging tissue of *Aplysina* sponges, we never observed an increase in the concentration of the postulated bioconversion products aeropylsinin-1 (**5**) and dienone (**6**) without a concomitant decrease in the concentrations of the isoxazoline alkaloids; (2) the structures of the postulated bioconversion products (**5** + **6**) are directly deducible from the structures of the brominated isoxazoline alkaloids (**1**–**3**) (Figure 1); (3) the bisoxazolidinone derivative (**4**) would be expected as a byproduct of the proposed cleavage of isofistularin-3 (**3**) into aeropylsinin-1 (**5**). When performing *in vitro* assays on the bioconversion of isofistularin-3 using a cell-free extract of *A. aerophoba*, we detected the formation of the bisoxazolidinone derivative (**4**) next to aeropylsinin-1 (**5**) and dienone (**6**) (Ebel et al., 1997).

The fact that Puyana et al. (2003) failed to detect alkaloid conversions is probably attributable to insufficient wounding of the sponges, as a mere stabbing of sponge tissue with a scalpel was insufficient to provoke a measurable conversion of isoxazoline alkaloids in our study as well (Table 2). More severe tissue damage as can be achieved by grinding are necessary (Table 2), as otherwise small changes in the alkaloid composition that will occur only in injured cells are likely to be masked by the unchanged alkaloid profiles remaining in the intact cells that will by far outnumber the former.

Activated chemical defense mechanisms, i.e., rapid conversions of inactive precursor compounds into ecologically active products (reviewed in Havel, 1986; Adler and Harvell, 1990; Paul and Puglisi, 2004), are typically catalyzed by enzymes that are usually separated from their substrates (precursors) by compartmentalization. Upon tissue damage, these compartments are disrupted, facilitating the contact between substrates and the enzymes, which in turn induces the bioconversion. In many cases, the compounds emanating from such reactions arise from cleavage of the precursors. Compartmentalization is also observed in the mesohyl tissue of sponges of the genus *Aplysina*: the brominated isoxazoline alkaloids are mainly stored in specialized cells, the so-

called spherulous cells, in the sponge tissue (Thompson et al., 1983; Turon et al., 2000).

The bioconversion reactions that are observed for living as well as for lyophilized tissue of *Aplysina* species appear to be specific for these sponges, as other marine invertebrates, the sponge *C. crambe* and the opisthobranch *T. perversa*, were unable to convert isoxazoline alkaloids. This is of special interest with regard to *T. perversa*, as this gastropod sequesters isoxazoline alkaloids from its prey *A. aerophoba* (Ebel et al., 1999; Thoms et al., 2003b). In contrast to *C. crambe*, the tissue of this gastropod “naturally” contains brominated isoxazoline alkaloids. However, after grinding and incubating lyophilized tissue of *T. perversa*, we observed no changes in alkaloid profiles or concentrations. Neither aeroplysinin-1 nor dienone was detected, indicating that no biotransformation reactions had occurred.

Earlier experiments with cell-free protein extracts from various sponges revealed that only extracts from the sponges *A. aerophoba*, *A. cavernicola* (both from the Mediterranean Sea), *A. archeri*, *A. cauliformis*, *A. fistularis*, *A. fulva*, and *A. lacunosa* (all from the Caribbean) were able to cleave isoxazoline alkaloids into aeroplysinin-1 and dienone. In cell-free protein extracts from other sponges, *Stelletta globostellata* (order Astrophorida), *Axinella carteri* (order Halichondrida), and *Theonella swinhoei* (order Lithistida) that do not accumulate brominated isoxazoline alkaloids, the concentrations of added isofistularin-3 or aerophobin-2 remained unchanged, and no aeroplysinin-1 or dienone occurred under otherwise identical conditions (Ebel et al., 1997). Alkaloid biotransformation by cell-free protein extracts from *Aplysina* sponges may be inhibited by addition of trichloric acid as well as by boiling (Ebel et al., 1997; Fendert, 2000). Substrate specificity studies that were carried out *in vitro* with naturally occurring isoxazoline alkaloids and with synthetic analogues indicated that the postulated alkaloid splitting enzyme(s) are specific for substrates containing spirocyclohexadienisoaxazoline moieties and acyl amide chains (Fendert, 2000; Goldenstein et al., 2000). Unfortunately, attempts to isolate the responsible enzyme(s) from this crude extract have been unsuccessful due to activity loss during purification (Ebel, 1998). Thus, while a final proof for the enzymatic nature of the bioconversion [e.g., the purification and detailed characterization of the responsible enzyme(s)] is still lacking, our observations point toward an involvement of enzymes in the bioconversion reactions.

The question remains whether the alkaloid conversion is ecologically advantageous for *Aplysina* sponges and can be considered an example of an activated chemical defense. Choice feeding experiments with the Mediterranean fish *B. sphinx* revealed that aeroplysinin-1 (**5**), as well as dienone (**6**), possesses considerably lower feeding deterrent properties against test fishes than their precursors (e.g., aerophobin-2 or isofistularin-3) when tested at their natural concentrations (Thoms et al., 2004). Even if in the course of the bioconversion

the feeding deterrent properties intensified, this could hardly be considered an activated protection against predatory fishes. First, the bioconversion rate (45 sec to 1 min; Figure 4 and Ebel et al., 1997) would probably be too slow to deter the predators in time, before serious wounding was caused. Second, the bioconversion products would presumably only arise at the surface of the sponge tissue piece bitten off, as only here does a decompartmentalization of the spherulous cells occur. The arising bioconversion product concentration would probably be too low to have a noteworthy effect on predators. It appears unlikely that the wound-induced bioconversion of alkaloids in *Aplysina* species will increase the fitness of sponges toward fish predators.

The situation is different, however, when a defense against microbial pathogens is considered. In this case, it might possibly be sufficient if the bioconversion products were formed only in injured cells, as penetration of pathogenic microorganisms is likely to occur through wounded tissue. It is difficult to determine the actual metabolite concentration in the thin surface layer covering the wounded tissue, because it is virtually impossible to harvest and analyze only wounded cells without adherent healthy tissue that will mask the results of a chemical analysis due to the “static” alkaloid profile that will remain unchanged. The only reasonable approximation for the concentrations of aeropylsinin-1 (**5**) and the dienone (**6**) that arise locally in wounded surface tissue are the amounts of bioconversion products that are formed in finely macerated sponge tissue. We determined the concentration of aeropylsinin-1 (**5**) in tissue of *A. aerophoba* after 5 min of grinding to amount to $4400 \pm 2036 \mu\text{g ml}^{-1}$ sponge tissue (Table 2). Teeyapant et al. (1993) determined the Minimum Inhibitory Concentrations (MIC) of aeropylsinin-1 and dienone against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* to range between 12.5 and $50 \mu\text{g ml}^{-1}$ depending on the bacterial species tested. Kelly et al. (2003) were able to show that seawater bacteria attachment to agar blocks treated with aeropylsinin-1 at a concentration of $1000 \mu\text{g ml}^{-1}$ was reduced to $1.4 \pm 0.1\%$ compared to untreated controls. Dienone at a concentration of $250 \mu\text{g ml}^{-1}$ reduced bacterial attachment to $4.1 \pm 0.1\%$. An amount of $5 \mu\text{g}$ aeropylsinin-1 showed activity against two out of six marine bacteria species tested in an agar diffusion assay (Weiss et al., 1996). Dienone at the same concentration inhibited the growth of five of six species in this experiment. One hundred μg of aeropylsinin-1 as well as $100 \mu\text{g}$ dienone were active against all eight marine bacteria species tested. Debitus et al. (1998) found activity for both compounds when they tested $10 \mu\text{g}$ of them against *S. aureus* and *Vibrio anguillarum* in agar diffusion assays. They proposed dienone (**6**) for use as an antibiotic in mariculture, as their investigation revealed that it rivals chloramphenicol with regard to decreasing mortality of scallop (*Pecten maximus*) larvae in culture. Thus, the concentrations of the bioconversion products arising in ground fresh *A. aerophoba* tissue exceed concentrations that have proven effective against

bacterial invasion, suggesting that the generation of aeroplysinin-1 (**5**) and subsequently of the dienone (**6**) at the site of wounding might protect injured sponge tissue against pathogenic bacteria. Consequently, in the course of the wound-induced bioconversion, the antipredatory isoxazoline alkaloids stored constitutively in tissue of *Aplysina* sponges are cleaved into compounds with pronounced antimicrobial activity. This process seems to correlate with the change in needs of the sponges after their tissue has been wounded and reveals the multiple ecological functions that marine natural products can possess.

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CHARACTERIZATION OF DOG REPELLENT FACTOR FROM CUTICULAR SECRETION OF FEMALE YELLOW DOG TICK, *Haemaphysalis leachi*

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Abstract—During its natural life cycle, the yellow dog tick, *Haemaphysalis leachi*, has three hosts, and it has to spend enough time on each of them to complete a blood meal. When irritated, the females of this tick species produce a cuticular secretion that contains a dog-repelling allomone. This improves the tick's chances of survival by deterring the dog from biting the tick off its body. Employing response-guided isolation techniques in conjunction with gas chromatography-mass spectrometry, the defensive allomone of *H. leachi* was found to consist of the six homologous aliphatic aldehydes from hexanal to undecanal. A mixture of synthetic versions of these six aldehydes in quantities corresponding to those secreted by one tick elicited strong aversion reactions in the majority of dogs of various breeds.

Key Words—Dog repellent, *Haemaphysalis leachi*, cuticular secretion, preparative GC, GC-MS, bioassay, sampling techniques.

INTRODUCTION

The yellow dog tick, *Haemaphysalis leachi*, is endemic to Southern Africa, Asia, and Australia, where it is a vector of *Rickettsia conori*, *R. rickettsii*, and *Coxiella burnetii*, which are responsible for tick bite fever, Rocky Mountain spotted fever, and Q fever, respectively, in humans (Howell et al., 1978). This tick is also the only known vector of *Babesia canis*, the bacterium responsible

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for biliary fever in dogs (Van Heerden et al., 1995). *H. leachi* is a tick where the female requires taking a blood meal from three hosts to complete the life cycle. Molting takes place on the ground because the female tick cannot cling to its host when she molts. Engorged female *H. leachi* have soft bodies that are completely unprotected from attack by their hosts. This is an especially significant handicap if the host is a dog that can use its teeth with considerable dexterity to remove the small parasites from its body. However, dogs do not attempt to remove female *H. leachi* from their bodies with their teeth (Oelofsen, unpublished data). Instead, when they are presented with *H. leachi* or with an extract of its cuticular secretion, dogs exhibit behaviors suggesting disgust (e.g., looking at the owner or handler in a reproachful way, turning the head away, straining at the leash, running away, hiding behind or under something) or even nausea (e.g., salivating copiously) (Oelofsen, unpublished data).

We hypothesize that a dog-detering allomone in the cuticular secretion of the tick elicits aversion in dogs and this probably constitutes a defensive mechanism used by the tick to prevent the host from removing the tick from its body during its blood meal. Even when engorged, the male dog tick has an average body mass of only about 2% of that of an engorged female. It would be difficult for a dog to find and remove these small animals. Therefore, the male probably does not produce a dog repellent. The female, on the other hand, is much more vulnerable and the secretion of a repellent would enable her to complete a blood meal on each of her three hosts (Arthur, 1962). In this article, we report the identification of the constituents of the cuticular dog-repelling allomone of the female yellow dog tick.

METHODS AND MATERIALS

General. Dichloromethane (Merck, Suprasolv Grade) was used for all extractions and as the solvent in which preparatively isolated fractions were collected. For the cleaning of syringes and glassware, dichloromethane (Riedel de Haën, pesticide analysis grade) was used. Synthetic chemicals were purchased from Aldrich (St. Louis, MO, USA).

Sample Preparation

Collection and Handling of Ticks. Live, engorged female *H. leachi* were collected by local dog pound personnel and veterinarians. To stimulate the female *H. leachi* to produce the cuticular exudates, we touched the tick with the tip of a soft artist's brush. This promoted the secretion of a varying number of less than about 50 tiny, barely visible droplets of liquid through pores in the integument of the engorged female. The production of the cuticular exudate can

also be stimulated by methods such as prodding the female *H. leachi* with a glass rod or pinching it with a pair of tweezers, but such methods can perforate the animal's integument.

Solvent Rinse of Cuticle. Female ticks were lightly held with a pair of tweezers and irritated as described above. Each irritated female tick was rinsed with about 1 ml dichloromethane. The combined rinses were stored at -18°C until analyzed or used for bioassays. For gas chromatography with flame ionization detection (GC-FID) and gas chromatography with mass spectrometric detection (GC-MS), the dichloromethane rinses of 10 ticks were combined and concentrated at room temperature (22°C) to a volume of about 10 μl by slow evaporation of the solvent in a static nitrogen gas atmosphere (Reiter et al., 2003). Samples were analyzed by injecting 2.5 μl of the concentrated extract in splitless mode, allowing for a splitless period of 150 sec.

Volatile Collection: Solid Phase Microextraction. The effluvium of 10 irritated, engorged ticks in a 25-ml glass bottle was sampled by solid phase microextraction (SPME; 100 μm polydimethylsiloxane fiber) for 5 hr at room temperature (Arthur and Pawliszyn, 1990). For GC-MS analysis, the sorbed volatiles were thermally desorbed for 150 sec at 220°C in the instrument's inlet. In this and all following experiments, volatiles trapped in a solventless manner were immediately analyzed after sample collection.

Volatile Collection: Adsorption on Activated Charcoal. The tip of a length of fused silica tubing (100 \times 0.7 mm o.d.) was coated to a length of 15 mm with polyimide monomer and carefully dusted with activated charcoal (particle size, 38–48 μm) of the type used in cigarette filters. The polyimide was cured overnight at 290°C to give a probe coated with a stable monoparticulate layer of activated charcoal. Sample enrichment by adsorption on the activated charcoal and GC-MS analysis of the enriched volatiles were performed as described above for SPME. In this technique, the septum was replaced by a septum carrying the probe (Burger et al., unpublished data). The carrier gas was turned off during the installation of the probe in the instrument's injector to avoid premature desorption of the collected volatiles.

Volatile Collection: Sorption in an Open Tubular Trap. The volatile organic constituents of the effluvium of irritated females were also collected on a phase-coated open tubular trap and analyzed according to the method described by Burger et al. (1995). Using helium as carrier gas, the effluvium of 10 irritated, engorged females was purged from a 50-ml screw-capped glass bottle with a Teflon-faced septum at 22°C , and the volatile organic compounds were trapped on the capillary trap (90 \times 0.3 mm) coated with 15 μm of the apolar phase PS-255 (Burger et al., 1991). The capillary trap was connected to the column using shrinkable Teflon (PTFE) tubing and inserted into the injector where the trapped material was thermally desorbed, transported to the column by the carrier gas, and analyzed by GC-MS.

Volatile Collection: Cryoprecipitation in Uncoated Capillary Trap. The previous experiment was repeated with an uncoated capillary trap. In this experiment, volatiles were immobilized from the effluvium of 10 irritated, engorged females in an uncoated capillary cooled with dry ice. The experiment was also repeated with liquid nitrogen as a cooling medium and helium as a purge gas. Desorption and analysis were performed according to the procedure used for the phase-coated trap described above.

Solventless Sampling. Attempts at collecting droplets of the secretion in capillary tubes were not successful. The following method was, therefore, used to collect material for solventless sample introduction. A strip (25 × 4.5 mm) of glass fiber filter paper (Whatman, Glass Microfibre Filter, Cat. 1820 090) was partly inserted into the 4-mm i.d. glass inlet liner of the GC-MS instrument. Two engorged females were irritated with the part of the filter strip protruding from the liner and the cuticular secretion absorbed by the filter paper. The strip of filter paper was fully inserted into the liner, and the liner was installed in the injector (220°C) of the instrument for splitless desorption (150 sec) at 220°C and GC-MS analysis of the desorbed volatiles.

Sample Analysis, Fractionation, and Bioassay

Chemical Analysis. Analytical and preparative GC separations were done with a Carlo Erba 4200 GC, equipped with a FID and split/splitless injector operated at 280 and 220°C, respectively, and a 40 m × 0.25 mm glass column coated with 0.25 μm of the stationary phase PS-089 (DB-5 equivalent). Hydrogen was used as carrier gas at a linear velocity of 50 cm/sec. The column was temperature programmed from 30 to 250°C at 2.0°C/min. Low-resolution electron impact mass spectrometry (LR-EIMS) was performed on a Carlo Erba QMD 1000 GC-MS system, using the same gas chromatographic parameters. Helium was used as carrier gas at a linear velocity of 32.25 cm/sec. The line-of-sight interface was kept at 250°C and the ion source temperature was set at 180°C. Mass spectra were recorded at 70 eV.

Sample Fractionation. A four-way effluent splitter was used to isolate fractions of the collected cuticular secretion of the tick. This splitter made provision for the introduction of make-up gas as well as splitting of the column effluent in a 1:3 ratio with 25% of the effluent directed to the detector. Uncoated, deactivated fused silica capillary tubing was used to connect the effluent splitter to the make-up gas supply, detector, and fraction collection port. The fraction collection outlet line was heated to its tip by an aluminum block thermostatted at 250°C. Fractions were collected manually by passing the effluent gas through 150 μl of dichloromethane in 1-ml conical vials. To avoid cross-contamination of fractions, each fraction was collected with a fresh glass

exit tip that was connected in a press-fit manner to the heated fused silica transfer line from the effluent splitter.

During the first fractionation series of the crude extract from the solvent rinse of the cuticle, 11 fractions were collected over 5-min retention time windows and then bioassayed. To keep the number of bioassay fractions within reasonable limits, the next fractionation was started at a retention time offset of 2.5 min. From this second fractionation series, 11 fractions were bioassayed. During additional series of fractionations, fractions were collected over smaller windows and only in those overlapping windows where activity had been detected in both of the initial fractionations, and then over incrementally smaller windows, which eventually corresponded to about the average width of peaks in the gas chromatogram. A total of about 80 fractions were collected.

Sample Bioassays. Only a limited number of appropriate dogs were available to us for the behavioral assays. Dogs from an animal health facility were not used for the behavioral assays because their craving for attention appeared to override the repellency of test samples. Similarly, untrained, young dogs were not used because they tended to run around uncontrollably. Instead, dogs that had undergone obedience training were used for the assays because they remained still when confronted with test samples. The limited availability of dogs for bioassay thus precluded replicated data collection and statistical analysis of the behavioral responses.

Aliquots (0.5 ml) of the crude unconcentrated solution of the cuticular secretion or of fractions were pipetted onto strips of filter paper (35 × 5 mm; Schleicher & Schüll GmbH, Dassel, Germany) and the solvent was allowed to evaporate for 5 min at 22°C. Controls were prepared by treating strips of filter paper in a similar manner with dichloromethane. The filter paper strips with the crude cuticular secretion, the fractions, or the controls, were handled with dressing forceps at all times and were placed individually in screw-capped glass vials with Teflon-faced septa and transported at the current daytime temperature to the sites where the bioassays were conducted. Bioassays were performed as soon as possible after the preparation of the test paper strips, normally with a delay of between 30 min and 3 hr, depending on whether bioassays were done locally or in neighboring towns. The samples were kept in the dark, but were not cooled to avoid the sublimation of the collected material from the paper strips onto the walls of the glass vials.

The vials with partly protruding paper strips were presented to the dogs by hand, and a distance of about 3 cm was maintained between each paper strip and the nose of the test animal. If a test strip came into contact with a dog's nose, it was discarded. With the exception of a few dogs (Table 1), the bioassays were the first encounters between each dog and the presenter of the test sample. In all cases, the bioassays were conducted in the presence of the dog's owner. The

TABLE 1. RESPONSE OF OBEDIENCE-TRAINED DOGS TO SOLVENT CONTROLS AND A MIXTURE OF SYNTHETIC ALDEHYDES^a

Dog no.	Breed	Name	Sex	Response ^b
1	Alaskan malamute × Wolf	Rex	♂	<i>c</i>
2	Alsatian	Rex	♂	<i>d</i>
3	Basset hound	Gigolo	♂	<i>e</i>
4	Basset hound	Poppie	♀	<i>d</i>
5	Belgian shepherd	Wolf	♂	<i>c</i>
6	Belgian shepherd	Duchess	♀	<i>d</i>
7	Boxer	Pilot	♂	<i>f</i>
8	Border collie	Lady	♀	<i>g</i>
9	Cocker spaniel	Lucy	♀	<i>d</i>
10	Corgi	Duke	♂	<i>d</i>
11	Corgi	Stoffel	♂	<i>d,f</i>
12	Dobermann × Spaniel	Beer	♀	<i>d</i>
13	Fox terrier	Vlooi	♀	<i>d</i>
14	Jack Russell	Nina	♀	<i>h</i>
15	Jack Russell	Jackie I	♀	<i>d</i>
16	Jack Russell	Jackie II	♀	<i>d</i>
17	Jack Russell	Jessie	♀	<i>h</i>
18	Labrador	Gunner	♂	<i>d</i>
19	Labrador	Bimbo	♂	<i>f</i>
20	Labrador	Rex	♂	<i>c</i>
21	Labrador	Baasjan	♂	<i>e,g</i>
22	Labrador	Linka	♀	<i>d</i>
23	Maltese poodle	Tinkels	♀	<i>e</i>
24	Mixed breed	Meisie	♀	<i>c,i</i>
25	Ridgeback	Rupert	♂	<i>e,j</i>
26	Rottweiler	Tequila	♀	<i>f,d</i>
27	Rottweiler × Spaniel	Bruno	♂	<i>d</i>
28	Rottweiler × St Bernard	Syrah	♂	<i>d</i>
29	Scottish terrier	Katy	♀	<i>h</i>
30	Shepherd's dog	Cloé	♀	<i>f</i>
17 ^k	Jack Russell	Jessie	♀	<i>h</i>
25 ^k	Ridgeback	Rupert	♂	<i>h,f</i>

^aComposition is given in the text.

^bAll dogs sniffed at or inspected solvent controls.

^cNo response. No difference between sample and solvent control.

^dTurned head away, avoided eye contact.

^eRan away and kept a safe distance.

^fModerate salivation.

^gBecame aggressive.

^hRan away but returned when called.

ⁱChewed the test strip.

^jExcessive salivation.

^kSecond assay with same dog, 8 weeks after the first one was completed.

situations in which the bioassays were done, whether at the home of the dog or after an obedience training session, and the handling of dogs just prior to bioassays, varied from dog to dog and was beyond our control.

In most cases, the assay dogs paid immediate attention to the test samples and would advance toward the sample, so that the 3-cm test distance could be established and the behavioral response could be described and recorded. In these instances, the test animal sometimes even attempted to lick the sample. In other cases, assay dogs remained uninterested when presented with samples, and the sample had to be brought within the 3-cm range by the presenter. Test animals sometimes turned their heads away from the samples, making it difficult to maintain the 3-cm test distance.

A solution of the synthetic aldehydes hexanal, heptanal, octanal, nonanal, decanal, and undecanal was prepared by dissolving these compounds in 1 ml dichloromethane in the quantitative ratio in which they are secreted by female *H. leachi*. Test filter paper strips were prepared by transferring 2.5 μl of this solution, equivalent to the quantities of the aldehydes present in the secretion of one female tick, to each of the paper strips. Assays were done by presenting 30 dogs, individually, first with a solvent control and then with an aldehyde-treated paper strip. The behavioral responses of the dogs to the controls and aldehydes were observed (e.g., degree of movement away from the stimulus, extent of salivation, movement of the head, etc.) and recorded.

RESULTS AND DISCUSSION

The cuticular secretion of female *H. leachi* was expected to contain some heavy lipid material that would not be amenable to gas chromatographic analysis. To avoid possible contamination of the capillary GC column with heavy material, methods were initially investigated for the enrichment of the volatile organic compounds directly from the effluvium of irritated female ticks. Sample enrichment by SPME, adsorption on activated charcoal, trapping in open tubular traps, and cryotrapping in capillary tubes with dry ice and liquid nitrogen as cooling media all produced poor results. With the exception of a considerable quantity of hexadecanoic acid trapped on activated charcoal, only a few peaks of compounds present in extremely low quantities were visible in the total ion chromatograms (TICs) of the material trapped with these sample enrichment methods. Some of the peaks could be attributed to thermal degradation of the stationary phase of the column. Other small peaks appeared at different retention times in the different TICs, and it was concluded that these compounds were either artifacts of unknown origin or, more likely, they

resulted from differences in selectivity toward compounds with different boiling points and polarities by the enrichment methods.

The collection of the cuticular secretion by absorption on a piece of glass fiber filter paper followed by insertion of the paper strip into a glass injector liner and introduction of the liner into the injector of the GC-MS, yielded a satisfactory TIC and mass spectra with ions of high abundance. TICs obtained by this procedure were used for identification of the components of the cuticular secretion. However, this technique could potentially overload the column with heavy lipid material and shorten its lifetime. This method also makes it difficult to control the quantity of material introduced into the injector and to obtain chromatograms with reproducible retention times, essential for the accurate and reproducible collection of fractions in preparative GC. Therefore, a more conventional method was employed for the collection of material for some GC procedures: irritated females were rinsed with dichloromethane and aliquots of the resulting extract were used for analytical and preparative work. Analysis of this extract by GC-MS showed that organic acids and aldehydes ranging in length from C₃ to C₁₁, as well as many other compounds that could not be characterized, were present in the extract (Figure 1).

We approached the fractionation of this extract by considering that there are at least two possible methods to locate the active constituent(s) in a crude extract. Following a conventional response-guided strategy, fractions can be collected, a certain percentage of each fraction bioassayed, and the rest of each fraction subjected to further fractionation and bioassay. This procedure can be

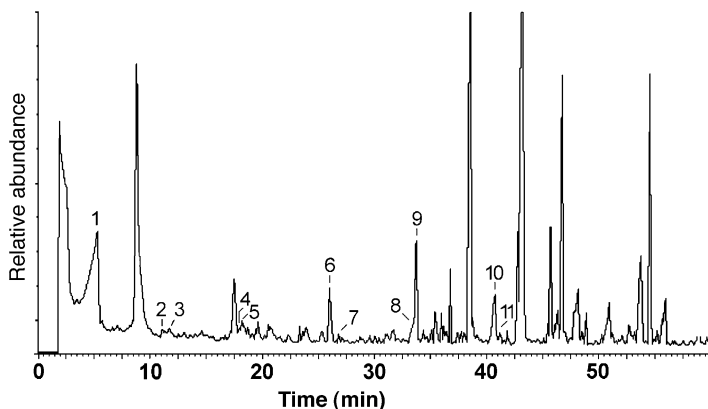


FIG. 1. Total ion chromatogram (TIC) of volatile organic compounds in rinses of engorged, irritated female yellow dog ticks. First 60 min of the chromatogram are displayed. 1 = Propanoic acid; 2 = hexanal; 3 = heptanal; 4 = hexanoic acid; 5 = octanal; 6 = nonanal; 7 = heptanoic acid; 8 = octanoic acid; 9 = decanal; 10 = nonanoic acid; 11 = undecanal. Analytical parameters are given in the text.

repeated until a fraction containing only one active constituent has been isolated. If, as in the present case, the active constituent(s) is present in such a low concentration in the starting extract that a large percentage has to be sacrificed for chemical detection, the consumption of material can be so high that insufficient material will eventually be available to continue with further fractionation steps. We used a second procedure in which preparative separations were all conducted on the original extract. In the first fractionation series, fractions were collected over relatively wide consecutive retention-time segments (windows) of 5 min and assayed for their repellent activity. In the following fractionation of a fresh aliquot of the original extract, fraction collection was initiated at a retention-time offset of 2.5 min, after which smaller fractions (1-min windows) were collected only in those windows that contained active material in the initial fractionation. The fractionation was repeated using incrementally smaller retention time windows, which eventually corresponded to about the width of the peaks in the gas chromatogram. In this approach, each fractionation is done with the original extract, and only one fractionation step had to be repeated to get back on track when bioassays gave inconclusive results. In one of the final fractionations, all the material collected during "inactive" time windows was pooled and bioassayed. This material did not show any activity, supplying corroborating evidence that all the active constituents had been correctly identified from the crude extract. In total, we conducted 10 series of iterative fractionations on the crude extract.

We experienced some technical limitations during the final stages of the fractionation procedure. The thin fused-silica transfer line had to be heated right up to its tip to prevent the condensation of the eluting constituents in a cold spot, and it was difficult to successfully couple the equally thin collection capillary, with its tip already in dichloromethane, to the transfer line at exactly the right moment for sample collection. The collection of material in very narrow windows was also complicated by the change of linear flow rates through the different components of the temperature-programmed preparative system, resulting in an increasing time lag between detection of a component and its arrival at the sample collection point. The minor constituents of the fractions were, therefore, collected in small quantities and low purities.

Approximately 100 samples of the crude cuticular secretion and the isolated fractions were bioassayed. We began the bioassay procedure by assessing the effect of the crude cuticular extract on four test animals: a docile Labrador, two well-behaved Corgis, and a Fox terrier. When we attempted to repeat the bioassays a few days later, the dogs refused to come near the presenter. The Corgis avoided this person for several weeks afterwards. Thus, we concluded that it would not be possible to use the same dog twice in this behavioral assay. Samples of the crude extract and the fractions were assayed only until the first clear indication of repellent activity had been obtained (data

not shown). In most cases, only one or two dogs were necessary to confirm the presence of activity in an extract or a fraction of the cuticular secretion, and these responses were compared to a previous assessment of the response to the solvent control by the same animal. The responses of the dogs to some of the fractions were also rather subtle. They were mostly reluctant to sniff at a specific test strip for a second time and/or they avoided eye contact with the presenter. The relatively indefinite results were interpreted as an indication that the defensive secretion contains several active constituents eliciting slightly different responses in the dogs, and that all of the active constituents have to be present in test samples to elicit the full aversive response.

We did not detect any behavioral activity in the major constituents of the cuticular secretion of female *H. leachi* (the later eluting peaks in Figure 1 and in the part of the TIC not shown). Upon fractionation, we found activity in several fractions collected over retention time windows that did not contain particularly prominent peaks. Comparative GC-MS analysis of the whole extract and the active fractions revealed the presence of an aldehyde in all of the active fractions. These aldehydes were tentatively identified as unbranched aldehydes. Because it is difficult to differentiate between the mass spectra of long-chain unbranched, iso- and anteiso-branched aldehydes, the active constituents were identified by GC-MS as the unbranched isomers by coelution with authentic unbranched synthetic compounds. On average, females secreted the aldehydes in the following approximate quantities (in ng/female): hexanal (0.1), heptanal (9.0), octanal (7.5), nonanal (43.0), decanal (34.0), undecanal (5.0). The shorter chain aldehydes eluted as broadened peaks due to the splitless sample introduction method (Figure 1). Partial coelution with some of the carboxylic acids also affected this analysis. Although these aldehydes have not been previously identified as constituents of arachnid defensive secretions, they are common constituents of insect defensive secretions (Dettner and Reissenweber, 1991; Dettner, 1993; Farine et al., 1993).

Synthetic versions of the six aldehydes were mixed for bioassays in a 1-ml solution of dichloromethane in the quantities noted above. The solvent control and this mixture were presented (in that order) to each of 30 dogs. In these assays, all dogs sniffed or inspected the solvent controls, but showed no negative behavioral responses (Table 1). Of the 30 dogs used in bioassays, four individuals exhibited no behavioral response differences to the solvent control and the synthetic mixture. However, 26 individuals displayed some type of aversion reaction to the synthetic mixture ranging from salivation (6 of 30 dogs) to turning the head away and avoiding eye contact with the presenter (15 of 30 dogs) to running away from the presenter with or without a return (7 of 30 dogs). In some cases, multiple behavioral responses were elicited from the same animal (e.g., salivation and turning of the head). One dog showed no aversive response to the test strip and attempted to chew on it; another dog

barked at the test strip and then tried to attack the presenter. Two dogs were reassayed 8 weeks later and both responded aversively again to the test strip. Since both ran away when they saw the presenter, even 8 weeks after the first test had been done, it was clear again that we could only assay each dog once.

The synthetic aldehyde mixture elicited the same range of aversive behaviors that we observed in the pilot study of the crude extract of the solvent rinse of the cuticle of *H. leachi*. However, because of the limited number of test animals available to us, we were not able to directly compare (i.e., in the same experiment) the behavioral response to the synthetic aldehyde mixture with the behavioral response to the crude extract of the solvent rinse of the cuticle of *H. leachi*. We note that the behavioral portion of this study is limited by the inherent difficulties of obtaining a sufficient number of test animals and by the differences in the behavioral experiences, personalities, and status of the test animals at the time of the assay. Under these circumstances, a statistical evaluation of the results of the bioassays was not feasible. Thus, we have presented the data in raw form (Table 1).

We observed that many different compounds inconsistently appeared in rinses collected from individual female *H. leachi* and in material obtained by batchwise rinsing of female *H. leachi*. Perhaps impurities picked up by ticks from their hosts and/or the environment could be responsible for the complexity of the extracted cuticular material. Propanoic acid and some of the other unbranched carboxylic acids from C₅ to C₁₅ were present in many of the rinses but, again, there was no consistent pattern. Some of these acids could have been picked up from the bodies of their hosts, but it is also possible that the C₆ to C₁₂ acids were formed by autoxidation of aldehydes previously secreted by irritated females.

We recognize that there are other possible interpretations of the results of the bioassays. The various reactions of dogs in our behavioral assays suggest that a dog's reaction may be influenced by its previous experiences with female *H. leachi*. Well-kept dogs or dogs from urban areas that have not had the unpleasant experience associated with tick infestation might, therefore, respond less aggressively to test substances than dogs from the outlying parts of town where they might be more prone to tick infestation. It is possible that the dogs are not inherently deterred by the smell of these aldehydes, which are not uncommon in nature, but rather that they associate it with the nasty taste and/or burning sensation caused by contact of the mammalian mucous membrane with the short-chain aldehydes. In addition, some of these aldehydes occur in rancid food where they are formed by autoxidation of unsaturated oils and fats. Thus, it is possible that the dogs used in the bioassays could have unpleasant memories of rancid food, which would explain the signs of nausea and excessive salivation displayed by some of the dogs in reaction to the mixture of aldehydes in bioassays. Finally, extracts of the cuticular exudate of female *H. leachi*

consistently contained several compounds in the higher mass range (not shown in Figure 1). These compounds apparently belong to different homologous series. Their mass spectra, which are not represented in any of the large libraries of spectra, could not be interpreted so far. These compounds could be the real poisonous defensive constituents of the secretion, whereas the aldehydes might constitute an aposematic odor associated by learning with the truly toxic material. These aspects might be investigated in future studies of this system.

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CALIFORNIA GROUND SQUIRREL (*Spermophilus beecheyi*) DEFENSES AGAINST RATTLESNAKE VENOM DIGESTIVE AND HEMOSTATIC TOXINS

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Abstract—Previous studies have shown that some mammals are able to neutralize venom from snake predators. California ground squirrels (*Spermophilus beecheyi*) show variation among populations in their ability to bind venom and minimize damage from northern Pacific rattlesnakes (*Crotalus oreganus*), but the venom toxins targeted by resistance have not been investigated. Four California ground squirrel populations, selected for differences in local density or type of rattlesnake predators, were assayed for their ability to neutralize digestive and hemostatic effects of venom from three rattlesnake species. In Douglas ground squirrels (*S. b. douglasii*), we found that animals from a location where snakes are common showed greater inhibition of venom metalloprotease and hemolytic activity than animals from a location where snakes are rare. Effects on general proteolysis were not different. Douglas ground squirrels also reduced the metalloprotease activity of venom from sympatric northern Pacific rattlesnakes (*C. o. oreganus*) more than the activity of venom from allopatric western diamondback rattlesnakes (*C. atrox*), but enhanced the fibrinolysis of sympatric venom almost 1.8 times above baseline levels. Two Beechey ground squirrel (*S. b. beecheyi*) populations had similar inhibition of venoms from northern and southern Pacific rattlesnakes (*C. o. helleri*), despite differences between the populations in the locally prevalent predator. However, the venom toxins inhibited by Beechey squirrels varied among venom from Pacific rattlesnake subspecies, and between these venoms and venom from allopatric western diamondback rattlesnakes. Blood plasma from Beechey squirrels showed highest inhibition of metalloprotease activity of northern Pacific rattlesnake venom, general proteolytic activity and hemolysis of southern Pacific rattlesnake venom, and

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hemolysis by allopatric western diamondback venom. These results reveal previously cryptic variation in venom activity against resistant prey that suggests reciprocal adaptation at the molecular level.

Key Words—*Spermophilus beecheyi*, *Crotalus oreganus*, *Crotalus atrox*, venom, natural resistance, coevolution, predator–prey.

INTRODUCTION

Some species of mammals are known to be resistant to the venom of the viperid snakes that hunt them (Thwin and Gopalakrishnakone, 1998; Pérez and Sánchez, 1999). Viper venoms contain toxins with a variety of effects (Meier and Stocker, 1995), including metalloproteases that break down the lining of blood vessels and extracellular matrix (Gutiérrez and Rucavado, 2000). Other venom components disrupt the normal processes of coagulation and induce hemolysis (Markland, 1998; Braud et al., 2000). In susceptible prey (and humans), venoms can lead to extensive hemorrhage, edema, tissue necrosis, and death (Russell, 1980; Ownby, 1982). Even if the prey successfully evades ingestion by a viperid predator, venom-induced shock and the digestive effects of toxins pose a serious threat. To maintain the ability to survive and reproduce in an environment that holds challenges from predators, competitors, and conspecifics, mammals must minimize hemorrhage, tissue destruction, and disruptions to hemostasis that follow envenomation.

The interactions of California ground squirrels (*Spermophilus beecheyi*) and northern Pacific rattlesnakes (*Crotalus oreganus oreganus*) may be the most intensively studied examples of the relationship between rattlesnakes and preferred prey. Ground squirrels are an important food resource for rattlesnakes throughout California (Linsdale, 1946; Fitch, 1948). Fitch (1949) estimated that *S. beecheyi* make up to 69% (by weight) of the diet for adult northern Pacific rattlesnakes (*C. o. oreganus*) at one site in the foothills of the Sierra Nevada. Field observations reveal that northern Pacific rattlesnakes are common near burrow systems during the reproductive season and periods of pup emergence. They are responsible for approximately 40% of pup and juvenile mortality in ground squirrel colonies (Linsdale, 1946; Fitch, 1949; personal observation). Sympatry between California ground squirrels and Pacific rattlesnakes is indicated in late Pleistocene fossil assemblages (Miller, 1912; Stock, 1918; Brattstrom, 1953). Elsewhere in the United States, middle Miocene fossil deposits indicate longer sympatry between rattlesnakes and the *Otospermophilus* ancestor of modern sciurids (Black, 1963; Holman, 1979). Therefore, the intensity of this predator–prey relationship is probably not a recent phenomenon, and represents an important biotic interaction for both ground squirrels and rattlesnakes.

It is not surprising, therefore, that *S. beecheyi* exhibits a variety of strategies to deter rattlesnake predation. Beyond a primary defense of vigilance, these squirrels will aggressively confront snake predators detected near their burrows (Owings and Coss, 1977; Coss and Owings, 1989; Rowe and Owings, 1990, 1996; Swaisgood et al., 1999). However, active harassment requiring close approach to rattlesnakes exposes squirrels to the risk of envenomation. To protect against this risk, California ground squirrels possess innate blood plasma factors that allow them to survive envenomation by northern Pacific rattlesnakes (Poran et al., 1987; Poran and Coss, 1990). Serum-venom binding in radioimmunoassays (RIA) has been used extensively to characterize variation in resistance among *S. beecheyi* populations (Poran et al., 1987; Coss et al., 1993). Unfortunately, RIA binding levels provide only indirect information about functional differences in venom neutralization among *S. beecheyi* populations. Although the RIA binding of pooled sera from squirrel populations correlates highly with the same sera used to protect envenomated mice in approximate LD₅₀ tests (Poran et al., 1987), this method does not reveal the underlying biochemical dynamics of toxicity and resistance. Enzyme-inhibitor binding, such as that described for the resistance of opossum to rattlesnake venom (Catanese and Kress, 1993), is indistinguishable from a variety of other protein-protein interactions that result in high binding levels (Mishell and Shiigi, 1980).

Experimental Questions. Adult northern Pacific rattlesnakes (*C. o. oreganus*) possess venoms with multiple proteolytic and hemolytic venom toxins that ground squirrel prey must defend against (Mackessy, 1988, 1993, 1996). Biardi et al. (2000) showed that plasma from California ground squirrels can reduce the activity of venom proteases on hide powder azure and gelatin. However, this study did not specifically measure the activity of venom metalloproteases or toxins affecting clotting and hemolysis.

A more detailed understanding of the biochemical basis of resistance is essential when attempting to clarify the relationship between RIA binding levels and functional resistance to venom. For example, previous research has revealed conflicting evidence about resistance in two populations of Douglas ground squirrels (*S. b. douglasii*) that vary in local rattlesnake density. One location (Winters, Yolo County, CA) is in oak woodland habitat in the foothills on the west side of the Sacramento Valley where rattlesnakes are common. This site also holds a winter hibernacula for rattlesnakes (W. Hamilton, personal communication). As expected, squirrels from this location show high RIA binding levels (Coss et al., 1993). A nearby site (Davis, Solano Co., CA) is on the floor of the valley less than 20 km to the east where rattlesnakes are rare (Poran et al., 1987). RIA binding levels in Davis squirrels are 59% lower, which Coss et al. (1993) interpreted as a decline in resistance that correlated with colonization of this rattlesnake-rare habitat when the populations diverged about 8.6 ± 5.9 thousand years ago. However, Biardi et al. (2000) showed that plasma samples

from these two populations are indistinguishable in their capacity to inhibit proteolytic activity of northern Pacific rattlesnake venom.

One interpretation of this discrepancy is that the correlation between RIA and approximate LD₅₀ dose, determined using squirrels from sites where snakes are common (Poran et al., 1987), may not be an informative measure of resistance in squirrel populations where encounters with rattlesnakes are rare. An alternative interpretation is that reduced inhibition of other venom toxins is responsible for lower binding in the Davis population. One goal of this study was to use three additional measures of venom activity (metalloprotease activity, fibrinolysis, and hemolysis) in order to determine whether decreased RIA binding reflects venom resistance or nonfunctional binding interactions.

Additional measures of venom biochemical activity also provide more detailed information on how ground squirrel plasma defenses deal with venoms from different rattlesnake lineages. Other mammal species seem to be broadly resistant to toxins from a variety of venom (e.g., Huang and Pérez, 1980; Soto et al., 1988; Tomihara et al., 1990). However, northern Pacific rattlesnakes (*C. o. oregonus*) are the only pit vipers sympatric with ground squirrels in northern and central California (Klauber, 1972). Folsom Lake in the foothills of the Sierra Nevada east of Sacramento lies within the core of this range, and has a high density of northern Pacific rattlesnakes (Poran et al., 1987). Ground squirrels (*S. b. beecheyi*) from this site show high resistance when measured by experimental envenomation, *in vivo* protection of venom-challenged mice (Poran et al., 1987), and RIA binding levels (Poran and Coss, 1990). Squirrels in the Santa Ynez Valley inhabit a snake-rich location southwest of the Central Valley within the zone of intergradation between northern (*C. o. oregonus*) and southern Pacific (*C. o. helleri*) rattlesnakes (Klauber, 1972). Squirrels from these two regions show differences in allozyme frequencies (Goldthwaite, 1989), reflecting divergence during the late Pleistocene (Coss and Goldthwaite, 1995; Coss, 1999). They have also been subject to predation from simultaneously diverging lineages of Pacific rattlesnake predators (Pook et al., 2000; Ashton and de Queiroz, 2001; Douglass et al., 2002).

Mackessy (1988) found no significant differences between venoms from northern and southern Pacific rattlesnake venoms collected from Santa Barbara, San Luis Obispo, and Ventura Counties across several assays of enzymatic activity and toxicity (to lizards). Similarly, Bee et al. (2001) detected only minor differences in metalloprotease activity between these two venoms. However, genetic divergence and geographic separation in squirrel populations suggests the opportunity for coevolutionary change (Thompson, 1994, 1997; Nuismer et al., 1999; Gomulkiewicz et al., 2000) that may affect the ability of plasma defenses to deal with venom from these two subspecies.

In other words, we expect plasma defenses in squirrels from Folsom Lake to be more effective against northern Pacific rattlesnake venom. Conversely, plasma

defenses in squirrels from the Santa Ynez valley should be more effective against southern Pacific rattlesnake venom. Finally, venom from western diamondback rattlesnakes (*C. atrox*) constitutes a novel challenge to plasma defenses of squirrels in both of these locations. This venom also shows proteolytic, hemorrhagic, and hemolytic activity (Soto et al., 1988), but western diamondback rattlesnakes are a lower Sonoran species restricted to the deserts of the southeastern part of California (Klauber, 1972). We know that California ground squirrel plasma inhibits *C. o. oregonus* proteolytic activity more than *C. atrox* activity (Biardi et al., 2000), and we expect a similar result when measuring the effects on other venom toxins. Therefore, a second goal of this study was to assess the specificity of plasma defenses by challenging them with venom from sympatric and allopatric predators.

METHODS AND MATERIALS

Blood Collection and Storage. We collected blood samples from ground squirrels in four locations that varied in the density or identity of rattlesnake predators. The method of determining local snake density (\approx predation intensity) is described in Poran et al. (1987), supplemented by information on rattlesnake distributions prior to extensive human disturbance of the Central Valley (Grinnell and Camp, 1917; Nussbaum et al., 1983).

Two populations of Douglas squirrels (*S. b. douglasii*) were sampled: Winters (Yolo Co.) is in the Coast Range foothills and has a high density of northern Pacific rattlesnake predators (*C. o. oregonus*); the Davis site (Yolo Co.) is on the floor of the Sacramento valley where rattlesnakes are rare (only two *C. o. oregonus* reported during the last 25 yr). Two populations of Beechey squirrels (*S. b. beecheyi*) were sampled: Folsom Lake State Recreation Area (El Dorado Co.) has a high density of northern Pacific rattlesnakes (*C. o. oregonus*); Sedgwick Natural History Reserve (Santa Barbara Co.) in the Santa Ynez valley also has a high density of rattlesnakes, and lies within the range of southern Pacific rattlesnakes (*C. o. helleri*). The number of individuals collected at each location is as follows: Davis = 11; Winters = 16; Folsom Lake = 10; and Sedgwick Reserve = 9.

Squirrels were live-trapped and anesthetized with ketamine-HCl (50 mg/kg, i.m.). Approximately 3 ml blood were collected from individual adults via cardiac puncture and stored in additive-free Vacutainer blood collection vials (Becton Dickinson, Irvine, CA, USA). Squirrels were released at the site of capture following their recovery from anesthesia. Samples were kept at 4°C overnight, then clots were removed and discarded. The supernatant was centrifuged at $3000 \times g$ for 10 min at 4°C to separate any remaining erythrocytes, then stored at -70°C until used.

Venom Source and Preparation. Lyophilized venoms were purchased from Sigma (St. Louis, MO, USA). Although venoms were not collected from the same sites as squirrel plasma samples, venoms from Pacific rattlesnakes were obtained from regions within the geographic range of California ground squirrels (Hall, 1981). According to the supplier, northern Pacific rattlesnake (*C. o. oreganus*) venom was obtained from multiple snakes originating in northern California and Oregon. Venom from southern Pacific rattlesnakes (*C. o. helleri*) was obtained from snakes originating in southern California. Information on the site(s) of origin for western diamondback (*C. atrox*) venom was unavailable. Venoms were reconstituted at a concentration of 10 mg/ml in a buffer containing 20 mM Tris-HCl + 1 mM CaCl₂, pH 8.0, and stored at -20°C until used.

Assays of Venom Toxicity. Four assays examined the effects of ground squirrel plasma in inhibiting the proteolytic, collagen hydrolysis, hemolytic, and fibrinolytic activity of rattlesnake venom. Agarose-gelatin plates were prepared according to the method described by Palmer (1993) and used as a measure of total venom proteolytic activity. A template was used to punch equally spaced 3-mm-diam. wells in the surface of the gel, and wells were loaded with 20 µl assay mixture. The gel was then incubated at 37°C for 24 hr in a humidified chamber. Following incubation, unhydrolyzed gelatin was precipitated in a saturated ammonium sulfate solution at 70°C for 10 min, and the area of hydrolysis was calculated from the product of two perpendicular diameters across the clear lysis zone.

Snake venom metalloprotease activity was quantified using Azocoll (Sigma) as a collagen substrate to model the effects on mammalian connective tissue and extracellular matrix. One-ml aliquots of 1% Azocoll (w/v) solution in 20 mM Tris-HCl, pH 8.0, were prepared in a microcentrifuge tube. Assay mixture (20 µl) was added, and the reaction mix was incubated at 37°C for 2 hr with occasional agitation. Following incubation, the tubes were centrifuged for 2 min at 16,000 × g to pellet unreacted substrate. The absorbance of the supernatant was determined at 520 nm using a Shimadzu UV160 spectrophotometer.

BBL Stacker plates (Carolina Biological Supply) containing 5% defibrinated sheep's blood in Columbia agar were used to assay for hemolytic activity. Wells (3 mm diam.) were punched in the substrate in a grid pattern. Assay mixtures (20 µl) were loaded into each well, then the plate was sealed with Parafilm and incubated at 37°C for 24 hr. The area of hemolysis, defined as the transparent zone around the well cleared of red blood cells, was calculated from two perpendicular measurements of well diameter.

Fibrin agarose plates prepared by a modification of the method described by Astrup and Müllertz (1952) were used to measure venom fibrinolytic activity. Bovine fibrinogen was dissolved in Tris-HCl, pH 8.0, to a concentration of 1 mg/ml using a sonicator bath. Ten ml of fibrinogen solution were placed in a 100 × 15 ml Petri dish and clotted by the addition of 100 µl thrombin (100 units/ml). Clot formation was completed within 30 min, as determined by the

opacity of the fibrinogen solution. Assay mixtures (20 μ l) were applied to the surface of the clot, and the plate was incubated at 37°C for 24 hr in a humidified chamber. Following incubation, the area of hydrolysis was calculated from the product of two perpendicular diameters across the liquefied lysis zone.

Relative Activity Scores and Statistical Analyses. Plasma samples from individual squirrels were screened against relevant venoms, and individual scores were recorded for each of the four assays as the mean of at least three replicates. Assay mixtures used in each of the four methods consisted of a venom-only treatment (10 μ l venom + 10 μ l buffer) and a venom + plasma treatment (10 μ l venom + 5 μ l plasma + 5 μ l buffer), as well as negative controls [(buffer only) and (plasma only)]. The difference in activity between venom-only and venom + plasma treatments was expressed as a ratio:

$$\text{Relative activity} = \frac{\text{activity of [venom + plasma]}}{\text{activity of [venom only]}}$$

This allows for testing qualitative differences in the outcome of interactions between venom toxins and plasma defensive factors across the four assays. It simultaneously adjusts for differences in scaling due to differences among venoms in baseline activity on these assays (Soto et al., 1988).

Since relative activity scores are proportions, they were subject to arcsine transformation prior to statistical analysis to yield measures that followed an approximately normal distribution (Zar, 1984). Data were analyzed using a one-factor between-subjects (populations), two-factor within-subjects (assay type, venom type) repeated-measures ANOVA. Specific comparisons of population and venom effects were tested with planned contrasts of the relevant group means.

RESULTS

Plasma Defenses in Douglas Ground Squirrels (S. b. douglasii). Effects of Douglas ground squirrel plasma on venom activity are shown in Figure 1. The two populations differed in their ability to inhibit venom from the northern Pacific rattlesnake ($F_{3,18} = 18.96$, $P < 0.001$). This was primarily due to higher inhibition by animals from Winters (where snakes are common) in the collagenase assay ($F_{1,20} = 56.97$, $P < 0.001$). Winters animals were also slightly more effective at reducing venom hemolytic activity ($F_{1,20} = 7.25$, $P = 0.014$), although the mean difference was quite small—a change in relative activity of only 2.4% of baseline venom activity. There were no significant differences between plasma samples from Davis and Winters squirrels in their effects on general proteolytic activity of northern Pacific rattlesnake venom. The two populations also did not differ in

their effect on fibrinolysis. In this case, relative activity increased to almost 1.8 times that of venom-only treatments (Figure 1a, b).

There were also differences in the effects of plasma samples on sympatric and allopatric venoms. Winters animals reduced proteolysis ($F_{1,25} = 17.21$, $P = 0.003$) and collagen hydrolysis ($F_{1,20} = 115.45$, $P < 0.001$) of northern Pacific rattlesnake venom more than western diamondback venom. Plasma samples from Davis animals were also more effective at reducing proteolytic activity of *C. o. oregonus* venom ($F_{1,25} = 6.44$, $P = 0.018$), but showed no difference in their effect on collagen hydrolysis by the two venoms ($F_{1,26} = 0.67$, $P > 0.05$).

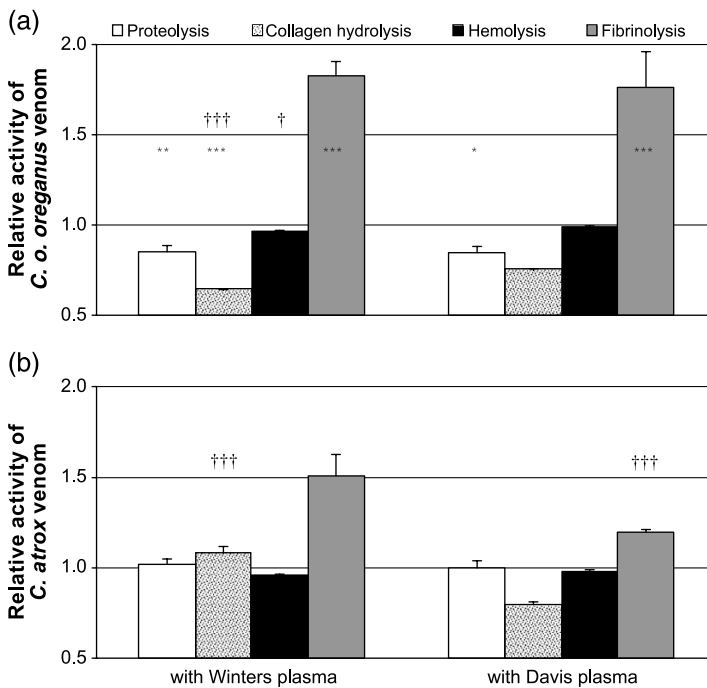


FIG. 1. Activity (mean \pm SE) of rattlesnake venoms incubated with plasma samples from two populations of Douglas ground squirrels (*S. b. douglasii*) that vary in local density of northern Pacific rattlesnakes (*C. o. oregonus*). Winters is a location where rattlesnakes are common, while Davis is a site where snakes are rare. Western diamondback rattlesnakes (*C. atrox*) are allopatric with both populations of squirrels. Activity is expressed relative to venom controls as described in Methods and Materials. Daggers indicate significant differences between the two squirrel populations ($\dagger P < 0.05$, $\dagger\dagger\dagger P < 0.001$). Asterisks indicate significant differences between the two venoms ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Plasma was less effective against *C. o. oregonus* venom than against *C. atrox* venom in the fibrinolytic assay ($F_{1,20} = 16.12$, $P < 0.001$). There was no significant difference between venoms in the hemolytic assay ($F_{1,25} = 1.24$, $P > 0.05$).

Plasma Defenses in Beechey Ground Squirrels (S. b. beecheyi). There were no differences between plasma from Folsom Lake and Sedgwick in their effects on venom proteolysis, collagen hydrolysis, or hemolysis (all tests: $P > 0.05$) (Figure 2). While plasma from Sedgwick squirrels generally induced higher

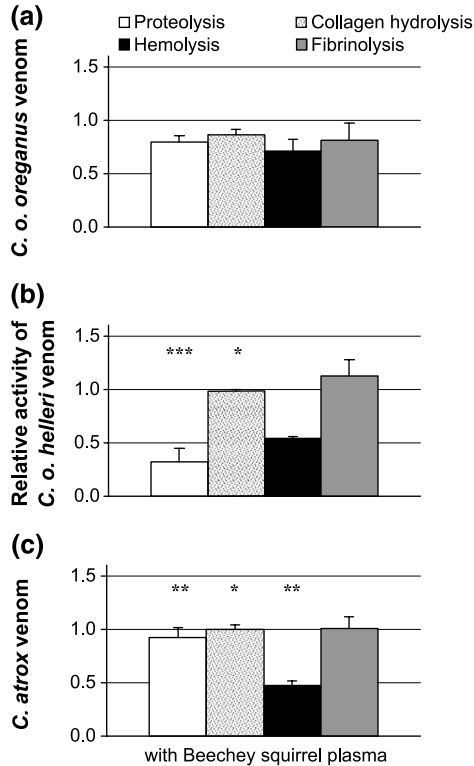


FIG. 2. Activity (Mean \pm SE) of rattlesnake venoms incubated with plasma samples from two populations of Beechey ground squirrels (*S. b. beecheyi*) that are subject to predation by Pacific rattlesnakes. Folsom Lake is a site where northern Pacific rattlesnakes (*C. o. oregonus*) are common, while the Sedgwick Reserve is a site within the zone of intergradation between northern and southern Pacific (*C. o. helleri*) rattlesnakes. Western diamondback rattlesnakes (*C. atrox*) are allopatric with both populations of squirrels. Activity is expressed relative to venom controls as described in Methods and Materials. Asterisks indicate significant differences from the effect on *C. o. oregonus* venom (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

relative activity in the fibrinolysis assays than Folsom plasma samples, this difference also was not statistically significant ($F_{1,10} = 1.43$, $P > 0.05$). Consequently, data from the two Beechey populations were combined for analysis of the effects of plasma defenses across different venoms.

Beechey squirrel plasma varied in effectiveness against general proteolytic activity of the three venoms ($F_{2,32} = 19.8$, $P < 0.001$)—Southern Pacific rattlesnake (*C. o. helleri*) venom was inhibited more than northern Pacific rattlesnake venom ($F_{1,16} = 11.09$, $P = 0.004$), which in turn was inhibited more than western diamondback venom ($F_{1,16} = 18.24$, $P < 0.001$). In contrast, Beechey plasma samples reduced northern Pacific rattlesnake metalloprotease activity more than southern Pacific ($F_{1,17} = 5.843$, $P = 0.032$) or western diamondback ($F_{1,17} = 5.536$, $P = 0.031$) venom. Hemolytic activity of western diamondback venom was reduced more than that of the two Pacific rattlesnake venoms ($F_{2,10} = 8.03$, $P = 0.008$). There were no significant differences in the way Beechey plasma samples affected fibrinolysis by the three venoms.

DISCUSSION

Although California ground squirrel sera-to-venom binding level in RIAs correlates highly with approximate LD_{50} in mice, this method does not directly measure the ability of prey sera to inhibit specific venom toxins. We observed distinct differences in the capacity of plasma proteins from California ground squirrels to affect the proteolytic and hemolytic activities of rattlesnake venoms. The protease, metalloprotease, and hemolytic activity of all three venoms were reduced in the presence of prey plasma from one or more ground squirrel populations. However, Douglas squirrels are unable to decrease venom fibrinolysis. In fact, fibrinolytic activity of northern Pacific rattlesnake venom is almost twice as high when incubated with plasma from Douglas squirrels (Figure 1a) than when assayed alone (relative activity = 1.0) or against Beechey squirrel plasma (Figure 2a). Fibrinolysis may result from plasmin-like activity of venom toxins or from activation of endogenous serum components (Hofmann and Bon, 1987; Braud et al., 2000). Increased activity of venom when incubated with serum suggests that venom not only maintains effectiveness in the face of prey defenses, but can also capitalize on the biochemical properties of prey tissues. RIA binding levels, therefore, may represent a multitude of interactions between venom and prey tissues that simultaneously reduce and enhance the toxic activities of venom. Radioimmunoassays have been valuable in screening for population differences in venom resistance in California ground squirrels (Poran et al., 1987; Poran and Coss, 1990; Coss et al., 1993). However, assays of venom + plasma interactions clarify the functional basis of differences in

binding levels, and will be more valuable in understanding the consequences of variation in prey defenses.

In contrast to enhanced fibrinolytic activity in the presence of Douglas blood plasma, samples from all four populations significantly reduced the metalloprotease activity of northern Pacific rattlesnake (*C. o. oreganus*) venom (Figures 1a and 2a). This is consistent with our understanding of antihemorrhagic factors identified in other mammalian prey of viperid snakes, which have been identified as snake venom metalloprotease inhibitors (SVMPI) (Pérez and Sánchez, 1999). The ability to inhibit hemorrhagic toxins appears to be a convergent response to envenomation, perhaps because the major threat of viper venoms, hemorrhage *in rhexis*, is thought to result from hydrolysis of collagen-rich connective tissue lining the capillary endothelium (Baramova et al., 1989; Tu, 1991). Minimizing the ability of venom toxins to rupture blood vessels can have a cascade of multiple subsequent protective benefits, including reduced access of other venom toxins to target tissues, reduced edema, and continued circulation of blood to regions near the bite, enhancing tissue repair and minimizing necrosis (Gutiérrez and Rucavado, 2000; Anai et al., 2002). However, it is surprising that the metalloprotease activity of southern Pacific and western diamondback venoms was relatively unaffected, since defenses specialized to one or a few venoms seems to be rare. For example, Soto et al. (1988) showed that sera from opossum (*D. virginiana*) and woodrats (*N. micropus*) were effective at preventing hemorrhage by all 47 species of snakes they examined. California ground squirrels encounter only a single rattlesnake species (*C. o. oreganus*) from central California northward to British Columbia. We suspect that coevolution may have favored plasma defenses in populations from the Central Valley that are especially effective against venom from this species.

Generalized resistance to venom metalloproteases is similar to the pattern observed in animals from the Davis site, where rattlesnakes are rare (cf. Figure 1a, b). Compared to the nearby Winters population, plasma factors were not as effective against the local rattlesnake predator (*C. o. oreganus*), but maintained some effectiveness against western diamondback (*C. atrox*) venom. Antihemorrhagic proteins isolated from other mammals show homology to proteins in the immunoglobulin supergene family (Perales and Domont, 2002), which includes plasma serpins and other protease and metalloprotease inhibitors. Catanese and Kress (1993) identified differences in the amino acid sequence of oprin (the antihemorrhagic factor from *Didelphis virginiana*) that distinguish it from other mammalian α_1 -proteinase inhibitors. Presumably because of these differences, oprin can bind and inhibit venom metalloproteases while these toxins rapidly hydrolyze human α_1 -proteinase. We suspect that consistent selection from northern Pacific rattlesnake toxins in the Winters, Folsom Lake, and Sedgwick Reserve animals has favored sequence changes in ground squirrel SVMPI that

maximize effectiveness against this venom. Since venom metalloproteases are also subject to selection favoring escape from inhibition by prey defenses, reciprocal changes may have led to SVMP and SVMPI variants unique to these species. Reactive center sequences in plasma serpins are hypervariable and affect protease recognition and inhibition effectiveness (Hill and Hastie, 1987; Goodwin et al., 1996). It will be interesting to see whether Davis individuals, and squirrels from other sites where snakes are rare or absent, possess plasma factors with a reactive site sequence similar to the precursor of SVMPIs in this species. Such sequences may confer some level of protection against xenologous metalloproteases, including those from a variety of snake venoms, but may not represent a form optimized to inhibit SVMP from the lone sympatric species, northern Pacific rattlesnakes.

The contrast between squirrel blood plasma effects on metalloprotease and fibrinolytic activity may reflect alternative solutions to venom toxicity by rattlesnakes and ground squirrels. Although ground squirrel plasma factors can reduce the activity of venom metalloproteases, rattlesnakes may still benefit by immobilizing prey as they disrupt hemostasis. Elevated fibrinolytic activity of *C. o. oregonus* venom may aid snakes in subduing prey by the induction of hypotensive shock if internal bleeding after envenomation is prolonged (Mackessy, 1993; Mackessy et al., 2003). This would increase the likelihood of obtaining a valuable meal, as long as sufficient SVMP activity is maintained to facilitate the rupture of prey viscera after ingestion (Thomas and Pough, 1979).

From the squirrel's perspective, avoidance of tissue damage from digestive venom toxins may be the crucial task of plasma defenses since behavioral defenses may buffer against the risk posed by venom-induced shock. Poststrike trailing by *C. oregonus* depends on a chemosensory profile of individual prey items developed from cues obtained during the strike and subsequent envenomation (Kardong and Smith, 2002). Chemosensory searching by vipers using this profile may last hours, and perhaps days after envenomation (see Diller, 1990), and may depend in part on tissue damage in prey after envenomation (Greenbaum et al., 2003). It is possible that inhibition of venom proteolytic activity may play a role in disrupting a snake's ability to search for prey. Certainly, recruitment of other colony members to mob rattlesnakes (Owings and Coss, 1977; Hersek and Owings, 1993) may minimize the opportunity for snakes to trail and ingest envenomated squirrels, even if they are temporarily immobilized by shock. In this case, the digestive effects of metalloproteases may pose a greater threat to squirrel fitness due to the potential for lasting damage to tissues. Studies of venom resistance in the rock squirrel, *Spermophilus variegatus*, a closely related species that also shows aggressive confrontation of rattlesnake predators (Owings et al., 2001), also indicate that metalloprotease inhibition is of primary importance (Biardi, 2000).

Our measures of venom activity also revealed significant differences between Pacific rattlesnake venoms when preying on the Beechey subspecies of California ground squirrels (*S. b. beecheyi*). We expected to find differences between squirrels from the Folsom and Sedgwick sites, but were unable to distinguish between them using any of the four assays of resistance. Instead, we observed striking differences between venoms. Northern Pacific rattlesnake venom maintained greater activity in the general proteolytic and hemolytic assays, whereas southern Pacific rattlesnake venom maintained greater effectiveness in the metalloprotease and fibrinolysis assays (cf. Figure 2a,b). This was surprising because previous studies have not identified clear differences in activity between these venoms (Mackessy, 1988; Bee et al., 2001). However, our results show that these venoms pose distinctly different threats to ground squirrels inhabiting a site (like the Sedgwick Reserve) where they are likely to encounter both snake species.

The role of Douglas squirrel plasma in enhancing fibrinolytic activity, inhibition of venom metalloproteases by ground squirrel SVMPI, and the detection of differences in northern and southern Pacific rattlesnake venoms in the presence of Beechey squirrel plasma all highlight the adaptive context of venoms and resistance. Our assays of venom activity in the presence of resistance factors revealed variation that is not detected in assays of venoms alone, and reinforce the idea that the outcome of envenomation will depend on both predator and prey, and will, in turn, affect the evolution of both venom toxicity and resistance.

Detection of previously cryptic differences between *C. o. oregonus* and *C. o. helleri* venoms may reflect an arms race that has generated variation within and between predators and prey. Relationships among species over evolutionary time can foster reciprocal adaptation of traits crucial to their interaction (Futuyma and Slatkin, 1983). Because populations of California ground squirrels vary in their ability to neutralize venom toxins (Biardi, 2000; Biardi et al., 2000, this study), and because this variation has important implications for the fitness of both predators and prey, both theoretical (e.g., Thompson, 1994, 1997) and modeling (e.g., Gomulkiewicz et al., 2000) approaches to coevolution predict corresponding local variation in venom from northern Pacific rattlesnakes. There are no published studies describing population differences in venom for this species; unfortunately, our use of pooled venoms also does not allow us to detect differences at the population level.

However, population variation in venoms related to diet is not unknown. For example, Daltry et al. (1996a,b) demonstrated that population variation in venom composition of the Malayan pit viper (*Calloselasma rhodostoma*) is strongly influenced by diet. However, this study related variation to the relative representation of different prey categories (amphibians, reptiles, and “endotherms”) in the diet, making it unclear whether the effect is attributable

to basic structural and physiological differences among prey categories, or more specific defenses to venoms such as we observe in ground squirrels. The subsequent debate between Sasa (1999a,b) and Wüster et al. (1999) centering on neutral vs. adaptive explanations for variability in snake venoms highlighted the importance of studying venoms in their natural context. To date, however, studies designed to test coevolution of venomous snakes and their prey remain exceedingly rare (e.g., Heatwole and Powell, 1998; Jorge da Silva and Aird, 2001).

Research on snake venom chemistry has an extensive history, driven primarily by interest in the clinical treatment of snakebite and the characterization of toxins in a therapeutic and research context. However, approaches to understanding venoms in a natural context (Mackessy, 1988; Daltry et al., 1996a,b; Kordis and Gubensik, 2000; Creer et al., 2003; Mackessy et al., 2003), especially considering venom resistance (Poran et al., 1987; Poran and Coss, 1990; Coss et al., 1993; Heatwole and Poran, 1995; Chiszar et al., 1999; Biardi et al., 2000; Heatwole et al., 1999) are becoming increasingly common. Future studies that simultaneously evaluate local populations of snakes and prey are likely to provide additional surprising insights into toxicity and resistance that arise from the coevolutionary arms race at the molecular level.

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IDENTIFICATION, SYNTHESIS, AND FIELD EVALUATION OF THE SEX PHEROMONE FROM THE CITRUS LEAFMINER, *Phyllocnistis citrella*[†]

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Abstract—Using male antenna as the sensing element, three electroantennographic detection (EAD)-active peaks were detected from pheromone gland extracts of the citrus leafminer, *Phyllocnistis citrella*. Based on gas chromatography (GC)-mass spectrometry and GC-infrared data, the semi-chemicals were tentatively identified as a novel pheromone, (Z,Z,E)-7,11,13-hexadecatrienal, a previously identified attractant, (Z,Z)-7,11-hexadecadienal, and (Z)-7-hexadecenal in a ratio of 30:10:1, respectively. Identification was confirmed with synthetic compounds, which gave retention times identical to those of the natural products on three capillary columns with polar and nonpolar phases. While traps baited only with the previously identified attractant alone did not catch any males in Brazil, binary and tertiary mixtures with the major constituents caught significantly more male moths than traps baited with five virgin females.

[†] This paper and the following paper (Moreira et al.) were submitted within a few days of each other. The editors and the authors agreed that they should be published in tandem.

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INTRODUCTION

Attractants for the citrus leafminer, *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae), are sorely needed not only for monitoring and controlling populations, but also for quarantine purposes as this serious pest of citrus is spreading rapidly. *P. citrella* was originally described in India (Stainton, 1856), but it is now established on five continents (Heppner, 1993). In the last decade, the citrus leafminer spread through Florida, Alabama, Louisiana, Texas, Brazil, and most Latin American countries (Heppner, 1999) and has reached California, Mexico, and Hawaii (Anonymous, 2004).

Field screenings of potential attractants for species in the Gelechiidae family led to the observation that Japanese populations of the citrus leafminer were captured in traps baited with (Z,Z)-7,11-hexadecadienal (Z7,Z11-16Ald) (Ando et al., 1985). However, subsequent field tests failed to catch males of *P. citrella* in traps baited with Z7,Z11-16Ald in China, Italy, Spain, USA, Turkey, and Brazil (reviewed in Sant'ana et al., 2003), although a related species, *P. wampella*, was captured with the same attractant in China (Du et al., 1989). These findings suggest there may be geographic variations in the pheromone system, prompting us to launch a collaborative project for the identification of the pheromone system of the citrus leafminer. In this work, we describe the isolation, identification, synthesis, and field evaluation of the sex pheromone of the Brazilian population of the citrus leafminer.

METHODS AND MATERIALS

Insects. Colonies, reared according to the protocol of Chagas and Parra (2000), were maintained at the Insect Biology Laboratory at Escola Superior de Agricultura Luiz de Queiroz (ESALQ), Universidade de São Paulo (USP) at $25 \pm 2^\circ\text{C}$, $65 \pm 10\%$ relative humidity, and a 14L:10D photoregime. Pupae were collected and held in individual glass vials (8×1 cm ID) containing a small piece of moistened filter paper. Adults were sexed using the criteria described by Jacas and Garrido (1996) and fed on a small drop of pure honey.

Pheromone Extraction and Isolation. Pheromone glands from groups of 200 2-d-old virgin females were excised during the last hour of the scotophase and the first hour of the photophase to coincide with the period of mating activity (Parra-Pedrazzoli et al., in press). The abdominal tips were immersed in hexane (200 μl) for ca. 1 hr. A total of 5000 gland-equivalent was pooled,

the extract was transferred to glass ampoules, which were sealed, and shipped to Davis. Crude extracts were subjected to flash column chromatography on silica gel (60–200 mesh; Fisher Scientific) by successively eluting with hexane–ether mixtures in the following order: 100:0 (hexane fraction), 99:1 (1% fraction), 98:2, 95:5, 90:10, 50:50, 0:100 (ether fraction). Aliquots of these fractions were shipped to Piracicaba, SP, Brazil for behavioral studies.

Electrophysiology. Gas chromatography-electroantennographic detection (GC-EAD) recordings were made with previously described methods and equipment (Cossé et al., 2005). The GC-EAD responses were amplified (500×) using an AC/DC UN-6 amplifier (Syntech) and analyzed on a computer equipped with an analog-to-digital conversion board (IDAC, Syntech) running GC-EAD software (Syntech). Samples were injected in splitless mode using a 6890 Series GC (Agilent Technologies, Palo Alto, CA, USA) fitted with a DB-1 column (J&W Scientific, Folsom, CA, USA; 15 m × 0.25 mm; 0.25 or 1.0 μm film thickness). GC-EAD sample analysis used temperature programs either from 50 to 280°C at 25°C/min (see Figure 1) or from 50 to 280°C in steps of 25°C/min to 180°C, 5°C/min until 215°C, and 25°C/min to 280°C (see Figure 4). Inlet temperatures were maintained at 280°C and GC-EAD effluent interface from postcolumn splitter was kept at 300°C.

Other Analytical Procedures. Gas chromatography-mass spectrometry (GC-MS) was performed either in EI or CI mode (methane) on a 5973 Network

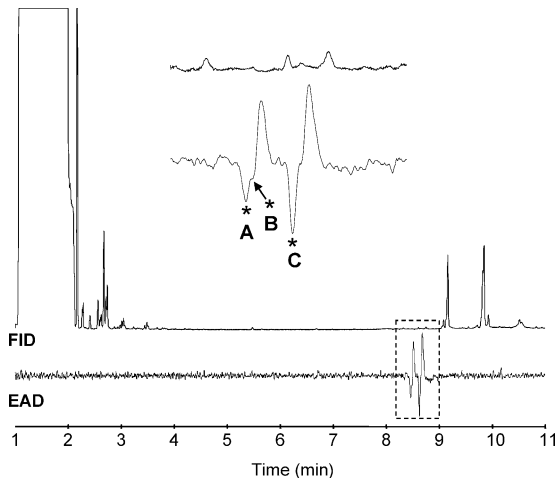


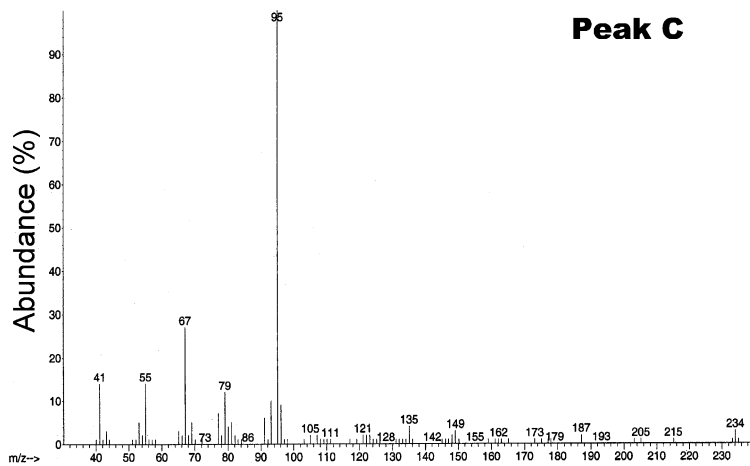
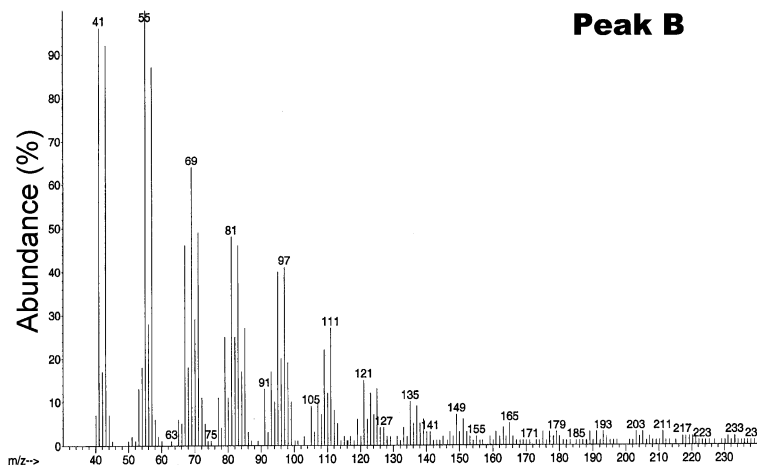
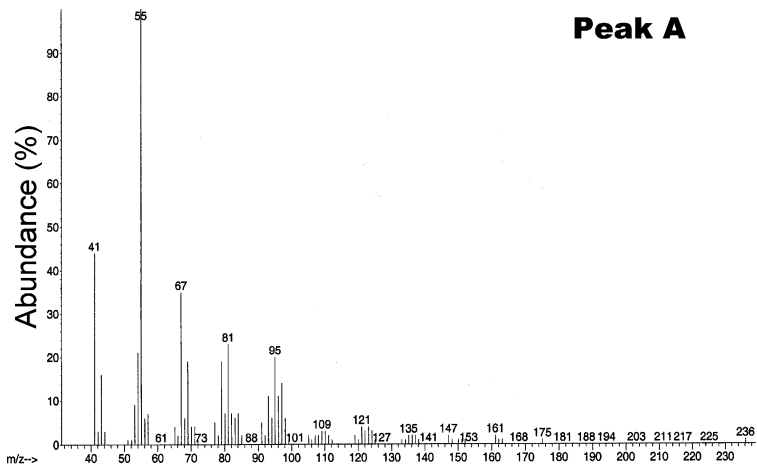
FIG. 1. Simultaneously recorded gas chromatogram (FID) and electroantennographic detection (EAD) of a male *P. citrella* antenna responding to a hexane extract of female *P. citrella* ovipositors (approx. 8 FE). Insert shows an enlarged section with antennal activity indicated by asterisk (*).

mass selective detector linked to a 6890 Network GC system (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation was done on three different columns: HP-5MS (Agilent; 30 m × 0.25 mm; 0.25 μm), HP-INNOWAX (Agilent; 30 m × 0.25 mm; 0.25 μm), and DB-1 column (15 m × 0.25 mm; 1.0 μm), which were operated at 70°C for 1 min, increased to 250°C (or 290°C) at a rate of 10°C/min (or 5°C/min), and held at the final temperature for 10 min, i.e., 70(1)-10-250(10), 70(1)-5-250(10), or 70(1)-10-290(10). Vapor phase infrared (IR) spectroscopy was done on a Win GC/IR Pro (Varian, Randolph, MA, USA) with a GC interface and a Scimitar FTS 2000 linked to a 6890 Network GC system (Agilent). Chromatographic separation was obtained with an HP-5 column (Agilent; 30 m × 0.32 mm; 0.25 μm) operated at 100(1)-20-250(5). Hydrogenation and dimethyl disulfide (DMDS) derivatizations were performed as previously described by Leal et al. (1996, 2001).

Synthesis of (Z,Z)-7,11-Hexadecadienal (Figure 3). A solution of **4** (10.8 g, 57.8 mmol) in THF (100 ml) was added to a THF solution (50 ml) of dibromopropane **3** (11.7 g, 57.8 mmol) and Li₂CuCl₄ (0.203 g, 0.925 mmol) at 5°C. After stirring for 2 hr, the mixture was gradually raised to room temperature and kept overnight. The reaction mixture was poured into diluted HCl and extracted with ether. The organic layer was washed with water, saturated aqueous NaHCO₃, brine, dried (MgSO₄) (hereafter referred to as the “washing protocol”), concentrated, and distilled to give **5** (6.16 g, 26 mmol). A solution of **5** (6.16 g, 26 mmol) in THF (10 ml) was added to a THF solution (30 ml) of lithium acetylide–ethylenediamine complex (3.11 g, 33.8 mmol) and HMPA (5 ml) at 5°C. After stirring for 2 hr, the mixture was gradually raised to room temperature and kept overnight under the same conditions. The reaction mixture was poured into diluted HCl and extracted with ether. After treatment (washing protocol), the organic layer was concentrated and distilled to give **6** (4.26 g, 23.4 mmol). *n*-BuLi (1.6 mol/l solution in hexane, 14.6 ml, 23.4 mmol) was added to a THF solution (50 ml) of **6** (4.26 g, 23.4 mmol) at 5°C. After 30 min, **7** (4.47 g, 23.4 mmol) in 10 ml HMPA was added to the solution at 5°C. After refluxing for 8 hr, the reaction mixture was poured into diluted HCl and extracted with ether. After the washing protocol, the organic layer was concentrated and distilled to give **8** (3.41 g, 11.7 mmol). A mixture of **8** (3.41 g, 11.7 mmol), 5% Pd/BaSO₄ (0.17 g), and quinoline (1.7 g) in 30 ml of MeOH was stirred for 3 hr in an atmosphere of hydrogen. The reaction mixture was poured into diluted HCl and extracted with ether. Following the washing protocol, the organic layer was concentrated *in vacuo*. The residual material was chromatographed on silica gel column (hexane–ether, 9:1) to give **9** (3.07 g, 10.5 mmol). **9** (3.07 g, 10.5 mmol) was stirred with *p*-toluenesulfonic acid monohydrate (PTS, 100 mg, 0.53 mmol) in 30 ml of MeOH at 60°C. After stirring for 2 hr, the reaction mixture was poured into water and extracted with ether, treated with the washing protocol, and concentrated *in vacuo* to give **10**

(2.65 g, 9.41 mmol). **10** (2.65 g, 9.41 mmol) was added to 100 ml THF and diluted HCl solution at room temperature. After stirring for 1 hr, the reaction mixture was poured into water and extracted with ether. After the washing protocol, the organic layer was concentrated *in vacuo*. The residual material was chromatographed on silica gel column (hexane–ether, 9:1) to give **1** (2 g, 8.47 mmol, >98% purity). IR (ν_{\max} , film, cm^{-1}): 2927, 2858, 2715, 1728, 1654, 1458, 1099, 1011, 914. ^1H NMR (300 MHz, CDCl_3): δ 0.90 (3H, t, $J = 7.5$ Hz), 1.27–1.42 (8H, m), 1.64 (2H, quintet, $J = 7.5$ Hz), 2.00–2.09 (8H, 2 broad peaks), 2.43 (2H, dt, $J = 2.1$ Hz, $J = 7.2$ Hz), 5.31–5.43 (4H, m), 9.77 (1H, t, $J = 1.8$ Hz). ^{13}C NMR (75.4 MHz, CDCl_3) δ 14.0, 22.0, 22.4, 27.0 (2C), 27.4 (2C), 28.8, 29.4, 31.9, 43.9, 129.1, 129.6, 129.8, 130.4, 202.8. GC-MS: m/z 236, 218, 175, 161, 147, 137, 123, 121, 109, 95, 93, 81, 69, 67, 55 (100%), 41.

Synthesis of (Z,Z,E)-7,11,13-Hexadecatrienal (Figure 6). **5** (2.37 g, 10 mmol) and NaI (2.98 g, 20 mmol) were added into acetone (50 ml) at room temperature. After the solution was refluxed for 2 hr, the reaction mixture was poured into water and extracted with ether. The organic layer was washed (see washing protocol) and concentrated *in vacuo*. The residual material was chromatographed on silica gel column (hexane–ether, 9:1) to give **11** (2.52 g, 8.91 mmol). *n*-BuLi (1.6 mol/l solution in hexane, 8.4 ml, 13.4 mmol) was added into THF solution (30 ml) of **12** (5-chloro-1-pentyne 1.37 g, 13.4 mmol) at 5°C. After 30 min, **11** (2.52 g, 8.91 mmol) in HMPA (5 ml) was added into the solution at 5°C. After the solution was refluxed for 2 hr, the reaction mixture was poured into diluted HCl and extracted with ether. Following the washing protocol, the organic layer was concentrated and distilled to give **13** (1.84 g, 7.13 mmol). **13** (1.84 g, 7.13 mmol) was stirred with CH_3COOK (1.37 g, 14 mmol) in HMPA (30 ml) at 60°C. After stirring for 2 hr, the reaction mixture was poured into diluted HCl and extracted with ether. The organic layer was washed (see washing protocol) and concentrated *in vacuo* to give **14** (1.62 g, 5.7 mmol). A mixture of **14** (1.62 g, 5.7 mmol), 5% Pd/BaSO₄ (0.081 g), and quinoline (0.81 g) in MeOH (10 ml) was stirred for 1 hr in an atmosphere of hydrogen. The reaction mixture was poured into diluted HCl and extracted with ether. The organic layer was washed (see washing protocol) and concentrated *in vacuo*. The residual material was chromatographed on silica gel column (hexane–ether, 9:1) to give **15** (1.45 g, 5.13 mmol). A solution of **15** (1.45 g, 5.13 mmol) in MeOH (5 ml) was added into MeOH solution (20 ml) of KOH (0.56 g, 10 mmol) and water (2 ml) at 5°C. After stirring for 1 hr, the reaction mixture was poured into water and extracted with ether. After treatment with the washing protocol, the organic layer was concentrated *in vacuo*. The residual material was chromatographed on silica gel column (hexane–ether, 5:5) to give **16** (1.1 g, 4.62 mmol). Pyridinium dichlorochromate (PDC) was added into CH_2Cl_2 solution of **16** (370 mg, 1.54 mmol) at 5°C. After stirring for 2 hr, the temperature was gradually raised to room temperature and maintained overnight



under the same conditions. The reaction mixture was chromatographed on silica gel column by ether and concentrated *in vacuo* to give **17** (294 mg, 1.23 mmol). *n*-BuLi (1.6 mol/l solution in hexane, 1.6 ml, 2.46 mmol) was added to a THF solution (20 ml) of **18** (1.01 g, 2.46 mmol) at -20°C . After stirring for 30 min, the temperature was gradually raised to room temperature. After 1 hr at room temperature, the solution was cooled to -100°C . **17** (294 mg, 1.23 mmol) in THF (2 ml) was added to the solution at -100°C . After stirring for 2 hr, the reaction mixture was poured into water and extracted with hexane. The organic layer was washed (see washing protocol) and concentrated *in vacuo*. The residual material was chromatographed on silica gel column (hexane–ether, 95:5) to give **19** (288 mg, 0.987 mmol). **19** (288 mg, 0.987 mmol) was stirred with PTS (9.6 mg, 0.05 mmol) in MeOH (30 ml) at 60°C . After stirring for 2 hr, the reaction mixture was poured into water and extracted with ether. Following the washing protocol, the organic layer was concentrated *in vacuo* to give **20** (249 mg, 0.888 mmol). **20** (249 mg, 0.888 mmol) was added to 10 ml THF and diluted HCl solution at room temperature. After stirring for 1 hr, the reaction mixture was poured into water and extracted with ether. The organic layer was washed (see washing protocol) and concentrated *in vacuo*. (7*Z*,11*E*,13*E*)-7,11,13-hexadecatrienal was removed as a Diels–Alder adduct with tetracyanoethylene. The residual material was chromatographed on silica gel column (hexane–ether, 9:1) to give **2** (110 mg, 0.47 mmol, >96% purity). IR (ν_{max} , film, cm^{-1}): 2927, 2854, 2715, 1728, 1651, 1458, 1076, 983. ^1H NMR (300 MHz, CDCl_3) δ : 1.02 (3H, t, $J = 7.5$ Hz), 1.31–1.41 (4H, m), 1.57–1.66 (2H, m), 2.00–2.26 (8H, m), 2.42 (2H, dt, $J = 1.8$ and 7.5 Hz), 5.26–5.43 (3H, m), 5.71 (1H, dt, $J = 6.6$ and 15.0 Hz), 5.96 (1H, t, $J = 11.1$ Hz), 6.29 (1H, ddt, $J = 1.5$, 11.1 and 15.0 Hz), 9.76 (1H, t, $J = 1.8$ Hz). ^{13}C NMR (75.4 MHz, CDCl_3) δ : 13.6, 22.0, 25.9, 27.0, 27.3, 27.8, 28.8, 29.4, 43.9, 124.6, 129.0, 129.2, 129.3, 130.0, 136.5, 202.8. GC-MS: m/z 234, 205, 173, 149, 135, 121, 107, 95 (100%), 79, 67, 55, 41.

(*Z*)-7- and (*Z*)-11-Hexadecenal were purchased from a commercial source (Shin-Etsu Chemical Co. Ltd., Tokyo, Japan).

Formulation and Field Evaluation. Hexane solutions of the synthetic pheromone constituents were shipped to Brazil and maintained at -80°C until use. Several hours before field experiments (August 3, 2004 to April 23, 2005), test blends were incorporated into rubber septa (Aldrich) at two different dosages (50 and 500 μg per septum) in the triene/diene/monoene ratio of 30:10:1. The lures were placed into delta traps, which were hung in a citrus orchard (Fazenda Quatrimãs, Botucatu, SP, Brazil) at a height of 1.6 m and with intertrap distance of 30 m. Pheromone traps were set in randomized blocks

FIG. 2. GC-MS data for peaks A, B, and C obtained from virgin female *P. citrella* pheromone glands.

(four replicated per block) along with control (empty traps) and those baited with five 2-d-old virgin females. Each set of experiments was conducted in two different locations with at least three temporal replicates. After daily inspection, virgin females were replaced, and traps were rotated to rerandomize the experimental block. Capture data were transformed to $\sqrt{(x + 0.5)}$ and analyzed by ANOVA (Tukey's test, 5%).

RESULTS AND DISCUSSION

Male attraction to female gland extracts was demonstrated in indoor bioassays conducted from 1 hr before to 1 hr after the onset of photophase (data not shown), coinciding with the peak of mating activity (Parra-Pedrazzoli et al., in press). After flash chromatography of the crude extract, behavioral activity was recovered in the 2% fraction, which was also EAD-active. Three EAD-active peaks (A, B, and C) were detected (Figure 1), with residual EAD activity of the peak C being also detected in a subsequent fraction (5%). This elution pattern suggests that the three compounds had the same functional group. Although not clearly identified in GC-MS analysis in the electron impact mode (Figure 2), CI-MS indicated that the molecular weights of A, B, and C were 236, 238, and 234, respectively. Based on the retention times of saturated hydrocarbon standards [GC-MS, HP-5MS, 70(1)-10-250(10); C₁₇, 13.11; C₁₈, 14.19; C₁₉, 15.21 min], we hypothesized that the three compounds were hexadecadienal, hexadecenal, and hexadecatrienal, respectively. To compare the EAD-active compounds with the previously identified attractant, we prepared a sample of (*Z,Z*)-7,11-hexadecadienal (**1**) in 14.7% overall yield, starting from dibromopropane (Figure 3). Synthetic *Z*7,*Z*11-16Ald was indistinguishable from peak A in the MS profile and gave identical retention times in polar and nonpolar capillary columns (HP-5MS, DB-1, and HP-INNOWAX). The *Z,Z* stereochemistry of the natural 7,11-hexadecadienal was further supported by GC-FTIR, which showed a characteristic *cis* C–H stretching band (medium intensity) at 3012 cm⁻¹ (Leal, 1998). Next, we tested whether the monoene B was biosynthetically related to the diene A. MS from both (*Z*)-7- and (*Z*)-11-hexadecenal were similar to that of the natural product. The retention times of (*Z*)-7-hexadecenal and B in three capillary columns were identical, but that of (*Z*)-11-hexadecenal differed (e.g., HP-5MS, 70(1)-10-250(5); Z11-16Ald, 14.27 min; B, 14.16 min). Given the small amount of sample available, it was not possible to determine analytically (GC-IR) the stereochemistry of the double bond. However, the retention time of the (*E*)-isomer differed from that of the natural product (e.g., HP-5MS, 70(1)-10-250(5); E7-16Ald, 14.21 min; natural product, 14.16 min). In addition, GC-EAD analysis further confirmed that peak B was indeed (*Z*)-7-hexadecenal (Figure 4).

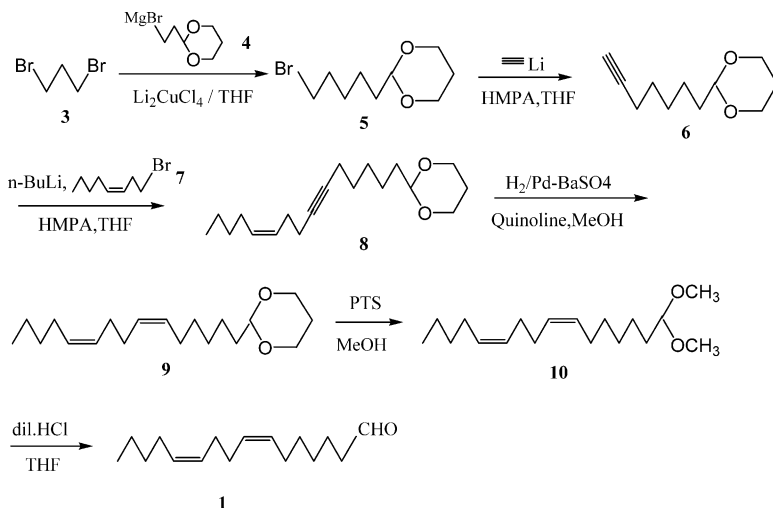


FIG. 3. Synthetic route for (Z,Z)-7,11-hexadecadienal.

Hydrogenation of a fraction containing peaks A, B, and C (50 gland-equivalent) generated the saturated 16Ald, thus suggesting that peak C was a related aldehyde with three unsaturations. Attempts to determine the positions of the double bonds by DMDS were unrewarding. With a large sample pooled

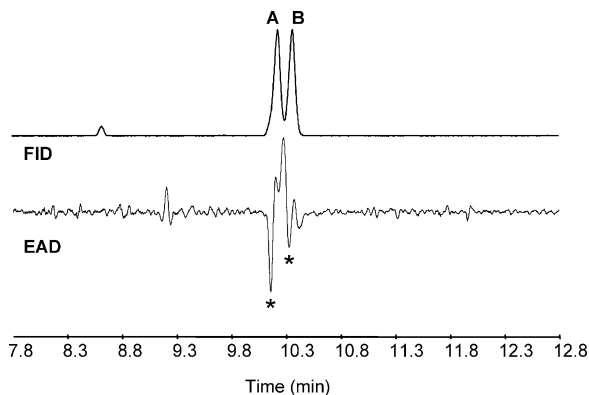


FIG. 4. Simultaneously recorded gas chromatogram (FID) and electroantennographic detection (EAD) of a male *P. citrella* antenna responding to synthetic (Z,Z)-7,11-hexadecadienal (A) and (Z)-7-hexadecenal (B). Asterisk (*) denotes antennal activity.

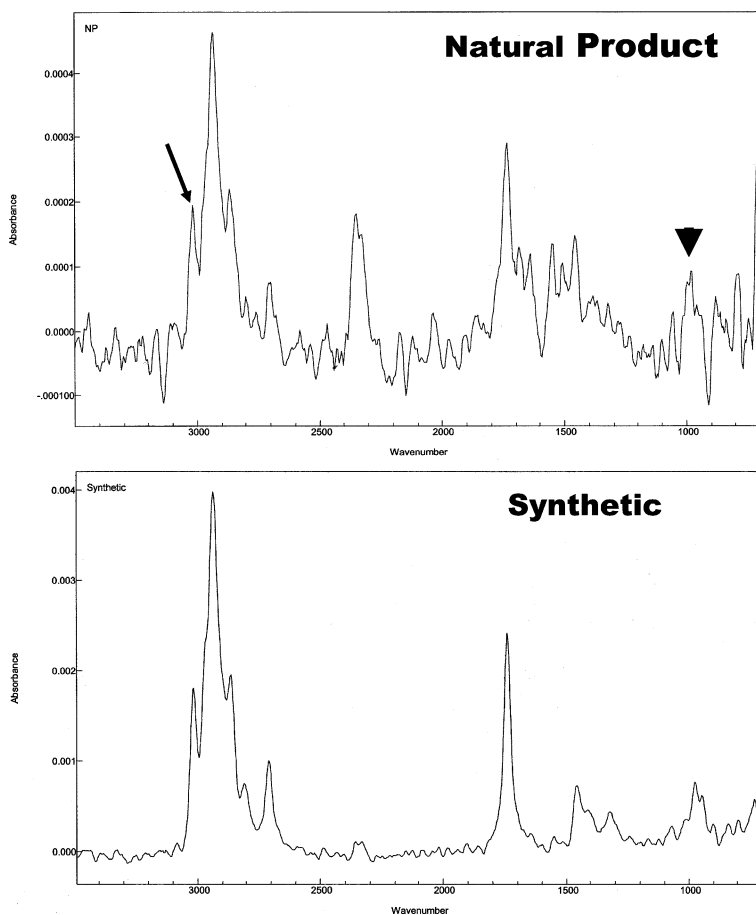


FIG. 5. GC-IR data for the natural (top) and synthetic (bottom) (*Z,Z,E*)-7,11,13-hexadecatrienal. Arrow and arrowhead indicate a = CH stretching and a CH bending of *cis* and conjugated *trans* configuration, respectively.

from ca. 2500 females-equivalent, we obtained a GC-IR trace (Figure 5). A band of medium intensity at ca. 3018 cm^{-1} suggests that C had two double bonds in *cis*-configuration as observed in A (data not shown), whereas a weak band at ca. 980 cm^{-1} indicated one conjugated double bond in the *trans*-configuration. Note also the carbonyl band at 1742 cm^{-1} , which is typical of aldehydes (Leal, 1998). In conjugated *Z,E*-dienes, the “*trans* band” ($940\text{--}990\text{ cm}^{-1}$) normally appears as a doublet, whereas a single band is characteristic of *E,E*-dienes (Leal, 1998). Given the limited amount of sample and consequently

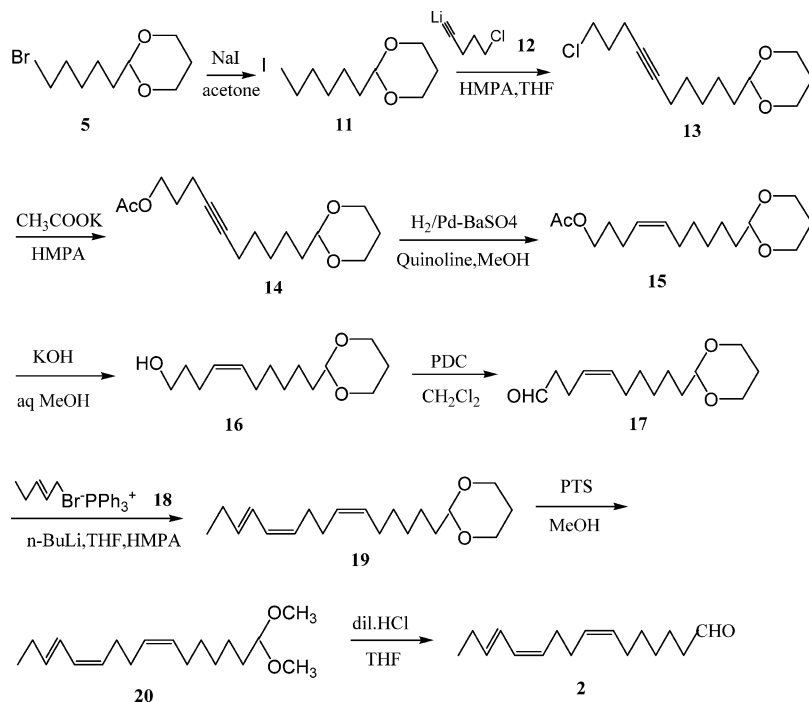


FIG. 6. Synthetic route for (Z,Z,E)-7,11,13-hexadecatrienal.

the poor resolution of small bands, it was not clear whether the *trans* band was a single or double peak. However, the medium intensity of the CH stretching band (Figure 5) suggests the occurrence of two double bonds in *cis* configuration, implying that the conjugated diene was in configuration *Z,E*. Considering that *P. citrella* biosynthesizes (*Z*)-7-hexadecenal, it is reasonable to assume that (*Z,Z*)-7,11-hexadecadienal is produced by the sequential action of a $\Delta 11$ desaturase on the 7-monoene (Jurenka, 2004). Similarly, the triene could be produced by the action of another desaturase on a 7,11-diene substrate. Given the conjugation in C, it could be produced by a $\Delta 5$, $\Delta 9$, or $\Delta 13$ desaturase. Based on the GC-IR data, we ruled out 7,9,11-hexadecatrienal, because an *E9* configuration flanked by (and conjugated to) two *Z*-double bonds would give a different ratio of the *cis/trans* bands, and probably shift the *trans* band. An examination of The Pherolist (Witzgall et al., 2004) indicated that, albeit common in pheromones with a shorter skeleton, it is unusual to have a double bond at position 5 in multiple unsaturated C-16 pheromones. It could be that $\Delta 5$ desaturases have a preference for C12 substrates, as demonstrated for the biosynthesis of (*E,Z*)-5,7-dodecadien-1-yl acetate (Ono et al., 2001). We then

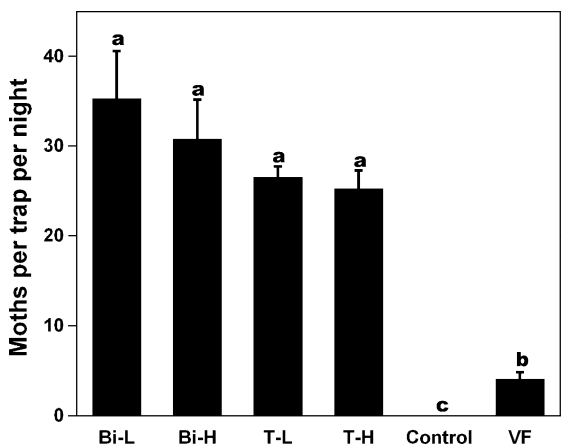


FIG. 7. Catches of male *P. citrella* in delta traps baited with five virgin females (VF), a binary mixture (triene + diene, 3:1), and a tertiary mixture (triene + diene + monoene, 30:10:1) at low (50 μg) and high (500 μg) doses. Experiments were run in randomized blocks for 3 d, with no catches in empty (control) traps. Bi-L: binary mixture, low dose; Bi-H: binary mixture, high dose; T-L: tertiary mixture, low dose; T-H: tertiary mixture, high dose. Treatments with the same letter are not significantly different (Tukey's test, 5%). Means of captures are untransformed, and error bars show 0.5 SE.

reasoned that C would be (*Z,Z,E*)-7,11,13-hexadecatrienal. A sample of authentic (*Z,Z,E*)-7,11,13-hexadecatrienal (**2**) was synthesized from **5** in 9% overall yield (Figure 6). MS and IR data (Figure 5) of the synthetic and natural products matched perfectly. Next, we compared the retention times of synthetic *Z7,Z11,E13*-16Ald and C in three different capillary columns. Both synthetic and natural products gave the same retention times in polar and nonpolar columns even when more shallow temperature programs were used [e.g., C and *Z7,Z11,E13*-16Ald appeared at 41.02 min in HP-INNOWAX with a ramp of 2.5°C/min, i.e., 70(1)-2.5-250(10)]. In summary, we identified three physiologically active compounds from the pheromone gland of *P. citrella*, namely, (*Z,Z*)-7,11-hexadecadienal, (*Z*)-7-hexadecenal, and (*Z,Z,E*)-7,11,13-hexadecatrienal, corresponding to peaks A, B, and C (Figure 1). These compounds appeared in crude extracts and a 2% fraction in the approximate ratio of 30:10:1, respectively.

In preliminary indoor Y-olfactometer bioassays, reported elsewhere (Parra-Pedrazzoli et al., in press), males responded to synthetic mixtures of the three compounds (but not to single constituents). They not only were attracted to the side-arm with pheromone, but also displayed the same courtship behavior observed when males approach virgin females. In the field, there were no

catches in traps baited with Z7,Z11-16Ald alone or in combination with Z7-16Ald (10:1). However, traps baited with mixtures containing the major constituent, Z7,Z11,E13-16Ald, caught significantly more males than virgin female-baited traps (Figure 7). These experiments indicate that mixtures of Z7,Z11,E13-16Ald and Z7,Z11-16Ald are attractants to males. However, the function of the minor EAD-active Z7-16Ald was not clear. One possibility is that the monoene is a behavioral antagonist to other species. However, as *P. citrella* is an exotic species in Brazil, this hypothesis must await field tests in the native habitat of *P. citrella*.

Activity of the pheromone samples, formulated in rubber septa with BHT, decreased dramatically over a 3-d period so experiments were run only in 3-d intervals before renewing the lures. We did not observe a significant difference in catches by traps loaded with low (50 µg) and high (500 µg) doses of pheromone. These results suggest that a long-lasting, low-releasing pheromone dispenser can be designed to monitor populations of the citrus leafminer.

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IDENTIFICATION, SYNTHESIS, AND FIELD TESTING OF THE SEX PHEROMONE OF THE CITRUS LEAFMINER, *Phyllocnistis citrella*

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Abstract—The citrus leafminer is an important vector of citrus canker in many of the major citrus production areas of the world. (7Z,11Z)-Hexadecadienal was reported as a sex attractant for this insect in the 1980s, based on trap catches during pheromone screening trials in Japan. However, attempts to reproduce this work in other areas of the world have not been successful. We report here that (7Z,11Z)-hexadecadienal is only one component of the pheromone, with the other critical component being the analogous trienal, (7Z,11Z,13E)-hexadecatrienal. Both compounds were identified in the effluvia from live female moths by coupled gas chromatography (GC)-electroantennography using nonpolar and polar GC columns, and the identifications were confirmed by comparisons of mass spectra with those of authentic standards. Stereoisomers of the two compounds, and a number of analogs, were synthesized to confirm the identifications. In field trials, neither compound alone was attractive to male moths, but blends of the two were highly attractive, with thousands of insects being caught per trial. Addition of the isomeric (7Z,11Z,13Z)-hexadecatrienal inhibited attraction to the two-component blend.

Key Words—Sex pheromone, (7Z,11Z)-hexadecadienal, (7Z,11Z,13E)-hexadecatrienal, 5,7,11-hexadecatrienal, citrus leafminer, (7Z,11Z,13Z)-hexadecatrienal.

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INTRODUCTION

The citrus leafminer (CLM), *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae), is a pest in most of the citrus-growing regions of the world (Heppner, 1993). It attacks all varieties of citrus and some related plant species, with grapefruit, tangerine, and pumello being among the most susceptible hosts (Legaspi and French, 2003). Although mature trees can tolerate some damage, growth of young trees in nurseries and newly planted orchards is reduced. Damage is caused by the larvae forming serpentine mines in the leaves, in which they are well protected from insecticide sprays, making them difficult to control. Even more important than direct damage is the potential for the adult moths to vector the highly contagious and lethal citrus canker bacterium, *Xanthomonas axonopodis* pv *citri*, where this pathogen occurs (Ando et al., 1985). The importance of this highly contagious disease to growers is reflected in the control program in Florida, USA, which calls for the destruction of all exposed citrus trees within 1900 ft of an infected one (Florida Department of Agriculture and Consumer Services, <http://www.doacs.state.fl.us/pi/canker/cankerflorida.pdf>).

There are currently no good methods for early detection and sampling of this insect to track the continued spread through California and other citrus-growing regions in the United States. Often, the first signs of its presence are the characteristic mines in the leaves, by which time the infestation may be well established. Although (7Z,11Z)-hexadecadienal (7Z,11Z-16:Ald) was reported as a sex attractant for this insect from screening trials 20 yr ago (Ando et al., 1985), in subsequent trials in other parts of the world, including China, Spain, Florida, and Italy (Jacas and Peña, 2002), this compound was not effective. Due to the threat of CLM to the California citrus industry, we undertook an investigation of its pheromone chemistry, and report that the pheromone of California populations consists of at least two components, the previously identified (7Z,11Z)-hexadecadienal, and the corresponding (7Z,11Z,13E)-hexadecatrienal (7Z,11Z,13E-16:Ald).

METHODS AND MATERIALS

Insects. Insects were obtained by collecting branches with infested leaves from lemon orchards in the Coachella Valley, Riverside Co., CA, USA and from Escondido, San Diego Co., CA. Branches with larvae were kept in humidified, vented plastic boxes [32 × 17 × 9 cm (*L* × *W* × *H*), with four 18-mm vents covered with fine brass screening, or for larger quantities, 40 × 30 × 14 cm with four 38-mm screen-covered vents) until the insects pupated. Pupae were removed from cocoons and transferred, using a damp camel-hair brush if necessary, into a Petri dish lined with a damp filter paper. The sex of pupae was

determined by examining the terminal abdominal segments (Garrido and Jacas, 1996). Pupae were then transferred to individual 1-dram glass shell vials (45×15 mm, $L \times \text{diam.}$) with a small disk of filter paper to prevent them from becoming trapped in condensation on the glass. The vials, closed with a plastic cap with a pinhole for ventilation, were held in groups in a vented, humidified plastic box. Vials were checked daily for adult emergence. Adults were fed a sugar-water solution (10 g sugar in 100 ml deionized water) absorbed on 2- to 3-mm cubes of cellulose sponge (ca. 10–20 $\mu\text{l}/\text{cube}$). Male moths were used for coupled gas chromatography-electroantennogram detection (GC-EAD) studies, and females were used for pheromone sampling (see below).

Collection of Pheromone from Live Females. Pheromone was collected from female CLM by placing groups of 1–30 virgin females in aeration chambers for 1–4 nights, collecting the emitted pheromone on solid phase microextraction (SPME) fibers. The aeration chambers were constructed from Swagelok[®] fittings (two 1/8 to 1/2 in. unions and one 1/2 to 1/2 in. union) connected to central 12-mm OD glass tubes (6.5 cm long). Activated-charcoal purified medical air was humidified by passage through a $\sim 5 \times 1$ cm ($L \times \text{diam.}$) wad of Soxhlet-extracted glass wool wetted with 1 ml of MilliQ[®] purified water, then it was passed through a 5×1 cm ($L \times \text{diam.}$) bed of 50–200 mesh activated charcoal, and finally into the aeration chamber through a steel frit, at a flow rate of ~ 8 –10 ml/min. An SPME fiber (100 μm polydimethylsiloxane coating, SPME portable field sampler; Supelco Inc., Bellefonte, PA, USA) was inserted into the 1-mm-diam. Teflon outlet tube at the downwind end of the chamber for collection of volatiles. Insects were added to the chamber prior to assembly, and were provided with sugar-water on a cellulose sponge cube during aerations. When there were >3 females, a 1×9 cm piece of clean, fine brass screening was provided as a perching substrate. Aerations were conducted in the laboratory with natural daylight, augmented with room fluorescent lighting during the day. Room temperatures varied from 20 to 25°C and were not precisely controlled.

Coupled Gas Chromatography-Electroantennography. Female-produced volatiles collected by SPME and synthetic standards were analyzed by GC-EAD on Hewlett-Packard 5890 series II gas chromatographs, with the first fitted with a DB-5 column (30 m \times 0.25 mm ID, 0.25 μm film, 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 \times 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 GC. Helium was used as the carrier and makeup gas, and all injections were made splitless. The column effluent was split equally with a glass X-cross with one branch going to the flame ionization detector (FID), another to the EAG detector (EAD), and the final branch 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 antennal preparation. Signals were recorded on either matched HP 3394 integrators or with SRI model 202 PeakSimple chromatography data system running on a personal computer with PeakSimple v. 2.83 software (SRI Instruments, Torrance, CA, USA).

Males (1–2 d old) used in GC-EAD analyses were not chilled or anesthetized prior to use. Males were held with fine forceps, the head removed with a scalpel, and the antennal tips cut off. The head was mounted on the ground electrode and both cut antennal tips were placed in contact with the saline-filled glass recording electrode.

Field Trials. In all trials, moths were counted under a stereomicroscope to ensure correct identification (based on distinctive wing patterns) because of the insects' small size (less than 3 mm) and the large numbers trapped. Moths were readily identified from their distinctive wing patterns. Voucher specimens, UCRC 92975-92982 (pinned) and 92983 (specimens in alcohol), have been deposited in the UC Riverside Entomology Museum.

Lures consisted of 11-mm gray rubber septa (West Pharmaceutical Services Co., Lionville, PA, USA) individually labeled with a treatment number and loaded with heptane solutions (75 or 100 μ l) of test blends. Field trials were conducted in September 2004, in a heavily infested lemon orchard in Escondido, CA. Green Delta traps were used [Pherocon IIID traps (Trécé Inc., Salinas, CA, USA) or Intercept D traps (IPM Tech Inc., Portland, OR, USA)]. In the first two trials, testing blend ratio and total dose, respectively, five replicate blocks containing a complete set of treatments (including a solvent control) were spaced four to seven rows apart. Treatments were randomly placed within the rows, four trees in from the end and on every other tree within a row. Traps were positioned just inside the canopy, 1.5–2 m above ground. Counts were taken 1–2 d after initial setup, and then the traps were re-randomized, with a second count made 2 d later. Traps with more than 10 moths were changed by transferring the rubber septum to a new trap, and the moths in the old trap counted. In the first trial, testing blend ratios, trap catches from the two counts were summed prior to transformation ($\sqrt{x + 0.5}$), and then subjected to two-way analysis of variance using SigmaStat v. 1.0 (Jandel Scientific, San Rafael, CA, USA). Significantly different treatment means were distinguished using the Student–Neuman–Keuls test (SNK, $\alpha = 0.05$). In the second trial, testing dose response, data could not be normalized by transformation, and so the data were analyzed by Friedman's two-way nonparametric ANOVA on the untransformed data, followed by Bonferroni's tests for separation of means ($\alpha = 0.05$).

A final trial testing the effects of 7Z,11Z,13Z-16:Ald as a possible synergist or antagonist was carried out on June 7–8 2005, using a standard blend of

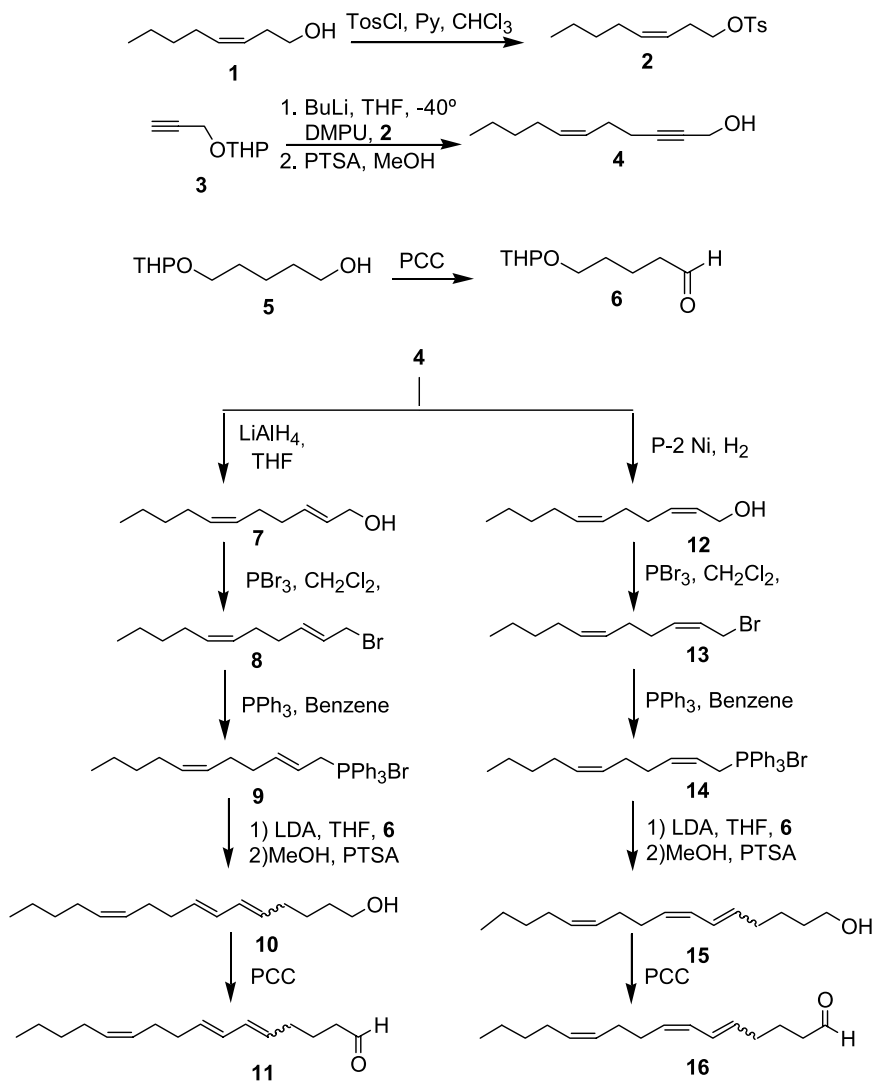
7Z,11Z,13E-16Ald and 7Z,11Z-16:Ald (100:33 μg) augmented with 0 to 100 μg 7Z,11Z,13Z-16:Ald. The experiment was set up in the same orchard as used previously. After $\log_{10}(x + 1)$ transformation to normalize the data, the transformed counts were analyzed by two-way ANOVA followed by SNK tests ($\alpha = 0.05$).

Synthesis of Pheromones and Related Compounds. Tetrahydrofuran (THF) was distilled from sodium/benzophenone under argon atmosphere. ^1H and ^{13}C NMR spectra were recorded with a Varian INOVA-400 (400 and 100 MHz, respectively) spectrometer, as CDCl_3 solutions. Chemical shifts are expressed in ppm relative to CDCl_3 (7.26 and 77.23 ppm for ^1H and ^{13}C NMR, respectively). Mass spectra were obtained with an HP 5890 GC equipped with a DB5-MS column (25 m \times 0.20 mm ID \times 0.33 μm film; J&W Scientific, Folsom, CA, USA), interfaced to an HP 5970 mass selective detector, in EI mode (70 eV) with helium carrier gas. Products were purified by flash or vacuum flash chromatography using silica gel (230–400 mesh; EM Science, Gibbstown, NJ, USA). Reactions with air- or water-sensitive reagents were carried out in dried glassware under argon atmosphere. Worked up reaction mixtures were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation under reduced pressure.

The syntheses of the 5,7,11-hexadecatrienal isomers (Schemes 1 and 2), and the previously known (7Z,11Z)-hexadecadienal (Scheme 5) are described in detail in the online supplement to this manuscript (Electronic Supplementary Material is available for this article at <http://dx.doi.org/10.1007/s10886-006-9359-6> and is accessible for authorized users).

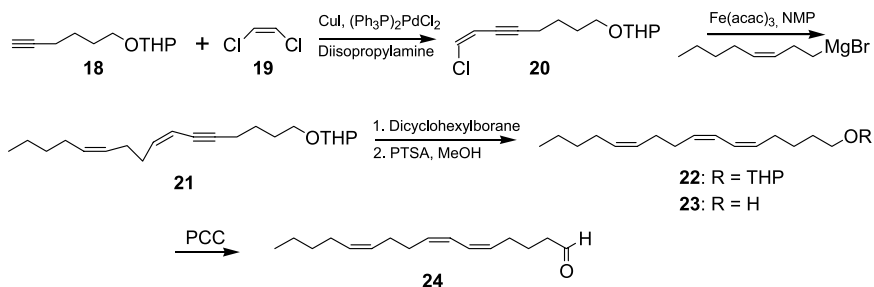
Short, Nonstereospecific Preparation of 7,11,13-Hexadecatrienal Isomers. (Z)-11-Acetoxyundec-4-enal (**28**). (Scheme 3) A solution of KMnO_4 (1.70 g, 10.7 mmol) in water (12.7 ml) was added dropwise to a stirred solution of (7Z,11E/Z)-hexadecadienyl acetate **25** (3.00 g, 10.7 mmol; Shin-Etsu Chemical Co., Tokyo, Japan) in ethanol (35 ml) at 0°C over 2.5 hr, and the resulting mixture was stirred at ambient temperature for 2 hr. The mixture was then filtered through a pad of Celite and the filtrate was concentrated. The residue was taken up in Et_2O (100 ml), washed with brine, dried, and concentrated. The crude product was filtered through a plug of silica gel (hexane/EtOAc, 9:1) giving 0.71 g of diols **26** and **27**. The diols (0.61 g, 1.94 mmol) were added to a solution of NaIO_4 (1.45 g, 6.8 mmol) in a mixture of THF/acetone/water (8:4:13–20 ml) and stirred for 4 hr at 0°C , then diluted with ethyl acetate (50 ml), washed with water and brine, dried, and concentrated. Purification by vacuum flash chromatography (hexane/EtOAc, 95:5 as eluent) afforded 0.21 g of aldehyde **28** (10.1% yield over two steps). MS: 226 (M^+ , trace), 148 (6), 122 (14), 109 (3), 95 (11), 93 (14), 84 (19), 81 (27), 80 (17), 79 (19), 69 (13), 68 (28), 67 (51), 55 (35), 54 (20), 43 (100), 41 (56).

(7Z,11E/Z,13Z)-Hexadecatrienyl Acetate (**31**). LDA (1.5 M in cyclohexane, 0.44 ml, 0.66 mmol) was added dropwise to a suspension of (Z)-2-pentenyl



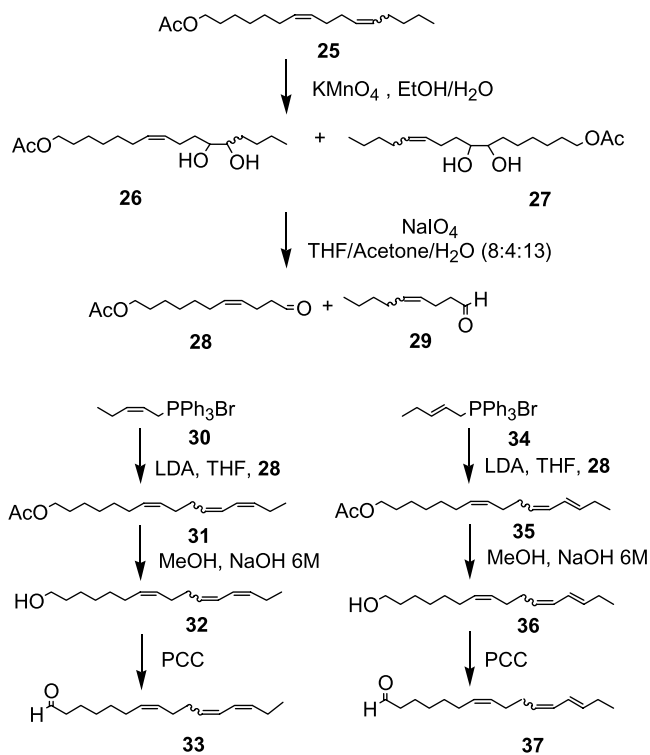
SCHEME 1. Synthesis of (5,7,11Z)-hexadecatrienals.

triphenylphosphonium bromide **30** (0.273 g, 0.66 mmol) in THF (2.0 ml) at -10°C . The mixture was stirred for 2 hr and then cooled to -20°C , and (Z)-11-acetoxyundec-4-enal **28** (0.100 g, 0.44 mmol) dissolved in THF (1.0 ml) was added dropwise. The resulting mixture was warmed to room temperature and stirred overnight, then quenched with water and poured into saturated aqueous



SCHEME 2. Synthesis of (5Z,7Z,11Z)-hexadecadienal **24**.

NH_4Cl solution. The organic layer was separated and the aqueous phase extracted with Et_2O ($3 \times 10 \text{ ml}$). The combined organic layers were washed with brine, dried, and concentrated. The residue was purified by flash chromatography (hexane/ethyl acetate, 95:5) affording (7Z,11E/Z,13Z)-hexadecatrienyl



SCHEME 3. Synthesis of (7Z,11,13)-hexadecatrienals.

acetate **31** (0.052 g) in 42.3% yield, in a 49:51 ratio of geometric isomers. MS: 7Z,11E,13Z (minor) isomer: 278 (M^+ , 10), 207 (1), 149 (3), 135 (8), 119 (5), 95 (100), 81 (16), 79 (23), 67 (48), 55 (21), 43 (51), 41 (30). MS: 7Z,11Z,13Z (major) isomer: 278 (M^+ , 8), 149 (2), 135 (7), 121 (7), 95 (100), 81 (17), 79 (24), 67 (58), 55 (24), 43 (54), 41 (31).

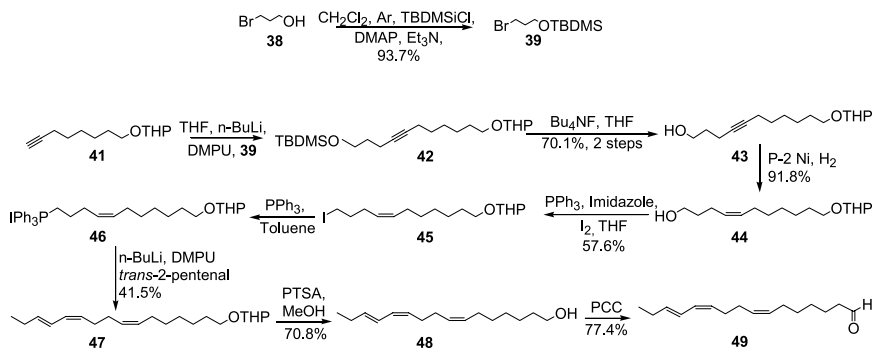
(7Z,11E/Z,13Z)-Hexadecatrien-1-ol (**32**). (7Z,11E/Z,13Z)-hexadecatrienyl acetate **31** (0.040 g, 0.14 mmol) was added to a solution of NaOH (6 M in water, 2 drops) in methanol (1.0 ml) and stirred for 4 hr at room temperature. After concentration, the residue was diluted with EtOAc, washed with water and brine, dried, and then concentrated. The residue was purified by flash chromatography (hexane/EtOAc, 5:1) affording (7Z,11E/Z,13Z)-hexadecatrien-1-ol **32** (0.030 g) in 88.2% yield. MS: 7Z,11E,13Z (minor) isomer: 236 (M^+ , 6), 163 (1), 149 (2), 135 (5), 121 (4), 95 (100), 81 (14), 79 (18), 67 (46), 55 (24), 41 (36). MS: 7Z,11Z,13Z (major) isomer: 236 (M^+ , 5), 149 (2), 135 (7), 121 (5), 95 (100), 81 (15), 79 (19), 67 (54), 55 (27), 41 (37).

(7Z,11E/Z,13Z)-Hexadecatrienal (**33**). (7Z,11E/Z,13Z)-Hexadecatrien-1-ol **32** (5.0 mg, 0.021 mmol) was oxidized with PCC and powdered 4 Å molecular sieve in CH_2Cl_2 as described above, yielding (7Z,11E/Z,13Z)-hexadecatrienal **33** in 56.6% yield (2.8 mg). MS: 7Z,11E,13Z (minor) isomer: 234 (M^+ , 3), 163 (1), 149 (2), 135 (5), 121 (2), 95 (100), 79 (16), 67 (50), 55 (25), 41 (36). MS: 7Z,11Z,13Z (major) isomer: 234 (M^+ , 3), 163 (1), 149 (1), 135 (3), 121 (1), 95 (100), 79 (13), 67 (44), 55 (21), 41 (34).

(7Z,11E/Z,13E)-Hexadecatrienyl acetate (**35**). In the same manner described for the preparation of (7Z,11E/Z,13Z)-hexadecatrienyl acetate **31**, the reaction of aldehyde **28** with the ylide from (*E*)-2-pentenyltriphenylphosphonium bromide **34** (0.273 g, 0.66 mmol) gave (7Z,11E/Z,13E)-hexadecatrienyl acetate **35** (0.071 g, 57.7% yield), in a 41:59 ratio of geometric isomers. MS: 7Z,11Z,13E (minor) isomer: 278 (M^+ , 7), 189 (1), 175 (1), 161 (2), 149 (2), 135 (5), 121 (4), 95 (100), 81 (13), 79 (22), 67 (47), 55 (23), 43 (47), 41 (29). MS: 7Z,11E,13E (major) isomer: 278 (M^+ , 7), 175 (1), 161 (1), 149 (1), 135 (4), 121 (3), 95 (100), 81 (10), 79 (13), 67 (40), 55 (18), 43 (40), 41 (23).

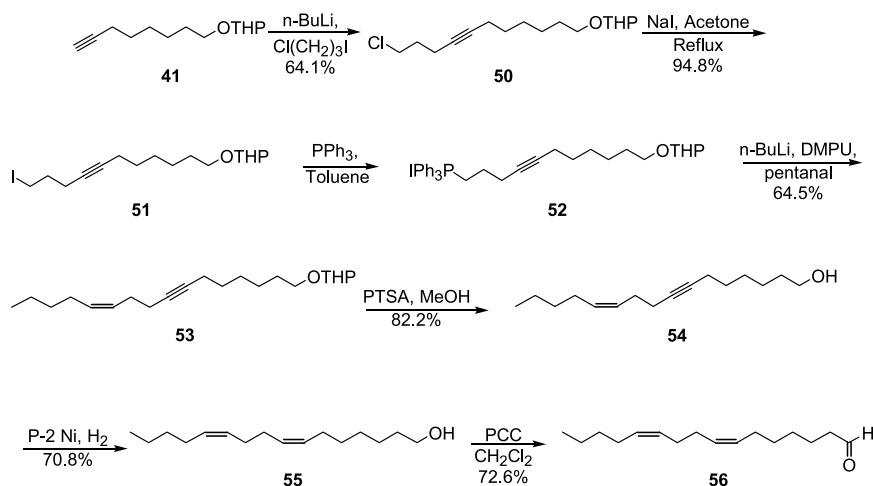
(7Z,11E/Z,13E)-Hexadecatrien-1-ol (**36**). (7Z,11E/Z,13E)-Hexadecatrienyl acetate **35** (0.060 g, 0.22 mmol) was hydrolyzed as described above for acetate **31**, giving (7Z,11E/Z,13E)-hexadecatrien-1-ol **36** (0.041 g, 80.6% yield). MS: 7Z,11Z,13E (minor) isomer: 236 (M^+ , 3), 207 (traces), 163 (1), 149 (2), 135 (4), 121 (3), 95 (100), 81 (10), 79 (16), 67 (45), 55 (24), 41 (34). MS: 7Z,11E,13E (major) isomer: 236 (M^+ , 4), 207 (traces), 149 (1), 135 (3), 121 (2), 95 (100), 81 (8), 79 (13), 67 (39), 55 (20), 41 (28).

(7Z,11E/Z,13E)-Hexadecatrienal (**37**). Alcohol **36** (5.0 mg, 0.021 mmol) was oxidized as described above for alcohol **32**, yielding (7Z,11E/Z,13E)-hexadecatrienal **37** (3.1 mg) in 62.7% yield. MS: 7Z,11Z,13E (minor) isomer: 234 (M^+ , 2), 149 (2), 135 (3), 121 (2), 95 (100), 79 (15), 67 (47), 55 (22), 41


 SCHEME 4. Synthesis of (7Z,11Z,13E)-hexadecatrienal **49**.

(32). MS: 7Z,11E,13E (major) isomer: 234 (M^+ , 3), 216 (traces), 149 (1), 135 (2), 121 (2), 95 (100), 79 (11), 67 (37), 55 (19), 41 (27).

Preparation of (7Z,11Z,13E)-Hexadecatrienal (**49**). 1-(*t*-Butyldimethylsilyloxy)-3-bromopropane (**39**). (Scheme 4) 4-(Dimethylamino)pyridine (70 mg, 0.57 mmol) and triethylamine (6.42 g, 63.4 mmol) were added dropwise to a -10°C , stirred mixture of 3-bromo-1-propanol **38** (8.00 g, 57.6 mmol) and *t*-butyl-dimethylsilyl chloride (9.11 g, 60.4 mmol) in CH_2Cl_2 (65 ml). After 1 hr, the mixture was warmed to room temperature and stirred for 16 hr. Water (50 ml) was added, and the mixture then was extracted three times with Et_2O (1 \times 80, 2 \times 50 ml). The combined organic phases were washed with 10%


 SCHEME 5. Synthesis of (7Z,11Z)-hexadecadienal **56**.

aqueous HCl (3 × 30 ml), saturated NaHCO₃ solution (2 × 50 ml), and brine, then dried and concentrated. The residue was Kugelrohr distilled (oven temp. 68–72°C, 10 mmHg) giving 13.66 g (93.7% yield) of bromide **39**. ¹H NMR: δ 0.07 (s, 6H), 0.90 (s, 9H), 1.93–2.00 (m, 2H), 3.51 (t, 2H, *J* = 6.4 Hz), 3.73 (t, 2H, *J* = 5.8 Hz). ¹³C NMR: δ -5.17, 18.51, 26.12, 30.89, 35.77, 60.64 ppm. MS: *m/z* 197 (M⁺ - 57, 48), 195 (45), 169 (61), 167 (60), 155 (5), 153 (6), 139 (100), 137 (97), 115 (65), 99 (7), 85 (8), 75 (23), 73 (28), 59 (27), 58 (16), 57 (24), 47 (19), 45 (31), 43 (20), 41 (43). The NMR spectrum matched literature values (Kerr et al., 1990).

1-(Tetrahydropyranyloxy)-oct-7-yne (41). Dihydropyran (16.00 g, 190.2 mmol) was added dropwise to a solution of 7-octyn-1-ol **40** (16.00 g, 126.8 mmol; prepared from 2-octyn-1-ol by the acetylene zipper reaction; Abrams and Shaw, 1988) and a few crystals of PTSA in CH₂Cl₂ (30 ml) at 0°C under argon. The reaction was allowed to warm to room temperature and stirred overnight. Most of the solvent was removed by rotary evaporation, the residue was diluted with hexane (150 ml), washed with saturated NaHCO₃ solution (2 × 50 ml) and brine, dried, and concentrated. The resulting oil was purified by vacuum flash chromatography (hexane, then hexane/ethyl acetate, 95:5) followed by Kugelrohr distillation (bp ~94–96°C, 1.0 mmHg) yielding 25.48 g (95.6%) of pure product. ¹H NMR: δ 1.32–1.48 (m, 4H), 1.48–1.65 (m, 8H), 1.67–1.75 (m, 1H), 1.78–1.88 (m, 1H), 1.93 (t, 1H, *J* = 2.7 Hz), 2.18 (td, 2H, *J* = 2.7 and 6.8 Hz), 3.38 (dt, 1H, *J* = 9.8 and 6.4 Hz), 3.46–3.53 (m, 1H), 3.73 (dt, 1H, *J* = 9.8 and 6.8 Hz), 3.83–3.90 (m, 1H), 4.57 (dd, 1H, *J* = 4.4 and 2.7 Hz). ¹³C NMR: δ 18.57, 19.92, 25.70, 25.99, 28.65, 28.79, 29.83, 31.00, 62.58, 67.75, 68.33, 84.90, 99.09 ppm. MS: *m/z* 209 (M⁺ - 1, 1), 125 (1), 109 (3), 101 (29), 85 (100), 67 (40), 55 (23), 43 (21), 41 (51).

1-(t-Butyldimethylsilyloxy)-11-(tetrahydropyranyloxy)-undec-4-yne (42). *n*-BuLi (1.6M in hexanes, 12.2 ml, 19.5 mmol) was added dropwise to a stirred solution of 1-(tetrahydropyranyloxy)-oct-7-yne **41** (3.90 g, 18.5 mmol) in dry THF (20 ml) at -40°C. The mixture was allowed to warm to -10°C and stirred for 4 hr. 1,3-Dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU, 9.0 ml, 74.2 mmol) was added over 10 min, the resulting mixture was stirred for 1 hr, and then cooled to -40°C. A solution of 1-(*t*-butyldimethylsilyloxy)-3-bromopropane **39** (4.70 g, 18.5 mmol) in dry THF (10 ml) was added dropwise, and the mixture was allowed to warm to room temperature over ~4 hr and then stirred overnight. The reaction was quenched with saturated NH₄Cl solution (20 ml) and extracted with ethyl acetate (4 × 20 ml). The combined organic phases were washed with saturated NaHCO₃ solution and brine, dried, and concentrated. The residue was purified by vacuum flash chromatography (hexane/ethyl acetate, 95:5) affording 6.83 g of product contaminated with unreacted 1-(*t*-butyldimethylsilyloxy)-3-bromopropane **39**. This impure material was used directly in the next step. ¹H NMR: δ 0.05 (s, 6H), 0.89 (s, 9H), 1.33–

1.88 (m, 16H), 2.18 (tt, 2H, $J = 7.0$ and 2.3 Hz), 2.26 (tt, 2H, $J = 7.0$ and 2.3 Hz), 3.38 (dt, 1H, $J = 9.6$ and 6.6 Hz), 3.47–2.53 (m, 1H), 3.68 (t, 2H, $J = 6.0$ Hz), 3.73 (dt, 1H, $J = 9.6$ and 6.8 Hz), 3.77–3.87 (m, 1H), 4.57 (dd, 1H, $J = 4.3$ and 2.7 Hz). ^{13}C NMR: δ -5.12, 15.36, 18.57, 18.91, 19.90, 25.72, 26.04, 26.16, 28.93, 29.29, 29.88, 30.99, 32.36, 61.98, 62.54, 67.80, 79.90, 80.51, 99.06 ppm. MS: m/z 325 ($\text{M}^+ - 57$, 2), 241 (5), 165 (3), 159 (12), 93 (9), 85 (100), 81 (12), 79 (10), 75 (29), 73 (13), 67 (19), 57 (10), 55 (13), 43 (12), 41 (22).

11-(Tetrahydropyranyloxy)undec-4-yn-1-ol (43). A solution of tetrabutylammonium fluoride (1.0 M in THF, 21.4 ml, 21.4 mmol) was added to a solution of 1-(*t*-butyldimethylsilyloxy)-11-(tetrahydropyranyloxy)undec-4-yne **42** (6.82 g) in dry THF (35 ml), at room temperature. The mixture was stirred until the starting material had been consumed (1.5 hr), then diluted with Et_2O (50 ml) and washed with water. The organic phase was separated, and the aqueous layer was extracted with Et_2O (2×20 ml). The combined organic phases were washed with brine, dried, and concentrated. The residue was purified by vacuum flash chromatography (hexane/ EtOAc , 4:1) to yield 3.49 g of **43** (70.1% over two steps). ^1H NMR: δ 1.31–1.64 (m, 10H), 1.66–1.88 (m, 4H), 1.73 (quint, 2H, $J = 6.3$ Hz), 2.13 (tt, 2H, $J = 6.8$ and 2.5 Hz), 2.27 (tt, 2H, $J = 6.8$ and 2.5 Hz), 3.38 (dt, 1H, $J = 9.6$ and 6.6 Hz), 3.46–3.54 (m, 1H), 3.73 (dt, 1H, $J = 9.6$ and 6.8 Hz), 3.74 (t, 2H, $J = 6.0$ Hz), 3.82–3.90 (m, 1H), 4.57 (dd, 1H, $J = 4.5$ and 2.5 Hz). ^{13}C NMR: δ 15.64, 18.88, 19.88, 25.71, 25.95, 28.84, 29.15, 29.82, 30.98, 31.80, 62.24, 62.56, 67.76, 79.61, 81.22, 99.08 ppm. MS: m/z 268 (M^+ , trace), 195 (3), 183 (3), 167 (3), 125 (2), 111 (3), 101 (18), 85 (100), 81 (13), 79 (15), 67 (21), 57 (14), 55 (26), 43 (19), 41 (44). Anal. calculated for $\text{C}_{16}\text{H}_{28}\text{O}_3$: C, 71.60; H, 10.52. Found: C, 71.85; H, 10.59. The ^1H NMR spectrum was in agreement with that reported in the literature (Yadav et al. 1988).

(Z)-11-(Tetrahydropyranyloxy)undec-4-en-1-ol (44). Alkynol **43** (3.40 g, 12.67 mmol) was reduced to the corresponding alkenol with hydrogen and P2-nickel catalyst as described above. The crude product was purified by vacuum flash chromatography (hexane/ethyl acetate, 9:1), yielding 3.14 g of **44** (91.8% yield). ^1H NMR: δ 1.24–1.42 (m, 6H), 1.44–1.64 (m, 8H), 1.64–1.73 (m, 1H), 1.73–1.85 (m, 1H), 1.96–2.07 (m, 2H), 2.07–2.14 (m, 2H), 3.36 (dt, 1H, $J = 9.6$ and 6.6 Hz), 3.44–3.51 (m, 1H), 3.62 (dt, 2H, $J = 5.3$ and 6.4 Hz), 3.70 (dt, 1H, $J = 9.6$ and 6.8 Hz), 3.81–3.88 (m, 1H), 4.55 (dd, 1H, $J = 4.3$ and 2.7 Hz), 5.30–5.41 (m, 2H). ^{13}C NMR: δ 19.89, 23.81, 25.70, 26.31, 27.30, 29.28, 29.79, 29.90, 30.97, 32.86, 62.57, 62.76, 67.87, 99.06, 129.18, 130.85 ppm. MS: m/z 270 (M^+ , trace), 186 (1), 168 (1), 150 (1), 109 (3), 101 (4), 95 (7), 85 (100), 81 (11), 67 (19), 57 (10), 55 (17), 43 (12), 41 (28). The ^1H NMR spectrum was in agreement with that reported in the literature (Sharma et al. 1994).

(4Z)-1-Iodo-11-(tetrahydropyranyloxy)undec-4-ene (45). Iodine (2.91 g, 11.46 mmol) was added in small portions to a -40°C solution of Ph_3P (3.00 g,

11.46 mmol), imidazole (1.56 g, 22.92 mmol), and (*Z*)-11-(tetrahydropyranyloxy)undec-4-en-1-ol **44** (3.10 g, 11.46 mmol) in dry THF (18 ml). The resulting mixture was warmed to 0°C and stirred for 3 hr. The mixture was diluted with Et₂O (100 ml), washed successively with saturated sodium thiosulfate (100 ml), water (2 × 50 ml), and brine, then dried and concentrated. The residue was suspended in hexane to precipitate most of the Ph₃PO and filtered. After concentration, the crude product was purified by vacuum flash chromatography (hexane/ethyl acetate, 9:1) yielding 2.15 g of **45** (57.6% yield) as colorless oil and 0.201 g of unreacted starting material. ¹H NMR: δ 1.22–1.42 (m, 6H), 1.45–1.64 (m, 6H), 1.64–1.75 (m, 1H), 1.77–1.87 (m, 1H), 1.87 (quint, 2H, *J* = 7.0 Hz), 2.05 (q, 2H, *J* = 6.6 Hz), 2.13 (q, 2H, *J* = 7.2 Hz), 3.18 (t, 2H, *J* = 6.8 Hz), 3.37 (dt, 1H, *J* = 9.6 and 6.6 Hz), 3.46–3.52 (m, 1H), 3.72 (dt, 1H, *J* = 9.6 and 6.8 Hz), 3.82–3.90 (m, 1H), 4.57 (dd, 1H, *J* = 4.3 and 2.7 Hz), 5.28 (dtt, 1H, *J* = 10.7, 7.2 and 1.4 Hz), 5.42 (dtt, 1H, *J* = 10.7, 7.4 and 1.4 Hz). ¹³C NMR: δ 6.87, 19.93, 25.73, 26.37, 27.56, 28.14, 29.35, 29.88, 29.95, 31.02, 33.64, 62.57, 67.86, 99.07, 127.51, 131.92 ppm. MS: *m/z* 183 (1), 155 (4), 109 (4), 101 (4), 95 (10), 85 (100), 81 (13), 69 (8), 67 (17), 55 (18), 43 (9), 41 (27). Anal. calculated for C₁₆H₂₉O₂: C, 50.53; H, 7.69. Found: C, 50.64; H, 7.73.

(*Z*)-11-(Tetrahydropyranyloxy)undec-4-enyl triphenylphosphonium iodide (**46**). A solution of Ph₃P (1.66 g; 6.31 mmol) and (*Z*)-1-iodo-11-(tetrahydropyranyloxy)undec-4-ene **45** (2.40 g; 6.31 mmol) in toluene (7.0 ml) was refluxed for 2 d (GC analysis showed no remaining iodide), then poured with vigorous stirring into 50 ml hexane, to produce a viscous oil. The mixture was stirred for 1 hr, the solvent was replaced with fresh hexane (50 ml), and then stirred for an additional 1 hr. The solvent was removed and the remaining phosphonium salt **46** was pumped under high vacuum for 8 hr, then used without further purification.

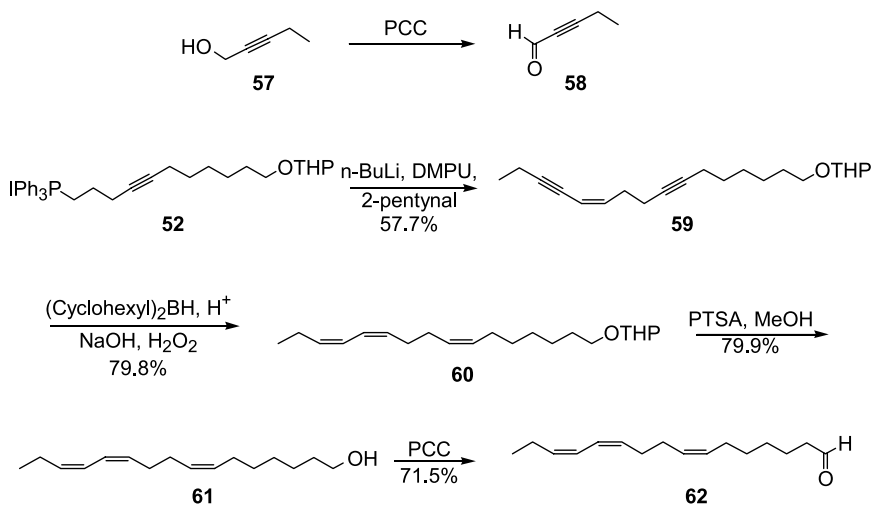
(7*Z*,11*Z*,13*E*)-1-(Tetrahydropyranyloxy)-hexadecatriene (**47**). Lithium hexamethyldisilazide (LiHMDS, 1.0 M in THF, 4.50 ml, 4.5 mmol) was added to a solution of dry DMPU (2.56 g, 20.0 mmol) in THF (12 ml) at 0°C. The resulting mixture was stirred for 30 min, then cooled to -78°C. A solution of (*Z*)-11-(tetrahydropyranyloxy)undec-4-enyl triphenylphosphonium iodide **46** (3.21 g, 5.00 mmol) in THF (18.0 ml) was added dropwise. After stirring for 1 hr, (*E*)-2-pentenal (0.560 g, 6.50 mmol) in THF (2 ml) was added dropwise. The resulting mixture was stirred for 2 hr at -78°C, then warmed to room temperature and stirred an additional 2 hr. The mixture was diluted with hexane/Et₂O (3:1–50 ml) and washed with saturated NH₄Cl solution and brine, dried, and concentrated. Purification by vacuum flash chromatography (hexane/EtOAc, 95:5) afforded a 93:7 mixture of the (7*Z*,11*Z*,13*E*)- and (7*Z*,11*E*,13*E*)-trienes, respectively (0.664 g, 41.5% yield). (7*Z*,11*Z*,13*E*)-isomer: ¹H NMR: δ 1.01 (t, 3H, *J* = 7.6 Hz), 1.22–1.40 (m, 6H), 1.46–1.64 (m, 6H), 1.66–1.80 (m, 2H), 1.96–2.06 (m, 2H), 2.06–2.16 (m, 4H), 2.16–2.26 (m, 2H), 3.37 (dt, 1H, *J* = 9.6 and 6.6 Hz), 3.45–3.55 (m, 1H), 3.72 (dt, 1H, *J* = 9.6 and 6.8 Hz), 3.84–

3.90 (m, 1H), 4.56 (dd, 1H, $J = 4.3$ and 2.7 Hz), 5.30 (dt, 1H, $J = 10.7$ and 7.4 Hz), 5.32–5.42 (m, 2H), 5.70 (dt, 1H, $J = 15.2$ and 6.6 Hz), 5.95 (t, 1H, $J = 10.9$ Hz), 6.25–6.33 (m, 1H). ^{13}C NMR: δ 13.85, 19.92, 25.73, 26.11, 26.38, 27.45, 27.58, 28.07, 29.37, 29.88, 29.95, 31.01, 62.56, 67.87, 99.06, 124.85, 129.18, 129.21, 129.51, 130.69, 136.64 ppm. MS: m/z 320 (M^+ , 2), 302 (trace), 236 (1), 235 (1), 175 (1), 161 (1), 135 (2), 122 (3), 95 (35), 85 (100), 79 (15), 67 (29), 55 (20), 43 (11), 41 (30).

(7Z,11Z,13E)-Hexadecatrien-1-ol (**48**). The THP protecting group was removed from (7Z,11Z,13E)-1-(tetrahydropyranyloxy)-hexadecatriene **47** (0.650 g, 2.02 mmol) with PTSA in methanol, as described above. The crude product was purified by vacuum flash chromatography (hexane/ethyl acetate 5:1) followed by Kugelrohr distillation (oven temp. $\sim 112^\circ\text{C}$, 0.03 mmHg), yielding (7Z,11Z,13E)-hexadecatrien-1-ol (0.338 g, 70.8% yield). The isomeric purity of the product was increased to 97% by recrystallization from hexane at -20°C . ^1H NMR: δ 1.01 (t, 3H, $J = 7.4$ Hz), 1.26–1.42 (m, 6H), 1.52–1.60 (m, 2H), 1.98–2.06 (m, 2H), 2.06–2.16 (m, 4H), 2.18–2.26 (m, 2H), 3.63 (t, 2H, $J = 6.7$ Hz), 5.30 (dt, 1H, $J = 10.9$ and 7.4 Hz), 5.35–5.42 (m, 2H), 5.70 (dt, 1H, $J = 15.2$ and 6.6 Hz), 5.96 (t, 1H, $J = 10.9$ Hz), 6.29 (ddq, 1H, $J = 15.2$, 10.9 and 1.6 Hz). ^{13}C NMR: δ 13.84, 25.86, 26.11, 27.41, 27.58, 28.06, 29.28, 29.87, 32.97, 63.25, 124.84, 129.18, 129.28, 129.49, 130.61, 136.67 ppm. MS: m/z 236 (M^+ , 3), 163 (1), 149 (2), 135 (4), 121 (3), 107 (3), 96 (10), 95 (100), 93 (12), 81 (10), 79 (18), 67 (48), 55 (24), 53 (9), 41 (37). Anal. calculated for $\text{C}_{16}\text{H}_{28}\text{O}$: C, 81.29; H, 11.94. Found: C, 80.11; H, 12.06.

(7Z,11Z,13E)-Hexadecatrienal (**49**). (7Z,11Z,13E)-Hexadecatrien-1-ol **48** (18.0 mg, 0.076 mmol) was oxidized to the aldehyde with PCC and powdered 4 Å molecular sieve in CH_2Cl_2 as described above, and the crude product was flash chromatographed (pentane/ Et_2O 9:1) yielding (7Z,11Z,13E)-hexadecatrienal **49** in 77.4% yield (13.8 mg). ^1H NMR: δ 1.01 (t, 3H, $J = 7.4$ Hz), 1.34–1.42 (m, 4H), 1.63 (quint, 2H, $J = 7.4$ Hz), 2.00–2.06 (m, 2H), 2.06–2.17 (m, 4H), 2.18–2.26 (m, 2H), 2.42 (td, 2H, $J = 1.8$ and 7.4 Hz), 5.30 (dt, 1H, $J = 10.8$ and 7.4 Hz), 5.34–5.42 (m, 2H), 5.71 (dt, 1H, $J = 15.2$ and 6.6 Hz), 5.96 (t, 1H, $J = 10.9$ Hz), 6.29 (ddq, 1H, $J = 15.2$, 10.9 and 1.6 Hz), 9.76 (t, 1H, $J = 1.8$ Hz). ^{13}C NMR: δ 13.84, 22.21, 26.11, 27.25, 27.59, 28.02, 29.00, 29.62, 44.10, 124.82, 129.23, 129.42, 129.53, 130.27, 136.71, 203.04 ppm. MS: m/z 234 (M^+ , 2), 216 (trace), 187 (1), 173 (1), 149 (2), 135 (3), 121 (2), 107 (2), 95 (100), 93 (11), 79 (14), 67 (45), 55 (22), 41 (32).

Preparation of (7Z,11Z,13Z)-hexadecatrienal (**62**)(Scheme 6). 2-Pentynal (**58**). 2-Pentyn-1-ol **57** was oxidized with PCC, as described above. The crude product was flash chromatographed (pentane/ Et_2O , 9:1), and the solvent was distilled off using a Vigreux column, giving 2-pentynal **58** (0.832g) in a ca. 15% mixture with solvent. MS: m/z 82 (M^+ , 34), 81 ($\text{M}^+ - 1$, 57), 67 (1), 66 (7), 54 (57), 53 (100), 52 (12), 51 (32), 50 (40), 49 (17).

SCHEME 6. Synthesis of (7Z,11Z,13Z)-hexadecatrienal **62**.

(*Z*)-1-(Tetrahydropyranyloxy)-hexadec-7,13-diyne-11-ene (**59**). The title compound was prepared by a *Z*-selective Wittig between the anion of 11-(tetrahydropyranyloxy)-undec-4-ynyl triphenylphosphonium iodide **52** and 2-pentynal **58** in THF/DMPU as described above. After workup, purification by vacuum flash chromatography (hexane/EtOAc, 95:5) gave endiyne **59** in 57.7% yield. ^1H NMR: δ 1.161 (t, 3H, $J = 7.6$ Hz), 1.26–1.59 (m, 12H), 1.61–1.69 (m, 1H), 1.72–1.82 (m, 1H), 2.08–2.17 (m, 2H), 2.17–2.26 (m, 2H), 2.33 (dq, 2H, $J = 7.6$ and 2.2 Hz), 2.45 (q, 2H, $J = 7.2$ Hz), 3.32 (dt, 1H, $J = 9.6$ and 6.4 Hz), 3.40–3.47 (m, 1H), 3.67 (dt, 1H, $J = 9.6$ and 6.8 Hz), 3.76–3.84 (m, 1H), 4.51–4.59 (m, 1H), 5.37–5.48 (m, 1H), 5.83 (dt, 1H, $J = 10.8$ and 7.2 Hz). ^{13}C NMR: δ 13.43, 14.26, 18.64, 18.92, 19.90, 25.72, 26.03, 28.90, 29.24, 29.87, 29.88, 31.00, 62.54, 67.80, 76.62, 79.55, 80.90, 96.50, 99.07, 110.54, 140.69. MS: m/z 287 (1), 231 (1), 215 (2), 201 (1), 185 (3), 171 (3), 157 (9), 145 (13), 143 (16), 131 (13), 129 (21), 117 (16), 91 (39), 85 (100), 77 (40), 67 (22), 57 (19), 55 (21), 43 (18), 41 (47).

(*Z*,11*Z*,13*Z*)-1-(Tetrahydropyranyloxy)-hexadecatriene (**60**). A solution of cyclohexene (0.549 g, 6.68 mmol) in dry THF (2.0 ml) was added dropwise under argon to a solution of $\text{BH}_3\cdot\text{DMDS}$ (solution 2.0 M in THF, 1.67 ml, 3.34 mmol) in dry THF (5.0 ml) at -10°C . The resulting mixture was stirred for 3 hr between -10 and 0°C , then allowed to warm to room temperature and stirred for 1 hr. The resulting white slurry of dicyclohexylborane was recooled to -20°C and a solution of (*Z*)-1-(tetrahydropyranyloxy)-hexadec-7,13-diyne-11-ene **59** (0.264 g, 0.834 mmol) in dry THF (2.0 ml) was added dropwise over

20 min. The resulting mixture was slowly warmed to 0°C (ca. 2.5 hr) and then stirred for 4 hr. Glacial acetic acid (0.84 ml) was added dropwise, and the mixture was warmed to room temperature and stirred overnight. The solution was cooled to 0°C and treated with aqueous NaOH (20% by wt., 6.0 ml) followed by dropwise addition of hydrogen peroxide (30% by wt., 0.40 ml), keeping the temperature below 15°C. The mixture was warmed to room temperature and stirred for 1 hr, diluted with water (10 ml), and extracted with hexane (4 × 15 ml). The combined organic layers were washed with brine, dried, concentrated, and purified by vacuum flash chromatography on silica gel (hexane/EtOAc, 95:5) giving (7Z,11Z,13Z)-1-(tetrahydropyranyloxy)-hexadecatriene **60** (0.213 g) in 79.8% yield as a 92:8 mixture of the (7Z,11Z,13Z)- and (7Z,11E,13Z)-trienes, respectively. ¹H NMR: δ 0.98 (t, 3H, *J* = 7.6 Hz), 1.26–1.43 (m, 6H), 1.46–1.65 (m, 6H), 1.66–1.74 (m, 1H), 1.76–1.80 (m, 1H), 1.96–2.06 (m, 2H), 2.06–2.26 (m, 6H), 3.36 (dt, 1H, *J* = 9.6 and 6.6 Hz), 3.44–3.52 (m, 1H), 3.71 (dt, 1H, *J* = 9.6 and 6.8 Hz), 3.81–3.89 (m, 1H), 4.53–4.58 (m, 1H), 5.20–5.30 (m, 2H), 5.30–5.39 (m, 2H), 6.06–6.20 (m, 2H). ¹³C NMR: δ 14.40, 19.91, 21.01, 25.73, 26.37, 27.43, 27.47, 27.83, 29.34, 29.86, 29.94, 31.00, 62.54, 67.85, 99.05, 123.17, 124.03, 129.13, 130.71, 131.47, 134.09 ppm. MS: *m/z* 320 (M⁺, 2), 236 (1), 235 (1), 175 (1), 161 (1), 135 (2), 121 (3), 95 (38), 85 (100), 79 (12), 67 (33), 55 (20), 43 (11), 41 (32).

(7Z,11Z,13Z)-Hexadecatrien-1-ol (**61**). The THP protecting group was removed from (7Z,11Z,13Z)-1-(tetrahydropyranyloxy)-hexadecatriene **60** (0.163 g, 0.51 mmol) with PTSA in methanol as described above. The crude product was purified by vacuum flash chromatography (hexane/ethyl acetate, 9:1), yielding (7Z,11Z,13Z)-hexadecatrien-1-ol (0.096 g, 79.9% yield). The isomeric purity of the product was increased to 95% by chromatography over silica gel with 10% AgNO₃ (hexane/Et₂O, 9:1). ¹H NMR: δ 0.99 (t, 3H, *J* = 7.6 Hz), 1.28–1.42 (m, 6H), 1.50–1.62 (m, 2H), 1.98–2.06 (m, 2H), 2.07–2.26 (m, 6H), 3.62 (t, 2H, *J* = 6.6 Hz), 5.32–5.40 (m, 2H), 5.40–5.48 (m, 2H), 6.16–6.30 (m, 2H). ¹³C NMR: δ 14.40, 21.02, 25.85, 27.39, 27.47, 27.83, 29.26, 29.85, 32.98, 63.26, 123.16, 124.03, 129.21, 130.64, 131.47, 134.14 ppm. MS: *m/z* 236 (M⁺, 7), 207 (1), 163 (1), 149 (3), 135 (8), 121 (5), 107 (5), 96 (11), 95 (100), 93 (17), 81 (19), 79 (23), 67 (65), 55 (31), 53 (13), 41 (46).

(7Z,11Z,13Z)-Hexadecatrienal (**62**). (7Z,11Z,13Z)-Hexadecatrien-1-ol **61** (13.0 mg, 0.055 mmol) was oxidized to the aldehyde with PCC and powdered 4 Å molecular sieve in CH₂Cl₂ as described above, and the crude product was flash chromatographed (pentane/Et₂O, 9:1) affording (7Z,11Z,13Z)-hexadecatrienal **62** in 71.5% yield (9.2 mg). ¹H NMR: δ 0.99 (t, 3H, *J* = 7.6 Hz), 1.28–1.42 (m, 4H), 1.63 (quint, 2H, *J* = 7.4 Hz), 1.99–2.06 (m, 2H), 2.06–2.26 (m, 6H), 2.41 (td, 2H, *J* = 2.0 and 7.4 Hz), 5.34–5.39 (m, 2H), 5.39–5.49 (m, 2H), 6.16–6.30 (m, 2H), 9.76 (t, 1H, *J* = 2.0 Hz). ¹³C NMR: δ 14.40, 21.02, 22.20, 27.23, 27.48, 27.79, 28.99, 29.61, 44.09, 123.13, 124.08, 129.46, 130.30,

131.39, 134.19, 203.01. MS: m/z 234 (M^+ , 6), 219 (trace), 187 (1), 173 (1), 149 (2), 135 (6), 121 (3), 107 (3), 95 (100), 93 (15), 79 (20), 67 (59), 55 (28), 41 (43).

RESULTS

The amounts of the pheromone components collected by SPME from overnight aerations of 1–30 female moths were usually below the detection limit of GC and GC-MS. Therefore, identification of the active compounds was primarily guided by a combination of GC-EAD, using the retention behavior of the compounds on GC columns of different polarity, and the relative responses of male moth antennae to various standards with different functional groups. Male moth antennae responded consistently to two compounds in the effluvia from females. Sporadically, there was a third, much smaller response visible when extracts were analyzed on the DB-5 column (Figure 1, Table 1). The earliest response on both columns (for DB-5, Figure 1, peak 1) matched the retention time of synthetic 7Z,11Z-16:Ald, the previously reported sex attractant

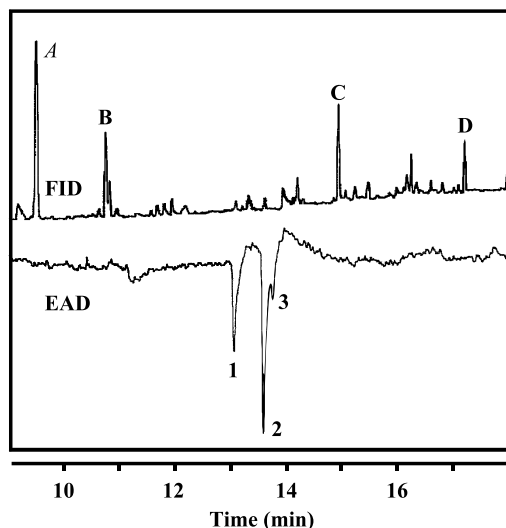


FIG. 1. Coupled gas chromatography-electroantennogram detection traces showing responses from the GC and a male moth antenna to volatiles collected from two citrus leafminer females over 2 nights on a SPME fiber. GC responses with letters were also present in blank runs. Peak 1: (7Z,11Z)-hexadecadienal; peak 2: (7Z,11Z,13E)-hexadecatrienal; peak 3: tentatively identified as (7Z,11Z,13Z)-hexadecatrienal. Column: DB-5 (30 m \times 0.25 mm ID, 0.25 μ m film), 100°C/1 min, 10°C/min to 275°C.

TABLE 1. COMPARISON OF RETENTION INDICES ON DB-5 AND DB-WAX GC COLUMNS OF INSECT-PRODUCED COMPOUNDS ELICITING ANTENNAL RESPONSES IN GC-EAD EXPERIMENTS, AND OF SYNTHETIC STANDARDS

Compound	Retention indices	
	DB-5 ^a	DB-WAX ^a
<i>Insect-produced compounds</i>		
Leafminer first response	17.96	22.19
Leafminer second response	18.43	23.71
Leafminer third response	18.58	— ^b
<i>Synthetic standards</i>		
Z11-16:Ald	18.10	21.86
Z11,E13-16:Ald	18.56	23.35
E11,Z13-16:Ald	18.65	23.47
Z11,Z13-16:Ald	18.74	23.57
Z7,Z11-16:Ald	17.95	22.20
Z7,E11-16:Ald	17.93	22.12
5Z,7E,11Z-16:Ald	18.29	23.46
5E,7Z,11Z-16:Ald	18.36	23.60
5Z,7Z,11Z-16:Ald	18.55	23.83
5E,7E,11Z-16:Ald	18.62	23.86
7Z,11Z,13E-16:Ald	18.43	23.70
7Z,11Z,13Z-16:Ald	18.57	23.89
7Z,11E,13E-16:Ald	18.56	23.97
7Z,11E,13Z-16:Ald	18.44	23.75

Matches are shown in boldface type.

^aGC conditions: DB-5 (30 m × 0.25 mm ID, 0.25 μm film, 100°C/1 min, 10°C/min–275°C/15 min); DB-WAX (30 m × 0.25 mm ID, 0.25 μm film, 100°C/1 min, 10°C/min–240°C/10 min). Because the final temperature was reached before the tetracosane standard eluted on DB-WAX, the retention indices on this column may differ slightly from true Kovat's indices.

^bThird response not seen on this column.

for this species (Ando et al., 1985). Furthermore, when challenged with synthetic standards of 7Z,11Z-16:Ald, male moth antennal responses were consistently stronger to the aldehyde than to the corresponding acetate or alcohol.

The second compound, which consistently elicited the largest antennal responses, eluted about 50 and 150 retention units later than 7Z,11Z-16:Ald from the DB-5 and DB-WAX columns, respectively (Table 1). Both retention time and antennal response data ruled out the possibility of the unknown being 7Z,11Z-16:OH or 7Z,11Z-16:Ac. Retention times on both the nonpolar and polar columns were markedly longer than those of 7Z,11Z-16:Ald, suggesting the presence of a conjugated diene or triene in the unknown. The presence of a conjugated triene (e.g., a 7,9,11-16:Ald) was ruled out by examination of retention time differences of standards available from other projects (10E,12E,14E-16:Ald, Chen and Millar, 2000; 9E,11Z,13Z-16:Ald, Millar

et al., 1996). On the DB-5 column, these standards eluted >100 retention units later than the unknown, whereas the difference was even greater on the DB-WAX column (208 and 179 retention units, respectively). Therefore, the most likely candidates for the unknown were the 5*E*/*Z*,7*Z*,11*Z*- or 7*Z*,11*Z*,13*E*/*Z*-16:Ald isomers. These isomers were synthesized, and only 7*Z*,11*Z*,13*E*-16:Ald matched the retention times of the unknown on both columns.

The identifications of both 7*Z*,11*Z*-16:Ald and 7*Z*,11*Z*,13*E*-16:Ald were subsequently confirmed from full-scan mass spectra of both compounds (Figure 2)

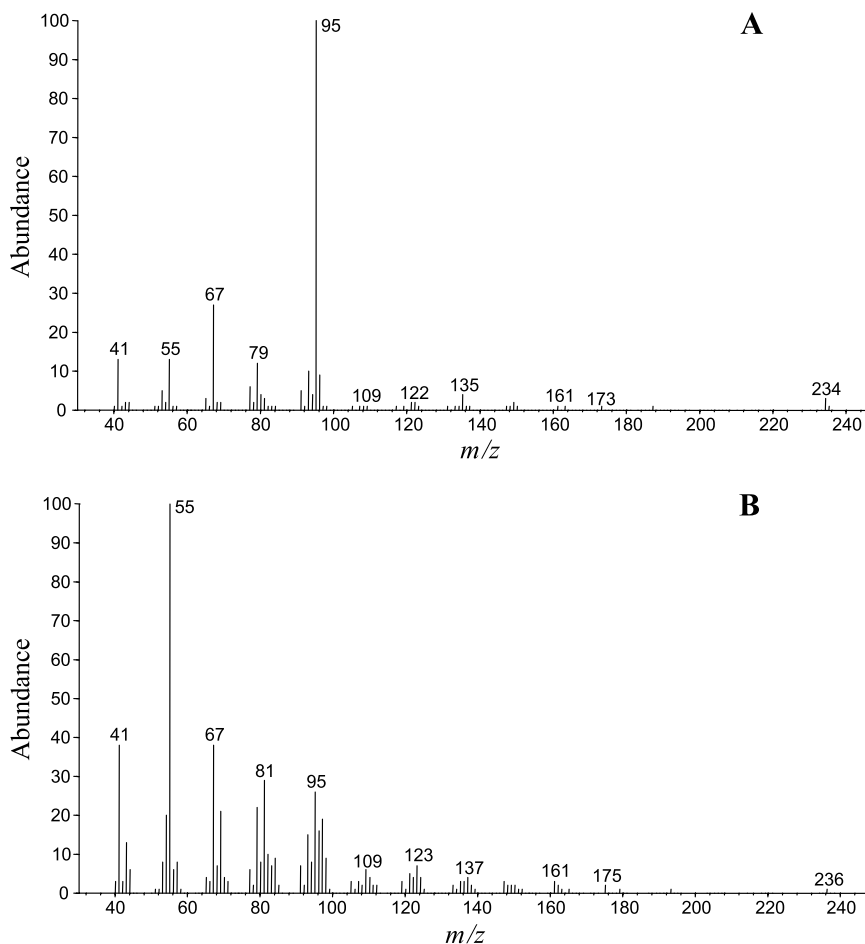


FIG. 2. EI mass spectra of (A) (7*Z*,11*Z*,13*E*)-hexadecatrienal and (B) (7*Z*,11*Z*)-hexadecadienal.

obtained from an SPME sample collected from two females aerated over five consecutive nights. In particular, the spectrum of 7Z,11Z,13E-16:Ald exhibits a diagnostic base peak at m/z 95, corresponding to a $C_7H_{11}^+$ ion arising from cleavage between allylic carbons 9 and 10, with the charge stabilized by the conjugated diene system. An analogous cleavage has been reported for 4,6,10-16:Ac isomers, which have a similar arrangement of double bonds (Beevor et al., 1986).

The third EAD response matched the retention time of 7Z,11Z,13Z-16:Ald on the DB-5 column. However, it was not seen on the DB-WAX column, nor was it possible to obtain a mass spectrum, and so the identity of this compound could not be confirmed.

Synthesis of the Pheromone Components and Analogs. The GC-EAD data indicated that the pheromone extracts contained 7Z,11Z-16:Ald, and we reasoned that the putative trienal, with a conjugated diene present, must either be a 5,7,11- or a 7,11,13-hexadecatrienal. Thus, we initially focused on short, nonstereoselective syntheses that would provide mixtures of isomers for comparisons of retention times with those of the insect-produced compound. These compounds enabled us to eliminate the 5,7,11-isomers, and confirmed that one of the 7,11,13-isomers matched the insect produced triene, so stereoselective syntheses were developed.

The syntheses of both pairs of (5E/Z,7E,11Z)-hexadecatrienals **11** and (5E/Z,7Z,11Z)-hexadecatrienals **16** started with the preparation of the key intermediate **4** (Scheme 1). Thus, (Z)-3-octen-1-ol **1** was transformed into its corresponding tosylate **2** (83% yield), which was then coupled with the terminal alkyne **3** followed by deprotection to give alcohol **4** in 31% yield in two steps. Enyne alcohol **4** was reduced to the corresponding (E)- and (Z)-allylic alcohols **7** and **12** by reaction with lithium aluminum hydride or P-2 Ni and H_2 , respectively. The alcohols were treated with PBr_3 to give bromides **8** and **13**. To introduce the third double bond into the alkyl chain, the bromides **8** and **13** were converted into the phosphonium salts **9** and **14**, followed by Wittig reactions of the corresponding ylides with protected hydroxyaldehyde **6**. Deprotection of the resulting trienes with PTSA in methanol gave (5E/Z,7E,11Z)-hexadecatrien-1-ol **10** (71% yield over two steps, 42:58 ratio of isomers) and (5E/Z,7Z,11Z)-hexadecatrien-1-ol **15** (75% yield, 55:45 ratio of isomers). Oxidation of alcohols **10** and **15** with PCC in dichloromethane gave the desired aldehydes **11** and **16** as pairs of isomers.

The synthesis of the (5E/Z,7Z,11Z)-hexadecatrienal mixture **16** gave an approximately 1:1 ratio of isomers. To determine the identities of the two isomers, one of which eluted very close to the insect-produced trienal, a stereospecific synthesis of the 5Z,7Z,11Z-isomer was carried out (Scheme 2). The key steps were the sequential alkylation of (Z)-1,2-dichloroethene **19** with the terminal alkyne **18** in the presence of CuI, $PdCl_2(PPh_3)_2$, and diisopropylamine, followed by a second alkylation of the resulting chloroenyne **20** with (Z)-3-octenyl magnesium bromide with $Fe(acac)_3$ catalysis and *N*-methylpyrrolidi-

none (NMP) as cosolvent (Cahiez and Avedissian, 1998). The latter coupling step was inefficient, returning mainly unreacted starting material, but it did furnish **21** in sufficient yield (13.5%) to proceed. Dienyne **21** was stereoselectively reduced to the corresponding triene **22** (76% yield) with dicyclohexylborane, in >99% isomeric purity by GC analysis. Removal of the THP group by acid catalysis in methanol gave trien-1-ol **23**, which was oxidized to (5*Z*, 7*Z*, 11*Z*)-hexadecatrienal **24** with PCC.

Although (5*Z*, 7*Z*, 11*Z*)-hexadecatrienal had retention times on both columns that were close to those of the insect-produced compound (Table 1), none of the four 5,7,11-isomers had retention times that exactly matched those of the insect-produced trienal on the two columns. Thus, we embarked on the syntheses of the other most likely isomers, the 7,11,13-trienals.

Initially, four of the eight possible geometric isomers of 7,11,13-hexadecatrienal were synthesized as two pairs of isomers, that is, (7*Z*, 11*E*/*Z*, 13*E*)-hexadecatrienal and (7*Z*, 11*E*/*Z*, 13*Z*)-hexadecatrienal, starting from commercially available (7*Z*, 11*E*/*Z*)-hexadecadienyl acetate **25** (pheromone of pink bollworm) (Scheme 3). Thus, oxidative cleavage of **25** gave aldehyde **28** (2 steps, 10.1% yield), which was reacted separately with the ylides generated from the phosphonium salts **30** and **34**. These reactions yielded the mixtures of trienes (7*Z*, 11*E*/*Z*, 13*Z*)-**31** and (7*Z*, 11*E*/*Z*, 13*E*)-**35** in 42 and 58% yield, respectively. After hydrolysis of the acetates, the corresponding alcohols **32** and **36** were oxidized with PCC, furnishing the desired mixtures of aldehydes **33** and **37**. With these mixtures in hand, the insect-produced trienal was identified as the 7*Z*, 11*Z*, 13*E*-16:Ald isomer. A more stereoselective route was developed to provide material for field trials, along with a parallel route to provide the diene component, 7*Z*, 11*Z*-16:Ald, in high isomeric purity as well.

The synthetic routes to 7*Z*, 11*Z*, 13*E*-16:Ald **49** and 7*Z*, 11*Z*-16:Ald **56** are shown in Schemes 4 and 5. The key step in both routes was a stereoselective Wittig reaction that placed a (*Z*) double bond in the 11 position of the desired compounds. The synthetic route to 7*Z*, 11*Z*, 13*E*-16:Ald **49** was based on a (C₈ + C₃) + C₅ approach. Thus, alkyne **41** (Waanders et al., 1987), prepared from 7-octyn-1-ol **40** by protection of the hydroxyl group as the tetrahydropyranyl (THP) ether, was deprotonated with BuLi, followed by addition of bromide **39** to give diprotected yne-diol **42**. Selective removal of the *t*-butyldimethylsilyl ether with tetrabutylammonium fluoride furnished monoprotected yne-diol **43** (Yadav et al., 1988) (70% over two steps). The alkyne was reduced by P-2 nickel-catalyzed reduction of **43** with H₂ (Brown and Ahuja, 1973) to produce **44** (Sharma et al., 1994) in 92% yield. Alcohol **44** was converted to the corresponding iodide **45** by reaction with iodine, triphenylphosphine, and imidazole (Gnädig et al., 2001) (58%), followed by reaction of **45** with triphenylphosphine to give the corresponding phosphonium salt. Wittig reaction of ylide **46** derived from this salt with (*E*)-2-pentenal in a THF–DMPU solvent

mixture, in a modification of the *Z*-selective olefination method of Labelle et al. (1990), afforded triene **47** as a 93:7 mixture of the 7*Z*,11*E*/*Z*,13*E*-trienes in 42% yield. The Labelle et al. (1990) method called for a THF–HMPA solvent mixture, whereas our results showed that DMPU was an effective and safer substitute for toxic and carcinogenic HMPA. Trienol **48** was obtained by removal of the THP protecting group with PTSA in methanol, and the isomeric purity of **48** was increased to 97:3 by recrystallization from hexane. Oxidation of **48** with pyridinium chlorochromate (PCC) then produced the desired aldehyde **49** in 77% yield.

The synthesis of 7*Z*,11*Z*-16:Ald was carried out by a modification of this route (Scheme 5). Thus, alkyne **41** was treated with BuLi followed by addition of 1-chloro-3-iodopropane to give chloride **50** (Sonnet, 1974) (64% yield). This was converted to the corresponding iodide **51** by refluxing with sodium iodide in acetone (95% yield). Reaction of **51** with triphenylphosphine gave the corresponding phosphonium salt **52**, which can be used as an intermediate for the syntheses of both the diene and the triene pheromone components. Wittig reaction of the ylide derived from **52** via treatment with lithium hexamethyldisilazide in THF–DMPU solvent gave the *Z* olefinic compound **53** (Yadav et al., 1988) in 65% yield as a 98.8*Z*:1.2*E* isomeric mixture. After removal of the THP group with PTSA in methanol, alcohol **54** (Yadav et al., 1988) was reduced with P-2 nickel and hydrogen, yielding dienol **55** in 71% yield. 7*Z*,11*Z*-16:Ald **56** was obtained by oxidation of **55** with PCC as before.

The synthetic route for the preparation of 7*Z*,11*Z*,13*Z*-16:Ald **62** is depicted in Scheme 6. Phosphonium salt **52**, previously used to prepare 7*Z*,11*Z*-16:Ald **56**, was deprotonated with lithium hexamethyldisilazide in THF–DMPU, and reacted with 2-pentynal to give protected endiynol **59** (58%), which was then stereoselectively reduced to the corresponding triene **60** with dicyclohexylborane (80%), yielding a 92:8 mixture of the (7*Z*,11*Z*,13*Z*)- and (7*Z*,11*E*,13*Z*)-trienes, respectively. After removal of the THP protecting group with PTSA in methanol (80%), the isomeric purity of trienol **61** was increased to 95:5 by chromatography on silica gel impregnated with 10% AgNO₃. Oxidation of **61** with PCC completed the synthesis, giving aldehyde **62** in 73% yield.

Field Trials. A preliminary, unreplicated field trial run for 1 d provided evidence that both 7*Z*,11*Z*-16:Ald and 7*Z*,11*Z*,13*E*-16:Ald were required for attraction of male moths, because traps baited with 100 μg of either component alone attracted no moths, whereas traps baited with 100:10, 100:50, or 100:100 μg ratios of 7*Z*,11*Z*,13*E*-16:Ald to 7*Z*,11*Z*-16:Ald caught 135, 192, and 146 moths, respectively. A subsequent, replicated field trial testing different blend ratios of the two components caught >13,000 moths, with a 3:1 ratio of the trienal and dienal being most attractive (Figure 3). A dose-response trial of the 3:1 ratio showed that trienal doses of 100–1000 μg were more attractive than lower doses of 1.0–33 μg/lure (Figure 4). Many thousands of moths were

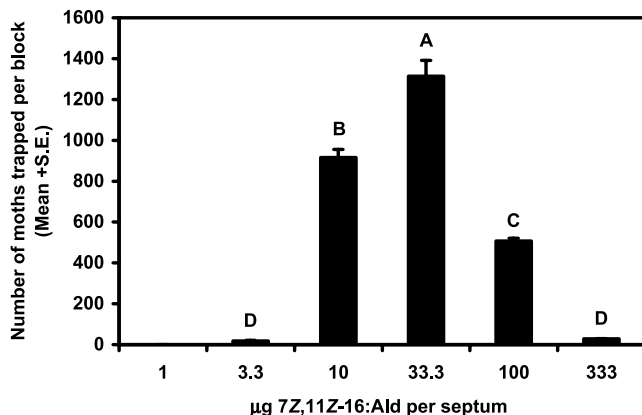


FIG. 3. Citrus leafminer field trial to determine the optimum ratio of the trienal to the dienal, September 8–12, 2004. Each septum was loaded with 100 µg *Z7,Z11,E13-16:Ald* and variable amounts of *Z7,Z11-16:Ald*. Bars with different letters are significantly different (two-way ANOVA followed by Student–Neumann–Keuls test, $\alpha = 0.05$, $F = 180.4$, $df = 4, 24$, $P < 0.001$). Total moths trapped = 13,915. Solvent controls and the 1 µg dose of *Z7,Z11-16:Ald* each caught 1 moth, and were not included in the statistical analysis.

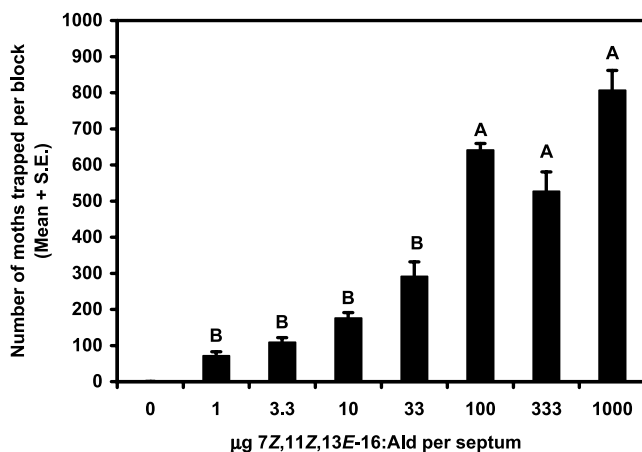


FIG. 4. Effect of pheromone dose on trap catches of citrus leafminer, October 15–22, 2004. The trienal/dienal ratio was fixed at 3:1. Bars with different letters are significantly different (two-way nonparametric ANOVA followed by Bonferroni's *t*-tests for means separation, $\alpha = 0.05$). For treatment, $F = 22.92$, $df = 6, 34$, $P < 0.001$; for block effect, $F = 0.0$, $df = 4, 34$, $P = 1.00$. Total number of moths trapped = 13,122. Control traps caught two moths, and were not included in the statistical analysis.

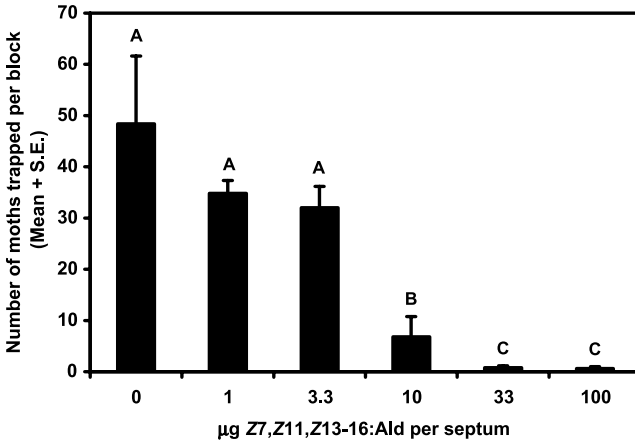


FIG. 5. Citrus leafminer blend ratio trial, June 2005. The trienal/dienal ratio was fixed at 100:33 µg, and variable amounts of 7Z,11Z,13Z-16:Ald were added to this base blend. Bars surmounted with different letters are significantly different (two-way ANOVA on $\log_{10}(x + 1)$ transformed data, followed by Student-Neumann-Keuls test, $\alpha = 0.05$). For treatment, $F = 44.0$, $df = 5, 29$, $P < 0.001$; for block, $F = 1.93$, $df = 4, 29$, $P = 0.14$. Total moths trapped = 617. Control traps caught no moths, and were not included in the statistical analysis.

caught, again indicating the effectiveness of the lure. The addition of increasing amounts of 7Z,11Z,13Z-16:Ald to the two component blend of 7Z,11Z,13E-16:Ald and 7Z,11Z-16:Ald (100:33) resulted in decreasing numbers of moths caught, indicating that this isomer was inhibitory at higher percentages of the blend (Figure 5).

DISCUSSION

Solvent extracts of pheromone glands were difficult to prepare because of the tiny size of these moths, and the pheromone components were not detected in these extracts by GC or GC-MS. In contrast, the SPME method of dynamic collection of pheromone from the effluvia from live female moths provided extracts in which the amounts of the pheromone components were detectable by GC and GC-MS, and these provided an accurate representation of the compounds released by the moths. However, even with overnight SPME collections made from groups of as many as 30 moths, and with the efficiency inherent in the desorption of the entire SPME sample directly into the injection port of the GC or GC-MS, we frequently could not detect the pheromone

components with either of these instruments. Thus, the preliminary identification of the two compounds was based solely on the combination of data from relative retention times on two GC columns of different polarities determined from EAD responses (Table 1), the relative sizes of antennal responses to different functional groups and double bond orientations, and latterly, the bioassay results. The sum total of these data presented a compelling case for the identification of the pheromone components. Confirmation of the pheromone structures was finally acquired after the field trials, when mass spectra of the two compounds were obtained from an SPME sample obtained from aeration of female moths for several sequential nights.

The analytical results and the field trials conclusively demonstrate that the citrus leafminer pheromone for California populations of this moth consists of two major synergistic components, with no attraction at all to either compound alone. This explains why researchers in several areas of the world had not been able to reproduce the results of Ando et al. (1985), who reported attraction of several hundred moths of a Japanese population of this species to 7Z,11Z-16:Ald as a single component. Furthermore, the marked difference between the attractiveness of 7Z,11Z-16:Ald between populations in Japan and other areas of the world suggests that the Japanese population may constitute a geographically distinct pheromone race, as has been reported for other lepidopteran species (e.g., turnip moth, *Agrotis segetum*, Tóth et al., 1992; moths in the genus *Hemileuca*, McElfresh and Millar, 2002).

The GC-EAD traces from the DB-5 column showed occasional weak antennal responses to another trace compound, tentatively identified as 7Z,11Z,13Z-16:Ald. However, addition of this isomer to the optimal two-component blend did not increase trap catches, and at higher doses, was actually inhibitory. Given that our field bioassays indicated that the two-component blend of 7Z,11Z,13E-16:Ald and 7Z,11Z-16:Ald was highly attractive to male moths, with many thousand moths being caught in each of the two bioassays in fall of 2004, it seems unlikely that this compound would be an important component of the pheromone blend.

To our knowledge, the citrus leafminer is the only lepidopteran species known to use 7Z,11Z-16:Ald, although the corresponding acetate has been reported as a sex attractant or sex pheromone component for one stathmopid, eight gelechiid, and one noctuid species (Witzgall et al., 2004). This is the first report of the trienal component, 7Z,11Z,13E-16:Ald, and to our knowledge, the 7Z,11Z,13E-16:X structural motif has not been reported before from any lepidopteran pheromone or other natural source. However, this may be a reflection of the few *Phyllocnistis* or related leafminer species' pheromones studied rather than a unique structural motif in nature.

In summary, this new pheromone blend will find immediate use in monitoring citrus leafminer populations, particularly in areas such as California

where the insect has been introduced fairly recently, and where it is still expanding its range.

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IDENTIFICATION OF COMPONENTS OF MALE-PRODUCED
PHEROMONE OF COFFEE WHITE STEMBORER,
Xylotrechus quadripes

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Abstract—The coffee white stem borer, *Xylotrechus quadripes* Chevrolat (Coleoptera: Cerambycidae), is the foremost pest of arabica coffee in India, Sri Lanka, China, Vietnam, and Thailand. Previous work showed that female beetles were attracted to traps baited with male beetles. Analyses of volatiles from male *X. quadripes* of Indian origin by gas chromatography (GC) linked to electroantennographic (EAG) recording from a female beetle antenna showed three male-specific components comprising more than 90% of the volatiles, two of which elicited EAG responses. The major EAG-active component was produced at up to 2 $\mu\text{g hr}^{-1} \text{insect}^{-1}$ and was identified as (*S*)-2-hydroxy-3-decanone (**I**) by comparison of GC data, and mass (MS), infrared, and nuclear magnetic resonance (NMR) spectra with those of synthetic standards. The second component was identified as 3-hydroxy-2-decanone (**II**) produced in part by isomerization of **I** under the conditions of the GC analysis, although the NMR spectrum suggested it is naturally produced at up to 7% of **I**. The minor component that elicited an EAG response, present at 7% of the amount of **I**, was identified as (*S,S*)-2,3-dihydroxyoctane (**III**) from GC and MS data. 2-Hydroxy-3-octanone (0.2–0.5% of **I**), 2,3-decanedione (2% of **I**), 2-phenylethanol (3% of **I**), and octanoic acid (4% of **I**) were also identified in volatiles from male beetles. A general, stereospecific synthetic route to the enantiomers of 2-hydroxy-3-alkanones from the enantiomers of ethyl lactate was developed. The enantiomers of **III** were synthesized from (*E*)-2-octene by Sharpless asymmetric dihydroxylation. (*S*)-(**I**) was attractive to male *X. quadripes* in

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laboratory bioassays, but addition of (*S,R,S*)-(III) at 10% of I reduced attractiveness. In field trials carried out in India with sticky, cross-vane traps, (*S*)- and (*R,S*)-(I) attracted male *X. quadripes* and addition of (*S,S*)-(III) at 10% of I reduced attractiveness. Significant numbers of female *Demonax balyi* Pascoe (Coleoptera: Cerambycidae) were sometimes caught in traps baited with (*S*)-(I) alone.

Key Words—*Xylotrechus quadripes*, coffee white stem borer, Coleoptera, Cerambycidae, male sex pheromone, (*S*)-2-hydroxy-3-decanone, 3-hydroxy-2-decanone, (*S,S*)-2,3-dihydroxyoctane, *Demonax balyi*.

INTRODUCTION

The coffee white stem borer, *Xylotrechus quadripes* Chevrolat (Coleoptera: Cerambycidae) is the foremost pest of arabica coffee in India, Sri Lanka, China, Vietnam, and Thailand (Le Pelley, 1968; Rhainds et al., 2002). In India, it is estimated that over 9 million trees have to be destroyed each year because of attack by *X. quadripes*, costing around \$40 m annually for replacement and loss of production.

The adults of *X. quadripes* have distinct flight periods, and the females lay eggs in crevices in the bark. The larvae tunnel into the trunk, blocking the tunnel with frass behind them to deter predators. The tunneling in the trunk and roots rapidly kills young plants of up to 7–8 yr old, whereas older plants may survive for a few seasons but eventually succumb. Current control measures include maintenance of good shade because the adults are more active in sunlight, but this can reduce photosynthesis and yield. Removal of loose bark or scrubbing the bark at the base of the tree discourages oviposition, and collar pruning of infested trees may be effective if the pest has not reached the roots. Benzene hexachloride applied to the trunk at critical times during the flight periods is still the only effective insecticide against young larvae of *X. quadripes*, but this has now been banned for agricultural use in India and many other countries. Once larvae have penetrated into the stem, trees should be removed and destroyed to prevent spread of infestation (Le Pelley, 1968; Sreedharan and Kumar, 2001; Rhainds et al., 2002).

Male *X. quadripes* were shown to attract female beetles in the field in India by Venkateshu et al. (1986). Production of a sex pheromone by males of a related species, the grape borer *X. pyrrhoderus*, was demonstrated by Iwabuchi (1982), and the chemical structures of the components were identified by Sakai et al. (1984). The same components were shown to be produced by the mulberry borer, *X. chinensis* (Iwabuchi et al., 1987; Kuwahara et al., 1987). Identification and synthesis of the male sex pheromone of *X. quadripes* could provide the basis for development of a monitoring system for this pest in order to determine its distribution, and to time control measures directed at young larvae more

effectively. Mass trapping with pheromone-baited traps could provide a method for control of this pest because it occurs at relatively low density and the pheromone attracts female beetles, thus directly affecting egg-laying.

We have reported the identification of (*S*)-2-hydroxy-3-decanone as the major component of the male sex pheromone of *X. quadripes* from India (Hall et al., 1998). This was confirmed by Rhains et al. (2001a), using insects from China, and those authors also reported the presence of 2,3-decanedione in volatiles from the male beetles. Here, we report in full the identification of the major component of the sex pheromone of male *X. quadripes* from India, identification of an alternative minor component, and laboratory and field bioassays of these components.

METHODS AND MATERIALS

Insects. *X. quadripes* adults were collected as they emerged from infested logs held in a netted room at the Central Coffee Research Institute (CCRI) under ambient temperature and humidity. Insects were collected several times during the morning emergence hours (Venkatesha et al., 1995) to ensure that they did not mate, and were sexed (Visitpanich, 1994) and maintained in separate tubes. These were hand-carried from India and separately maintained in an insectary at 70% RH with 12:12 h L/D light and 27:23°C temperature cycles.

Pheromone Collection. Volatiles were collected from 15 virgin male beetles held in a silanized, 2-liter round-bottomed flask containing a filter paper (15 cm diam; Whatman No. 52). Air was drawn into the flask through a filter of activated charcoal (10 × 2 cm; 10–18 mesh) at 2 l/min⁻¹, and volatiles were trapped on a filter of Porapak Q (50–80 mesh; 200 mg; Waters Corp., USA) packed between silanized glass wool plugs in a Pasteur pipette (i.d. 4 mm). The Porapak Q was purified by Soxhlet extraction with chloroform for 8 hr, and filters were rinsed well with dichloromethane before use. Volatiles from 15 virgin female beetles were collected similarly. Trapped volatiles were eluted from the filter after 24–72 hr with dichloromethane (3 × 0.5 ml) or alternatively with CDCl₃ (1 ml) or ether (1 ml) for nuclear magnetic resonance (NMR) analysis or microreduction, respectively. For the latter, a few grains of lithium aluminum hydride were added to the ether solution, and the mixture was shaken at intervals for 1 hr. Water (0.1 ml) was then carefully added and the mixture was shaken. The upper ether layer was removed, dried with magnesium sulfate and analyzed by GC.

A container wash was obtained by maintaining nine male beetles in a conical flask (250 ml) with a filter paper (7 cm diam; Whatman No. 52) for 48 hr and then removing the beetles and washing flask and filter paper with hexane (5 + 5 ml). A female beetle container wash was obtained similarly.

Whole-body washes were obtained by placing nine male or nine female beetles in hexane (2.5 ml) 3 hr after lights on. After 10 min, the solvent was removed, and the residue was rinsed with a further aliquot of hexane (1 ml).

Gas Chromatographic Analyses. Gas chromatographic (GC) analyses were carried out on Carlo Erba Mega instruments with fused silica columns (25 m \times 0.32 mm i.d.) coated with polar CP Wax 52CB (Carbowax 20M equivalent; Chrompack, London, UK) or nonpolar CP Sil 5CB (methyl silicone; Chrompack). Carrier gas was helium at 0.5 kg cm⁻² and oven temperature was programmed at 50°C for 2 min, then at 6°C min⁻¹ to 230°C. Injection was splitless or split (50:1) (200°C), and detection was by flame ionization detection (FID; 220°C). GC retention times are reported as retention indices (RI) relative to those of normal hydrocarbons.

Enantiomeric purity was determined by GC analysis on cyclodextrin GC columns (CP-Chirasil-DexCB, 25 m \times 0.32 mm i.d., Chrompack; or β -Cyclodextrin, 50 m \times 0.22 mm i.d., SGE, Milton Keynes, UK.). These were operated isothermally at 130 or 135°C, respectively, with helium carrier gas at 0.5 or 1.5 kg cm⁻², respectively, and injector and detector temperatures as described above.

Coupled Gas Chromatography-Mass Spectrometry. Gas chromatography-mass spectrometry (GC-MS) analyses were carried out on a fused silica column (25 m \times 0.2 mm i.d.; CP Wax 52CB) directly linked to a Finnigan ITD 700 ion trap detector (Thermoquest, Hemel Hempstead, Herts., UK) operated in EI or CI (*iso*-butane) mode. GC conditions were as described above.

Coupled Gas Chromatography-Electroantennography. Linked gas chromatography-electroantennography (GC-EAG) analyses were carried out as described by Cork et al. (1990) with effluent from the GC column split equally between the FID and a small glass reservoir in the column oven, the contents of which were expelled at 15-sec intervals with nitrogen (500 ml min⁻¹ for 3 sec) over an EAG preparation. In the GC analysis, we used a fused silica column (25 m \times 0.32 mm i.d.; CP Wax 52CB) programmed at 50°C for 2 min, then 20°C min⁻¹ to 100°C, and then 4°C min⁻¹ to 260°C. For EAG preparations, the head of the beetle was excised and impaled on the indifferent glass capillary electrode. The tip of one antenna was then inserted into the recording glass electrode and the preparation positioned so that the stimulus was directed at the terminal segments of the antenna. The electrodes contained 0.1 M aqueous KCl solution with 10% polyvinylpyrrolidone to reduce evaporation, and were inserted into stainless steel electrode holders (Syntech, Hilversum, The Netherlands) mounted on micromanipulators (Leica Microsystems, Wetzlar, Germany). Electrical contact was made with chloridized silver wires to the amplifier (UN-06, Syntech), and both EAG and GC signals were collected and processed with Turbochrom 4 software (Perkin Elmer, Beaconsfield, Bucks., UK).

Spectrometry. NMR spectra were recorded in CDCl_3 on a JEOL EX270 machine at 270 MHz for ^1H and 67.8 MHz for ^{13}C . Enantiomeric purity was determined after addition of the chiral cosolvent, (*R*)-(-)-2,2,2-trifluoro-1-(9-anthryl)ethanol (10 mg; Sigma-Aldrich, Gillingham, Dorset, UK). Infrared (IR) spectra were recorded as thin films with a Perkin Elmer 298 grating spectrophotometer (Perkin Elmer, Beaconsfield, Bucks., UK).

Synthetic Compounds. (*S*)-2-Hydroxy-3-decanone was synthesized from (*S*)-ethyl lactate by a short, general route (Figure 1). The *R* enantiomer was synthesized similarly from (*R*)-ethyl lactate. In initial experiments, (*S*)-ethyl lactate was protected as the benzyl ether by reaction with NaH in THF at room temperature followed by addition of benzyl bromide (65%; b.p. $82^\circ\text{C}/0.6$ mmHg). After hydrolysis to the acid (88%; b.p. $96^\circ\text{C}/0.75$ mmHg), reaction with heptyl lithium gave 2-benzyloxy-2-decanone (62%; b.p. $90^\circ\text{C}/0.2$ mmHg) (cf. Figure 1). The benzyl group was removed by catalytic hydrogenation over 10% palladium on charcoal in ethyl acetate (98%). Examination of the 2-hydroxy-3-decanone product by chiral GC (below) showed that the product was completely racemized. Analysis of the reaction intermediates by chiral GC showed that this racemization had occurred during the initial benzylation step. The (*R,S*)-2-hydroxy-3-decanone was used in some field trials as indicated.

Use of 2-tetrahydropyranyl or *tert*-butyl dimethylsilyl protecting groups gave 2-hydroxy-3-decanone without detectable racemization as shown in Figure 1. The former protecting group was preferred for large-scale synthesis, as described below.

Ethyl 2-(2-tetrahydropyranyloxy)propanoate. Freshly distilled 3,4-dihydro-2H-pyran (50.4 g, 0.6 mol) was added dropwise to a solution of (*S*)-ethyl lactate (59 g, 0.5 mol; Sigma-Aldrich, Gillingham, Dorset, UK) and a few crystals of *p*-toluenesulfonic acid (PTSA) in dry diethyl ether (250 ml) cooled to

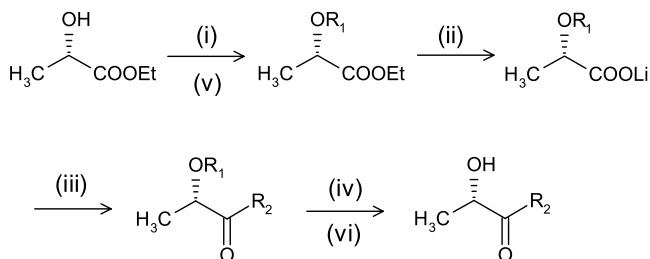


FIG. 1. Synthesis of (*S*)-2-hydroxy-3-decanone. [R_1 = tetrahydropyranyl, R_2 = C_7H_{15} : (i) 2,3-dihydropyran/PTSA (92%); (ii) $\text{LiOH}\cdot\text{H}_2\text{O}/\text{EtOH}$ (100%); (iii) $\text{C}_7\text{H}_{15}\text{Li}/\text{ether}$ (90%); (iv) MeOH/PTSA (68%). R_1 = *tert*-butyldimethylsilyl, R_2 = C_7H_{15} : (v) tBDMSCl/imidazole/THF (93%), (ii) (94%), (iii) (57%), (vi) $\text{Bu}_4\text{NF}/\text{THF}$ (36%).

0°C under nitrogen. The reaction mixture was stirred for 4 hr at room temperature. Solid K_2CO_3 was added to the solution, and the mixture was extracted twice with saturated $NaHCO_3$ solution. The organic phase was dried over $MgSO_4$, filtered, and the solvent was removed on a rotary evaporator. The residue was distilled to give the product in 92% yield (92 g; b.p. 76–81°C/0.5 mmHg). 1H NMR: δ 1.27 (t, $J = 7.2$ Hz, 3H, CH_2CH_3), 1.45 (d, $J = 7.2$ Hz, 3H, $CHCH_3$), 1.55–1.80 (m, 6H, CH_2), 3.68 (m, 2H, $O-CH_2$), 4.16 (q, $J = 7.2$ Hz, 2H, CH_2CH_3), 4.20 (q, $J = 3.3$ Hz, 1H, CH_3CH-OH), 4.40 (d, $J = 6.9$ Hz, 1H, $O-CH-O$); MS: m/z (%) 200 (2), 184 (1), 157 (3), 144 (2), 130 (3), 119 (trace), 101 (10), 85 (100), 67 (5), 57 (5), 43 (10); GC analysis on the polar column showed the two diastereoisomers in a 68:32 ratio.

Lithium 2-(2-tetrahydropyranyloxy)propanoate. Lithium hydroxide monohydrate (4.6 g, 0.1 mol) was added to a stirred solution of ethyl 2-(2-tetrahydropyranyloxy)propanoate (20.2 g, 0.1 mol) in ethanol (50 ml) under nitrogen. After 4 hr of stirring, GC analysis showed no trace of the starting material. Ethanol was removed under reduced pressure and the residue was washed twice with petroleum ether (100 ml) to give lithium 2-(2-tetrahydropyranyloxy)propanoate as a white solid in 100% yield (18.0 g). 1H NMR: δ 1.40 (d, $J = 7.3$ Hz, 3H, $CHCH_3$), 1.65–1.80 (m, 6H, CH_2), 3.60 (m, 2H, $O-CH_2$), 4.20 (q, $J = 7.3$ Hz, 1H, $O-CHCH_3$), 4.40 (d, $J = 6.9$ Hz, 1H, $O-CH-O$).

2-(2-Tetrahydropyranyloxy)-3-decanone. Heptyl lithium was prepared from heptyl bromide (17.9 g, 0.1 mol) and lithium (1.75 g, 0.25 mol) in dry ether (100 ml) under argon. The heptyl lithium (95 ml, 66 mmol) was added dropwise to a mechanically stirred suspension of lithium 2-(2-tetrahydropyranyloxy)propanoate (10.8 g, 60 mmol) in dry ether (50 ml) under nitrogen and cooled in an ice bath. After stirring at room temperature overnight, the contents were poured onto ice and saturated NH_4Cl solution. The aqueous layer was extracted twice with ether and the extracts were washed with saturated NH_4Cl , dried with $MgSO_4$, and filtered through a pad of Florisil. The solvent was evaporated under reduced pressure to give the crude 2-(2-tetrahydropyranyloxy)-3-decanone (14.7 g; 95%). Kugelrohr distillation of an analytical sample gave the pure product as a colorless oil (90%), b.p. 98°C/0.06 mmHg; MS: m/z (%) 211 (2, $M-C_2H_4O$)⁺, 182 (2), 173 (5), 155 (3), 137 (2), 127 (5), 114 (3), 95 (trace), 85 (100), 67 (3), 57 (5), 41 (10); GC analysis on the polar column showed the two diastereoisomers in a 68:32 ratio.

(S)-2-Hydroxy-3-decanone. The crude 2-(2-tetrahydropyranyloxy)-3-decanone (14.7 g) was dissolved in methanol (120 ml), a few crystals of PTSA were added, and the solution was stirred at room temperature. GC analysis on the polar column indicated reaction was complete after 6 hr. A small amount of solid K_2CO_3 was added and most of the methanol was removed on a rotary evaporator under reduced pressure. The residue was dissolved in 1:1 diethyl ether/hexane, washed with saturated $NaHCO_3$ and dried over $MgSO_4$. After

removal of solvents under reduced pressure, the residue was distilled to give a main fraction in 68% yield (b.p. 57–60°C/0.04 mmHg; 7.0 g). IR (film): ν 3420 cm^{-1} (O–H), 1710 cm^{-1} (C=O); ^1H NMR: δ 0.88 (t, $J = 6.9$ Hz, 3H, CH_2CH_3), 1.27 (bm, 8H, CH_2), 1.38 (d, $J = 6.9$ Hz, 3H, CH_3CHOH), 1.61 (m, 2H, $\text{CH}_2\text{—CH}_2\text{—C=O}$), 2.41 (dt, $J = 16.8, 7.6$ Hz, 1H, $\text{CH}_2\text{—CH=CH—C=O}$), 2.52 (dt, $J = 16.8, 7.6$ Hz, 1H, $\text{CH}_2\text{—CH=CH—C=O}$), 3.55 (d, $J = 4.5$ Hz, 1H, CH—OH), 4.24 (dq, $J = 7.1, 4.5$ Hz, 1H, CH_3CHOH); ^{13}C NMR: δ 14.05, 19.86, 22.59, 23.63, 29.00, 29.18, 31.63, 37.54, 72.56, 212.72; MS: m/z (%) 173 ($\text{M} + 1$)⁺ (15), 155 (3), 137 (5), 127 (40), 57 (100), 45 (60), 29 (30).

By GC analysis on the polar GC column, all samples of 2-hydroxy-3-decanone contained 5–10% of the isomeric 3-hydroxy-2-decanone. GC analysis of the enantiomers on the 25-m cyclodextrin column at 130°C gave baseline separation (retention times R 23.7 min, S 24.0 min). Both synthetic enantiomers showed 98% enantiomeric excess (ee), similar to those of the starting ethyl lactates. A high level of enantiomeric purity was also indicated by the ^1H NMR spectrum of the S enantiomer in the presence of a chiral cosolvent using either the signals due to the $\text{CH}_3\text{—CH—OH}$ doublet or the $\text{CH}_3\text{—CH—OH}$ multiplet.

(S)-2-Hydroxy-3-octanone was synthesized similarly from (S)-ethyl lactate and 1-bromopentane in 62.5% yield (b.p. 110°C/15 mm). Spectroscopic data were identical with those of Mori and Otsuka (1985).

2,3-Dihydroxyoctane was first synthesized as a 40:60 mixture of the S,S and S,R diastereoisomers by reduction of (S)-2-hydroxy-3-octanone with lithium aluminum hydride in ether. The *erythro* S,R diastereoisomer was predicted to be the major product of this reduction, assuming coordination of the aluminum by the 2-hydroxy group, and this was confirmed by synthesis of the separate enantiomers (below). This diastereoisomeric mixture was used without further purification in laboratory bioassays.

(S,S)-2,3-Dihydroxyoctane was synthesized by reaction of (E)-2-octene (Sigma-Aldrich) with the Sharpless reagent “AD-mix- α ” (Sigma-Aldrich) in *tert*-butyl alcohol in the presence of methanesulfonamide (Sharpless et al., 1992). The product was obtained in 91% yield after chromatography and Kugelrohr distillation (b.p. 135°C/4 mmHg). Similar reaction of (E)-2-octene with the “AD-mix- β ” (Sigma-Aldrich) gave (R,R)-2,3-dihydroxyoctane in similar yield. Both compounds were completely free ($\leq 0.2\%$) of *erythro* diastereoisomers by GC analysis. ^1H NMR: δ 0.89 (t, $J = 6.6$ Hz, 3H, $\text{CH}_3\text{—CH}_2\text{—}$), 1.18 (d, $J = 6.3$ Hz, 3H, $\text{CH}_3\text{—CH(OH)—}$), 1.50–1.22 (m, 8H, $4 \times \text{CH}_2$), 2.47–2.41 (br m, 2H, $2 \times \text{OH}$), 3.48–3.46 (br m, 1H, CHOH), 3.58 (quint, $J = 6.3$, 1H, $\text{CH}_3\text{—CHOH}$); ^{13}C NMR: δ 14.0, 19.4, 22.6, 25.2, 31.8, 33.3, 70.8, 76.2. These data were identical with literature values (e.g., Mori and Otsuka, 1985).

Analyses of the synthetic 2,3-dihydroxyoctanes on the 50 m cyclodextrin GC column at 135°C showed good separation of the two enantiomers. The S,S enantiomer (retention time, 28.2 min) had an ee of only 83%, whereas that of

the *R,R* enantiomer (28.7 min) was 90%. The lower ee of the former was thought to be due to the prolonged reaction time (24 hr at 0°C and 17 hr at room temperature) compared with that used in the second reaction (15 hr at RT).

2,3-Decanedione was prepared in 50% yield by oxidation of (*S*)-2-hydroxy-3-decanone with pyridinium chlorochromate and sodium acetate in dichloromethane (Corey and Suggs, 1975) followed by flash chromatography and Kugelrohr distillation (b.p. 80°C/0.06 mm). ¹H NMR: δ 0.86 (t, $J = 7$ Hz, 3H, $\text{CH}_3\text{-CH}_2$), 1.27 (m, 8H, $4 \times \text{CH}_2$), 1.56 (quint, $J = 7$ Hz, 2H, $\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-C=O}$), 2.31 (s, 3H, $\text{CH}_3\text{-C=O}$), 2.71 (t, $J = 7$ Hz, 2H, $\text{CH}_2\text{-C=O}$); ¹³C NMR: δ 14.0, 22.6, 23.1, 23.7, 29.0, 29.1, 31.6, 35.7, 197.7, 199.6; MS m/z (%) 127 (22), 109 (8), 83 (1), 81 (1), 67 (9), 58 (4), 57 (100), 55 (11), 43 (79), 42 (12), 41 (61) (c.f. slightly different data in Rhainds et al., 2001a).

Pheromone Dispensers. Controlled release dispensers for the synthetic pheromone were heat-sealed polyethylene vials (22 \times 8 \times 1.5 mm thick; Just Plastics Ltd., London, UK) or polyethylene sachets (2.5 cm \times 2.5 cm \times 120 μ thick) made from heat-sealed layflat tubing (Transatlantic Plastics, Southampton, UK) and contained 100 μ l (approximately 80 mg) or 200 μ l pheromone. Release rates from dispensers were measured by periodic weighing of duplicate dispensers maintained in a wind tunnel at 27°C and 8 km hr⁻¹ wind speed.

Bioassays. Bioassays were carried out in an insectary maintained at 27°C with 5000 lux lighting, between 10:00 and 12:00 hours coinciding with the period of maximum activity of the beetles in the field (Venkatesha et al., 1995). Two types of bioassay apparatus were used, both constructed of Plexiglas.

The "swastika" bioassay consisted of a central circular chamber (19 \times 5 cm high) with four equally spaced entry/exit openings in the side wall. The openings led to tubes (3.8 cm diam, total length 55 cm) with a right angle bend in the middle to give a swastika appearance. Plexiglas sample bottles (6 \times 4 cm) with fine mesh tops and bottoms were fixed to the distal ends of the tubes. Air was drawn into the apparatus through the arms and out through a tube attached to the center of the top of the central chamber at 20 l/min⁻¹. Initial studies were carried out at NRI with 25 male and 25 female insects so that insects were used as lures and/or test individuals more than once, but never on the same day. A single insect was placed at the center of the central chamber with appropriate treatments in the sidearms. Insect behavior was observed for 10 min: if an insect entered one of the sidearms this was scored, otherwise it was scored as remaining in the central chamber. The apparatus was rotated through 90° between each observation to avoid any possible effects due to uneven lighting or visual cues. Ten insects were tested in each experiment and data were analyzed by a χ^2 test assuming equal distribution of responding beetles between the arms as null hypothesis.

The tube bioassay utilized a Plexiglas tube (1 m \times 3.8 cm i.d.) with a Plexiglas sample bottle (6 cm \times 4 cm) with fine gauze top and bottom attached

at one end. The test source was put in the sample bottle and a single test beetle placed at the opposite end of the tube. Air was drawn in through the source bottle and out through the other end of the tube at 10 l min^{-1} . The tube was marked at 10-cm intervals, and the nearest approach to the source by the test insect during 10 min of observation was scored according to the interval mark crossed (i.e., 1.0 = reached source, 0.5 halfway, 0.0 = no upwind movement). Each lure was tested with 10 insects, the data were subjected to arcsin transformation before analysis of variance, and differences between means were tested for significance by Duncan's multiple range test (DMRT).

Field Trials. Field trials were carried out in estates round the Central Coffee Research Institute, Chikmagalur, Karnataka, the Coffee Research Sub-Station, Chettalli, Karnataka, and the Regional Coffee Research Station, Thandigudi, Tamil Nadu, India, during the two flight seasons, April–June and October–December, in 1997 and 1998. Traps were sticky delta traps (20×20 floor, 15-cm walls; Agrisense, Treforest, UK) or sticky white cross-vane traps (60×30 cm; Agrisense) fastened to wooden poles at a height of approximately 1.5 m. Treatments were tested in randomized complete blocks with 20 m between traps, and catches were recorded and discarded every 2–4 d. Total catches were ranked and analyzed by the nonparametric Kruskal–Wallis or Mann–Whitney *U* tests. Critical differences were calculated to test differences in catches for significance.

RESULTS

Pheromone Identification. GC analyses of volatiles from male *X. quadripes* beetles on a polar column showed two main components (A) and (B) representing up to 90% of the volatile material (Figure 2). These were produced at rates up to $2 \mu\text{g hr}^{-1} \text{ beetle}^{-1}$ of (A) measured over a 24-hr period and quantified by comparison of peak area with that of a 1-octen-3-ol standard. The two components cochromatographed on a nonpolar column, and retention indices (RI) are shown in Table 1. The same two components were observed in male container washes and male body washes, but they were not detected in volatiles collected from female beetles. In initial analyses, the ratio of (A)/(B) in volatile collections varied between 1:0.14 and 1:0.75, and different results could be obtained from the same sample, suggesting that (A) and (B) were interconverting under the conditions of the analyses.

In linked GC-EAG analyses of the male volatiles (Figure 2), an EAG response was elicited from antennae of female beetles by the major component (A) and also by a smaller peak (C) eluting after (B) on the polar GC column. GC-MS analyses in EI mode of components (A) and (B) showed the two

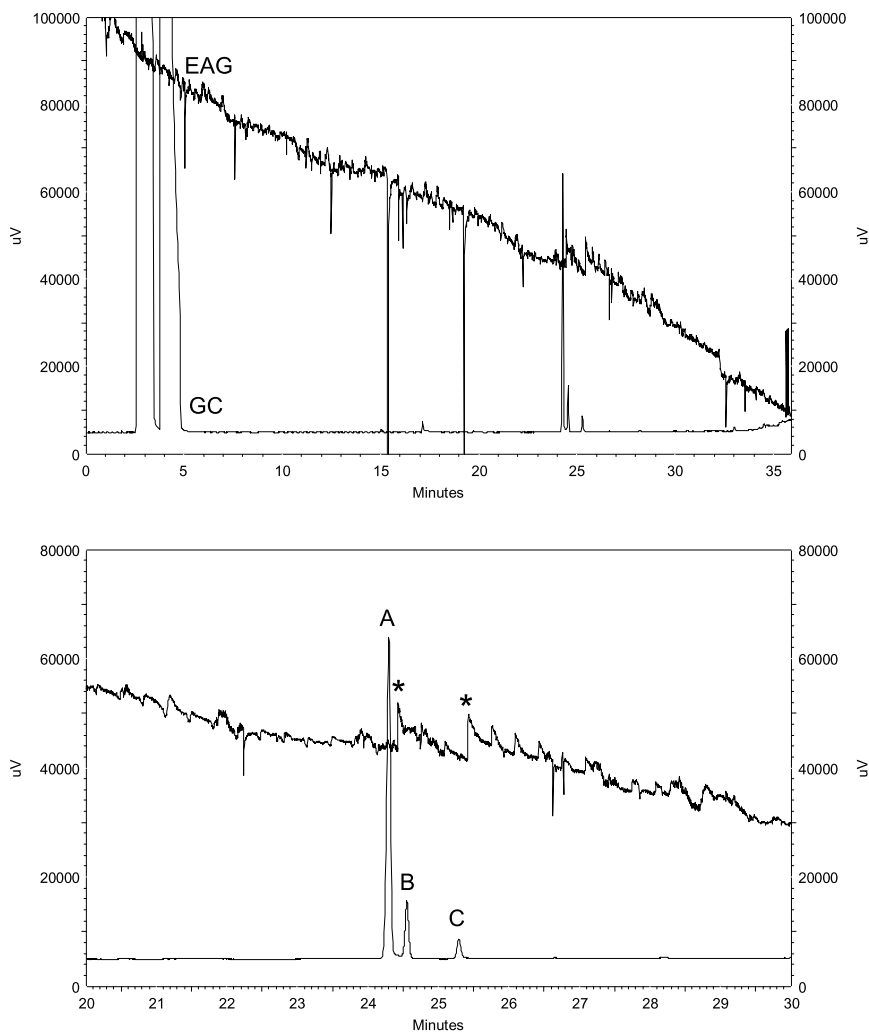


FIG. 2. Linked GC-EAG analysis of volatiles from male *X. quadripes* analyzed on polar CP Wax52CB column; lower traces are expansions of upper traces.

components had mass spectra (Figure 3) with highest mass ions at m/z 173. These indicated a possible molecular weight of 172 for both compounds, as the ion trap instrument used can give $M + 1$ ions due to self-induced chemical ionization, particularly at high concentrations. Molecular weight assignments were confirmed by CI (*iso*-butane) mass spectra, which both showed strong $M + 1$ ions at m/z 173. Comparison of the EI mass spectra with those reported

TABLE 1. GC RETENTION INDICES (RI) OF COMPONENTS IN VOLATILES FROM MALE *X. quadripes* AND SYNTHETIC COMPOUNDS

Compound	Retention index	
	CPSil5CB	CPWax52CB
Component A	1272	1848
Component B	1272	1862
Component C	1144	1902
(<i>S</i>)-2-Hydroxy-3-decanone (I)	1272	1848
3-Hydroxy-2-decanone (II)	1272	1862
2,3-Dihydroxydecane	1375, 1390	2105, 2144
(<i>S,S</i>)-2,3-Dihydroxyoctane (III)	1144	1902
(<i>S,R</i>)-2,3-Dihydroxyoctane	1155	1943
(<i>S,S</i>)-2,3-Diacetoxyoctane	1377	1823
(<i>S,R</i>)-2,3-Diacetoxyoctane	1366	1779
(<i>S</i>)-2-Hydroxy-3-octanone	1069	1631
3-Hydroxy-2-octanone	1069	1642
2,3-Decanedione	1161	1520

by Kuwahara et al. (1987) for 2-hydroxy-3-octanone and 3-hydroxy-2-octanone, pheromone components of *X. chinensis*, suggested that (A) and (B) might be the 10-carbon analogs, 2-hydroxy-3-decanone (**I**) for (A) and 3-hydroxy-2-decanone (**II**) for (B) (Figure 4). Thus, the spectrum of (A) has a prominent ion at m/z 127 ($M^+ - \text{CH}_3 - \text{CH} - \text{OH}$) and an ion at m/z 109 ($127 - \text{H}_2\text{O}$), corresponding to those in the spectrum of 2-hydroxy-3-octanone at m/z 99 and 81, respectively. The spectrum of (B) has ions at m/z 129 ($M^+ - \text{CH}_3 - \text{C} = \text{O}$) and 111 ($129 - \text{H}_2\text{O}$) corresponding to those at m/z 101 and 83 in the spectrum of 3-hydroxy-2-octanone.

Further evidence for these assignments was obtained from the IR spectrum of a volatile collection assayed at approx. 1.5 mg and evaporated onto a sodium chloride plate. The IR spectrum showed a broad hydroxyl absorption at 3400 cm^{-1} and a sharp carbonyl absorption at 1710 cm^{-1} . NMR spectra were obtained from a volatile collection eluted with CDCl_3 and assayed by GC at 0.5 mg of (A), the ratio of (A) to (B) being 1:0.14. The ^1H and ^{13}C spectra were consistent with those for 2-hydroxy-3-decanone (**I**) by comparison with corresponding data for 2-hydroxy-3-octanone reported by Bel-Rhlid et al. (1989) and Mori and Otsuka (1985), respectively. In the ^1H NMR spectrum, a singlet at δ 2.20 corresponding to the $\text{CH}_3 - \text{C} = \text{O}$ in isomer (**II**) (cf. Bel-Rhlid et al., 1989) integrated approximately 7% relative to the $\text{CH}_3 - \text{CH} - \text{OH}$ doublet at δ 1.38 for the major component (**I**). This suggested that **II** was indeed produced by the insect, although some isomerization of **I** to **II** also occurred during GC analysis.

A collection of volatiles from male beetles was eluted from the Porapak with ether and reduced with LiAlH_4 . GC analysis showed components (A) and

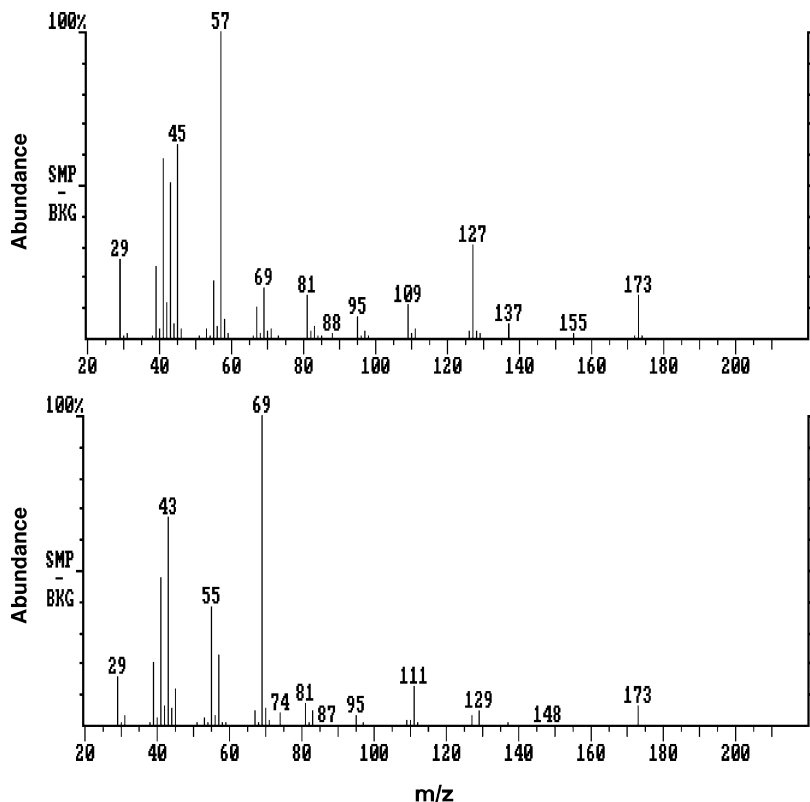


FIG. 3. EI mass spectra of component (A) (upper) and (B) (lower) in volatiles from male *X. quadripes*.

(B) had reacted to give two new components in 40:60 ratio at RI 2105 and 2144 on the polar GC column. The two components had identical mass spectra, which were analogous to that reported for 2,3-dihydroxyoctane by Kuwahara et al. (1987), with ions at m/z 129 and 111 compared with m/z 101 and 83 in the latter

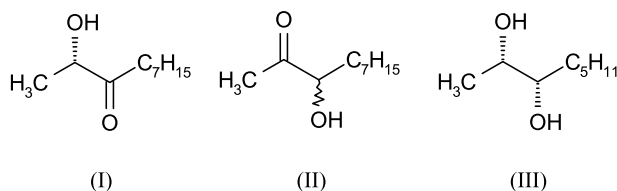


FIG. 4. Proposed structures of components in volatiles from male *X. quadripes*, (*S*)-2-hydroxy-3-decanone (I), 3-hydroxy-2-decanone (II), and (*S,S*)-2,3-dihydroxyoctane (III).

compound. These two products were proposed to be two diastereoisomers of 2,3-dihydroxydecane (Table 1), and identical results were obtained on similar reduction of synthetic (*S*)-2-hydroxy-3-decanone (**I**).

The EI mass spectrum of component (C) is shown in Figure 5. The CI (*iso*-butane) spectrum showed a strong $M + 1$ ion indicating a molecular weight of 128. GC-EAG analyses were only carried out on a polar GC column, but component (C) could be detected in GC-MS analyses on a nonpolar column by its characteristic CI mass spectrum, and retention data are shown in Table 1. The mass spectra suggested that the structure of (C) was related to those of (A) and (B), although probably with a lower molecular weight. The retention data in terms of the differences between the relative retention indices on polar and nonpolar columns (Δ) showed that (C) ($\Delta = 758$) was significantly more polar

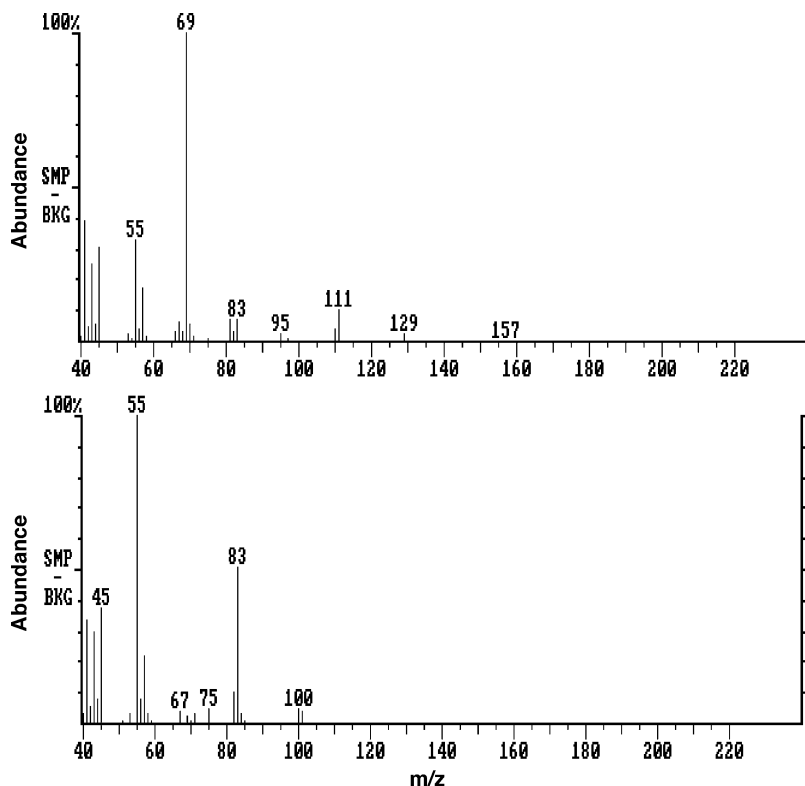


FIG. 5. EI mass spectrum of product of reduction of major component (A) in volatiles from male *X. quadripes* (upper) and spectrum of the minor component (C) in the volatiles (lower).

than either (A) ($\Delta = 570$) or (B) ($\Delta = 590$). Comparison of the EI mass spectrum with the literature mass spectrum of 2,3-dihydroxyoctane (Kuwahara et al., 1987) and the mass spectrum (Figure 5) and retention data (Table 1) for the product of reduction of major component (A) proposed as 2,3-dihydroxydecane, suggested that component (C) was 2,3-dihydroxyoctane (**III**) (Figure 4).

Synthesis and Configuration of Proposed Pheromone Components. The (*S*)- and (*R*)-enantiomers of 2-hydroxy-3-decanone were synthesized from (*S*)- and (*R*)-ethyl lactate, respectively (Figure 1). The synthetic 2-hydroxy-3-decanone had identical GC retention times, mass spectra, and ^1H and ^{13}C NMR spectra to the natural component (A) in volatiles collected from male *X. quadripes*. Comparison of retention times on a chiral GC column showed that the insect-produced compound cochromatographed with the *S* enantiomer (**I**) (Figure 6). Furthermore, in coupled GC-EAG analyses, the *S* enantiomer elicited a significant EAG response from the antenna of a female *X. quadripes* beetle, whereas the *R* enantiomer did not (data not shown).

The enantiomers of *threo* 2,3-dihydroxyoctane were each synthesized by Sharpless asymmetric dihydroxylation of (*E*)-2-octene. These *threo* enantiomers had identical mass spectra and retention times on polar and nonpolar GC columns to those of the minor component (C) in volatiles from male *X.*

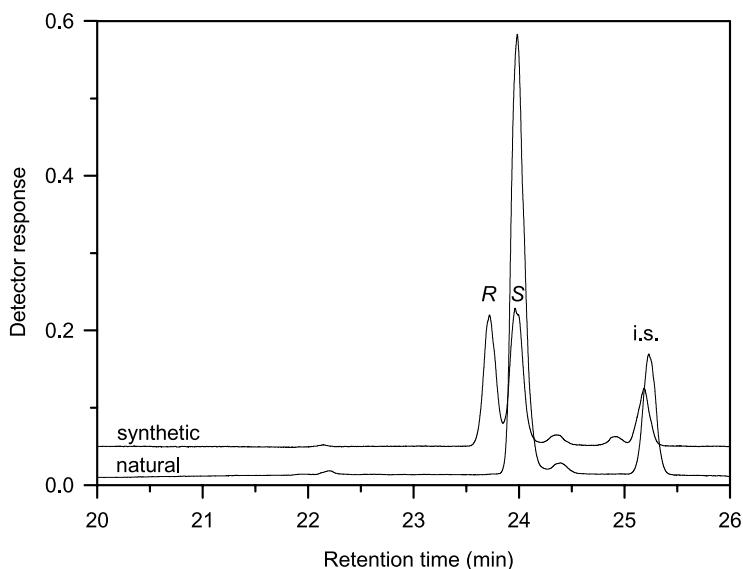


FIG. 6. GC analysis of natural and synthetic enantiomers of 2-hydroxy-3-decanone on a chiral cyclodextrin GC column (25 m \times 0.32 mm i.d. at 130°C; i.s. = internal standard, decyl acetate).

quadripes, and none (<0.5%) of the *erythro* diastereoisomer could be detected. Mass spectra and GC retention times of the corresponding acetates were also identical to those of a minor component in a collection of volatiles from male *X. quadripes* after acetylation with acetic anhydride and pyridine (Table 1).

Analysis of the volatiles collected from male *X. quadripes* on the cyclodextrin GC column showed a peak at the retention time of (*S,S*)-2,3-dihydroxyoctane (**III**) (28.05 min), and addition of the synthetic compound confirmed cochromatography (Figure 7). The diastereoisomeric diols were not detected ((*S,R*)-2,3-dihydroxyoctane eluted at 31.11 min). Thus, the minor EAG-active component (C) in volatiles collected from male *X. quadripes* was confirmed as (*S,S*)-2,3-dihydroxyoctane (**III**) (Figure 4). The amount present assayed at 7.8% of the major component (**I**) on the nonpolar GC column, 7.0% on a polar column, and 2.6% on the cyclodextrin column. It was observed that these 2,3-diols chromatograph poorly on polar GC columns, and, moreover, that the *threo* diastereoisomers seem to chromatograph worse than the *erythro*, such that the former can be completely lost on an older column.

During this work, the corresponding eight-carbon analogue of the major pheromone component (**I**), i.e., (*S*)-2-hydroxy-3-octanone was found to be present at 0.2–0.5% of **I** in the volatiles collected from male *X. quadripes* by

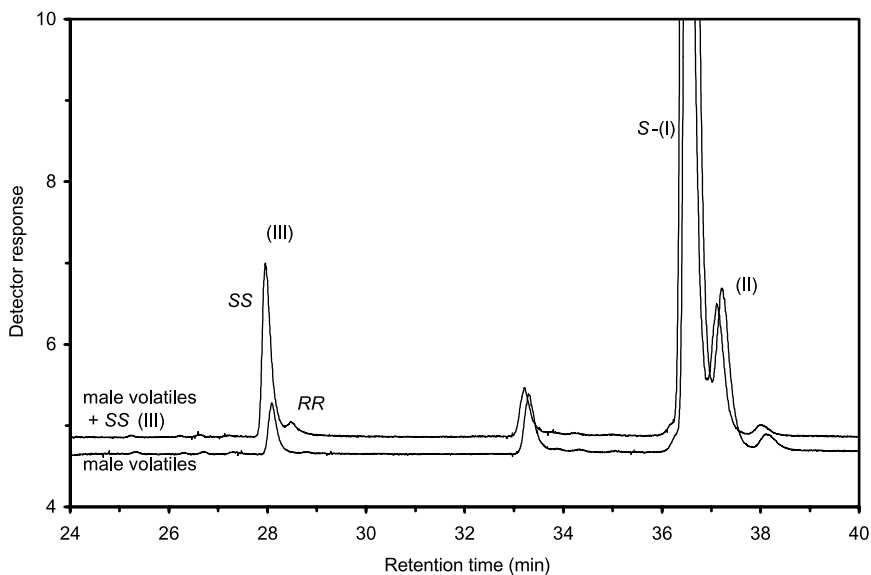


FIG. 7. GC analyses of volatiles from male *X. quadripes* on a cyclodextrin GC column, alone and coinjected with synthetic (*S,S*)-2,3-dihydroxyoctane (**III**) (50 m \times 0.22 mm i.d. column at 135°C).

comparison of GC retention times and mass spectra with those of the synthetic material. Also, following the report of Rhainds et al. (2001a), 2,3-decanedione was synthesized, and reexamination of GC-MS results showed that this compound was present at 2% of the major component (**I**). The only other significant components detected were 2-phenylethanol (3% of **I**), octanoic acid (4% of **I**), and two unidentified components at RI 1894 and 2384, respectively, on the polar GC column (each approximately 1% of **I**) having mass spectra similar to that of 2,3-decanedione. No significant EAG responses from female *X. quadripes* were observed to these components in GC-EAG analyses of male volatile collections.

Pheromone Dispensers. Release of synthetic (*S*)-2-hydroxy-3-decanone (**I**) from both polyethylene vials and sachets was zero order under constant laboratory conditions. The release rate from the sachets was 0.32 mg hr^{-1} at 27°C , essentially commencing immediately after the sachets were made up and continuing at a constant level until the contents were exhausted. Release from the vials took 3 d to start, while the material permeated through the wall of the vial, but then remained constant at 0.018 mg hr^{-1} for the period of measurement (60 d).

Bioassays. Numerous attempts to develop a suitable bioassay were made during work in India in April and October 1997. A wind tunnel, pitfall bioassay, and simple observational cages were investigated, but no meaningful quantitative results were obtained, although mating behavior was observed in the cages (Venkatesha et al., 1995). Subsequently, satisfactory responses were obtained using swastika and tube bioassays measuring the responses of crawling beetles. Results with the swastika bioassay are shown in Figure 8. Female beetles were attracted to volatiles from male beetles but not to those from female beetles (Figure 8a, $\chi^2 = 14.56$, $df = 3$, $P < 0.01$; Figure 8c, $\chi^2 = 13.67$, $df = 3$, $P < 0.01$), whereas male beetles were not attracted to volatiles from males or females (Figure 8b, $\chi^2 = 0.33$, $df = 3$, $P > 0.05$). For the results shown in Figure 8d, only two arms were utilized, one with a polyethylene vial dispenser containing (*S*)-2-hydroxy-3-decanone (**I**) and the opposite arm with a vial containing a 10:1 mixture of (*S*)-2-hydroxy-3-decanone (**I**) and (*S,RS*)-2,3-dihydroxyoctane (diastereoisomers of **III**). Female beetles were attracted to **I** alone, but addition of **III** completely inhibited this attraction (Figure 8d, $\chi^2 = 8.00$, $df = 1$, $P < 0.01$).

These results were confirmed with the tube bioassay in which female *X. quadripes* beetles were tested against a blank control, a single male beetle, a vial containing (*S*)-2-hydroxy-3-decanone (**I**) alone, and a vial containing a 10:1 mixture of **I** and (*S,RS*)-2,3-dihydroxyoctane (**III**) as described above. Mean distances of attraction of the female beetles along the tube are shown in Figure 9 ($F = 7.13$, $df = 3,27$, $P = 0.001$). The male beetle or (*S*)-2-hydroxy-3-decanone (**I**) alone was equally attractive ($P > 0.05$) and more attractive ($P < 0.05$) than

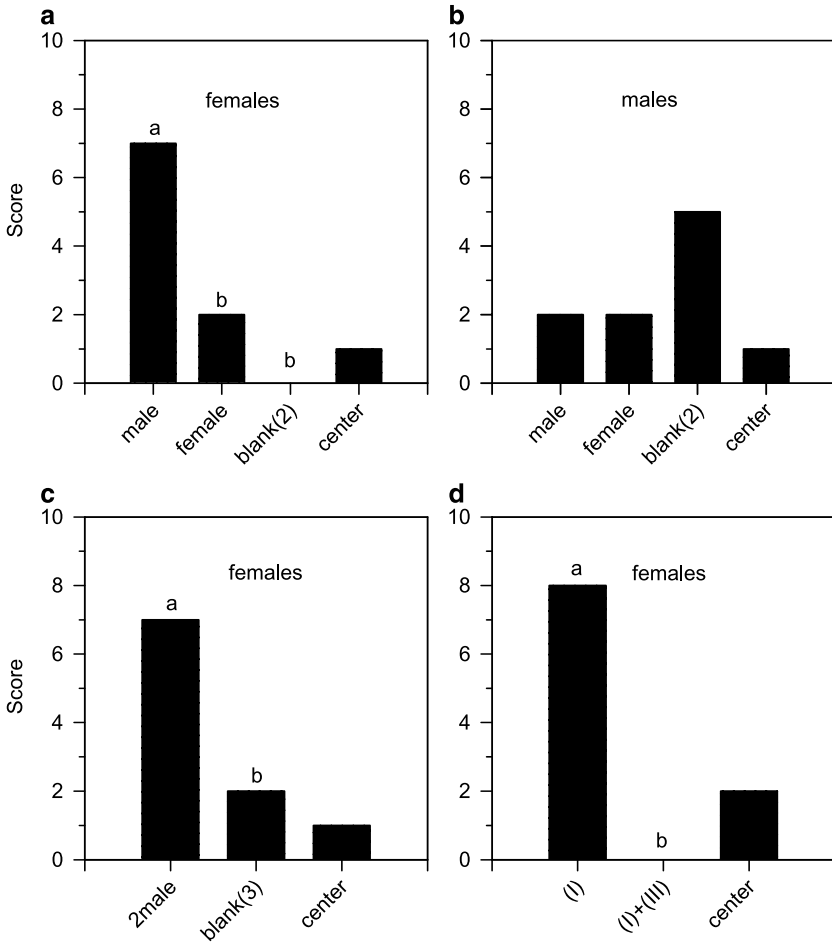


FIG. 8. Responses of female and male *X. quadripes* beetles in swastika bioassay to natural and synthetic sources of pheromone (each figure shows scores for 10 replicates; scores with different letters are significantly different, $P < 0.05$).

no lure or the 10:1 mixture of **I** and (*S,R,S*)-2,3-dihydroxyoctane (**III**). Mean levels of attraction of the latter two were not different ($P > 0.05$).

Field Trials. In a preliminary field test, the sachet dispensers were found to be inconvenient to use because the rapid release rate meant that the 100- μ l loading was exhausted within 7 d under field conditions. Polyethylene vial dispensers were used in all the experiments described here. In field tests during October–December 1997, delta and cross vane traps were compared directly.

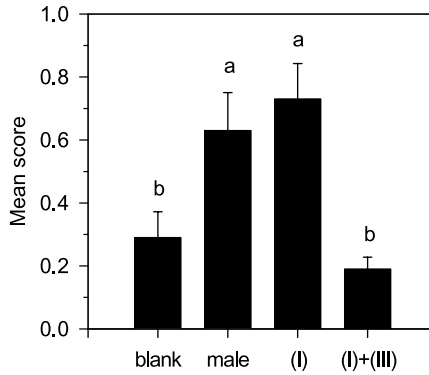


FIG. 9. Attraction of female *X. quadripes* beetles in a tube bioassay to natural and synthetic sources of pheromone (mean distance in meters \pm SE for 10 replicates; means with different letters are significantly different ($P < 0.05$) after transformation of means to arcsin, ANOVA, and DMRT).

The racemic mixture and the separate *S* and *R* enantiomers of 2-hydroxy-3-decanone (**I**) were dispensed from polyethylene vial dispensers. No beetles were caught in the delta traps, and results with cross-vane traps are shown in Table 2. There were significant differences in catches of female *X. quadripes* by the different treatments (Kruskal–Wallis H statistic = 9.77, $df = 3$, $P = 0.02$). Catches were higher in traps baited with (*S*)-2-hydroxy-3-decanone (**I**) or the racemic *RS* mixture than in traps baited with the *R* enantiomer or in unbaited traps ($P < 0.05$). (*R*)-2-Hydroxy-3-decanone showed some attractiveness ($P < 0.05$), although the catches observed may have been due to the small amount of the *S* enantiomer (approximately 1%) present in the material used. Similar

TABLE 2. TOTAL CATCHES^a OF *X. quadripes* IN CROSS-VANE TRAPS BAITED WITH ENANTIOMERS OF 2-HYDROXY-3-DECANONE (**I**) DURING OCTOBER 1997 (FOUR REPLICATES AT EACH OF THREE SITES; 30 D)

Lure	<i>X. quadripes</i>		Other cerambycids	Total
	Female	Male		
<i>R</i> -(I)	3 b	2	4	11
<i>S</i> -(I)	10 a	0	8	18
<i>R,S</i> -(I)	10 a	2	5	17
None	1 c	2	2	5

^aCatches within a column followed by a different letter are significantly different ($P < 0.05$) by Kruskal–Wallis H test.

TABLE 3. TOTAL CATCHES^a OF CERAMBYCID BEETLES IN CROSS-VANE TRAPS BAITED WITH (S)-2-HYDROXY-3-DECANONE (I) (APRIL–MAY 1998; THREE REPLICATES AT THREE SITES)

Treatment	<i>X. quadripes</i>		<i>Demonax balyi</i>	Other cerambycids
	Female	Male		
I	19 a	3	120 a	6
Unbaited	1 b	6	7 b	4

^aCatches within a column followed by a different letter are significantly different ($P < 0.05$) by Mann–Whitney U test.

numbers of male *X. quadripes* were caught in baited and unbaited traps. Small numbers of other, unidentified cerambycid beetles were also captured.

Field trials carried out during April–May 1998 compared catches in cross-vane traps baited with (S)-2-hydroxy-3-decanone (I) with those in unbaited traps. Combined results from sites in and around the Central Coffee Research Institute are shown in Table 3. Catches of female *X. quadripes* were higher in the baited than in the unbaited traps (Mann–Whitney $U = 9.00$, $P = 0.05$). Catches of male *X. quadripes* were low and numerically higher in the unbaited than the baited traps. During this time of the year, larger numbers of another cerambycid were caught at all sites in the baited traps (Mann–Whitney $U = 9.00$, $P = 0.05$), and these were identified as *Demonax balyi* Pascoe. Examination of the catch of 21 beetles from one trap showed 19 females and 2 males ($\chi^2 = 13.76$, $df = 1$, $P < 0.001$), the females being clearly distinguishable by their prominent ovipositor.

Trials held later in 1998 were set up to investigate the effect of adding 10% of the minor pheromone component, (S,S)-2,3-dihydroxyoctane (III) to the major component (I). Combined results from four sites in Karnataka and two in Tamil Nadu are shown in Table 4. More female beetles were caught in traps

TABLE 4. CATCHES^a OF *X. quadripes* BEETLES IN CROSS-VANE TRAPS BAITED WITH (S)-2-HYDROXY-3-DECANONE (I) OR A BLEND OF I WITH 10% (S,S)-2,3-DIHYDROXYOCTANE (III) AT SIX SITES IN INDIA (NOVEMBER 1998–JANUARY 1999)

Lure	No. trap days	Females		Males	
		Total	Mean/trap \pm SE	Total	Mean/trap \pm SE
I	1516	36 a	1.50 \pm 0.50	5	0.21 \pm 0.16
I + III	758	6 b	0.50 \pm 0.26	1	0.08 \pm 0.08
Unbaited	758	1 b	0.08 \pm 0.08	0	0.08 \pm 0.08

^aCatches within a column followed by a different letter are significantly different ($P < 0.05$) by Kruskal–Wallis H test.

baited with the major pheromone component (**I**) alone than in traps baited with the blend or unbaited traps (Kruskal–Wallis H -statistic = 8.51, $df = 2$, $P = 0.01$). Catches of male beetles in traps baited with **I** or the blend were similar to those in unbaited traps (Kruskal–Wallis H -statistic = 0.82, $df = 2$, $P = 0.66$).

DISCUSSION

Pheromone Identification. As previously reported (Hall et al., 1998), the major component in volatiles from male *X. quadripes* beetles from India eliciting an EAG response from antennae of female beetles was (*S*)-2-hydroxy-3-decanone (**I**), and this was confirmed by Rhains et al. (2001a) using insects from China. However, in collections from Indian insects this compound represented up to 90% of the volatile materials and was produced at up to 2 $\mu\text{g hr}^{-1}$ beetle⁻¹, whereas the GC traces shown in Rhains et al. (2001a) contained at least six other peaks of similar size to the major component.

GC analysis with splitless or split injection of natural or synthetic 2-hydroxy-3-decanone produced variable amounts of the isomeric 3-hydroxy-2-decanone (**II**), although this did not elicit a response from female beetles in GC-EAG analyses. Rhains et al. (2001a) reported that this isomerization did not occur with cool, on-column injection and that this isomer is not produced by the male beetle. However, the ¹H NMR spectrum of crude material from the Indian insects showed a signal corresponding to this isomer integrating at approximately 7% of the major isomer, suggesting that 3-hydroxy-2-decanone was naturally produced or isomerized during the collection process.

Neither 2-hydroxy-3-decanone (**I**) nor 3-hydroxy-2-decanone (**II**) has previously been reported from insects although analogues are produced by other cerambycid species. The eight-carbon analogue of **I**, (*S*)-2-hydroxy-3-octanone, is produced by males of the grape borer, *X. pyrrhoderus* Bates (Iwabuchi, 1982; Sakai et al., 1984), and the mulberry borer, *X. chinensis* Chevrolat (Kuwahara et al., 1987; Iwabuchi et al., 1987). A six-carbon analogue of **II**, (*R*)-3-hydroxy-2-hexanone, is produced by the old house borer, *Hylotrupes bajulus* L. (Fettköther et al., 1995), *Pyrrhidium sanguineum* L. (Schröder et al., 1994), and *Anaglyptus subfasciatus* Pic. (Leal et al., 1995). The latter species also produces the eight-carbon analog, (*R*)-3-hydroxy-2-octanone (Leal et al., 1995).

The Indian insects were shown to produce a third component that caused at least as large an EAG response from females as the major component in GC-EAG analyses, although it was only present at approximately 7% of the major component. This was identified as (*S,S*)-2,3-dihydroxyoctane (**III**), found previously as the major component in volatiles from male *X. pyrrhoderus* (Iwabuchi, 1982; Sakai et al., 1984). This was not reported by Rhains et al.

(2001a), although it would have eluted before the major component in their analyses. These authors did find a second component present in relatively small amounts but highly EAG-active, and this was identified as 2,3-decanedione. This compound was present at approximately 2% of the major component (**I**) in volatiles from Indian insects. Small amounts [0.2–0.5% of the major component (**I**)] of 2-hydroxy-3-octanone were also detected, but neither of these two compounds elicited an EAG response from female *X. quadripes* in GC-EAG analyses.

Synthesis. At least three syntheses of (*S*)-2-hydroxy-3-octanone have been reported, but these were not suitable for large-scale production of 2-hydroxy-3-decanone (**I**), because of the unavailability of starting materials (Mori and Otsuka, 1985) or low yields (Bel-Rhlid et al., 1989). Sakai et al. (1984) reported the synthesis of 2-hydroxy-3-octanone from lactic acid without any details, but it was thought that this could provide a good route to **I** from readily available starting materials. Numerous attempts to react heptyl lithium with free (*S*)-lactic acid under a variety of conditions in a range of solvents failed to give any significant quantity of the desired product. However, protection of the hydroxyl group and hydrolysis of the ester followed by reaction with heptyl lithium in diethyl ether and deprotection gave the desired product (**I**).

Initially, it was considered that the deprotection step should be as mild as possible to avoid isomerization of **I** to **II**, and the benzyl protecting group was selected ($R_1 = \text{benzyl}$ in Figure 1). The reaction scheme proceeded well, the benzyl group being put on with sodium hydride and benzyl bromide in THF at room temperature and removed smoothly by hydrogenation over palladium on charcoal in ethanol. However, the product (**I**) was essentially racemic, and examination of intermediates by chiral GC showed that the starting (*S*)-ethyl lactate was optically pure but that conversion to the benzyl ether had caused racemization. However, use of the tetrahydropyranyl or *tert*-butyldimethylsilyl protecting groups gave (**I**) without any significant racemization, the former being favored because the reagents were cheaper and yields higher. A further useful modification of the procedure involved hydrolysis of the protected ester with one equivalent of lithium hydroxide in aqueous ethanol and isolation of the lithium salt. In the case of the tetrahydropyranyl-protected derivative, the lithium salt was a white amorphous solid, and this reacted with one equivalent of heptyl lithium rather than the two equivalents that would be required for the free acid.

Several multistep syntheses of (*S,S*)-2,3-dihydroxyoctane (**III**) have been reported (Mori and Otsuka, 1985; Bel-Rhlid et al., 1989; Chattopadhyay et al., 1990; Kang et al., 1990; Bonini and Righi, 1992; Takahata et al., 1994; Bonini et al., 1995; Paolucci et al., 1995), but a single-step synthesis applicable to both enantiomers was used in this work. Reaction of (*E*)-2-octene with the Sharpless reagents “AD-mix- α ” or “AD-mix- β ” in *tert*-butyl alcohol in the presence of

methanesulfonamide (Sharpless et al., 1992) gave (*S,S*)- or (*R,R*)-2,3-dihydroxyoctane (**III**), respectively, in 91% yield. Both compounds were completely free ($\leq 0.2\%$) of *erythro* diastereoisomers by GC analysis. Conducting the reaction at room temperature for 15 hr gave product with 90% ee, although this could probably be improved further. An analogous synthesis of the enantiomers of 2,3-dihydroxyhexane was recently described by Lacey et al. (2004).

Behavioral Bioassays. Attraction of female *X. quadripes* to male beetles or synthetic lures was demonstrated with crawling insects in swastika or tube bioassays in the laboratory. Attempts to carry out bioassays with a large wind tunnel, a similar swastika bioassay, or in cages under field conditions in India were less successful, although mating was observed as described by Venkatesha et al. (1995) and Rhainds et al. (2001a). These observations were consistent with those of Rhainds et al. (2001a), who were able to demonstrate weak attraction of female *X. quadripes* to natural or synthetic pheromone in a room or field cage but only by introducing large numbers of insects and after patient and detailed observations. These authors reported that mating on the ground was common, providing a possible explanation why bioassays with crawling beetles were more successful than those with flying beetles.

We also demonstrated the attraction of female *X. quadripes* beetles to synthetic lures in the field. The (*S*)- or racemic (*RS*)-2-hydroxy-3-decanone was attractive, but addition of (*S,RS*)- or (*S,S*)-2,3-dihydroxyoctane (**III**) at 10% of **I** reduced attractiveness in laboratory bioassays and field trapping trials, respectively. However, numbers trapped were extremely low. Rhainds et al. (2001a) essentially failed to trap any beetles in field tests using multifunnel or delta traps, although beetles were trapped in the funnel traps during the laboratory tests, presumably with much higher population densities. Rhainds et al. (2001a) suggested that our field trapping results (Hall et al., 1998) were attributable to higher population densities or favorable positioning of traps. Unfortunately, no independent estimations of population are available that can be compared with those of Rhainds et al. (2001a), but these were not thought to be particularly high, and traps were positioned at random. The most likely explanation for the difference in results is that we used much larger, sticky cross-vane traps, consistent with the mating behavior described by Venkatesha et al. (1995) and Rhainds et al. (2001a), in which females are attracted to the vicinity of males after which short range chemical or visual signals probably take over. Similar conclusions were reached by Morewood et al. (2002), who reported that cross-vane traps with a large silhouette were more effective than multifunnel traps for trapping cerambycid beetles.

Although small numbers of male insects were also trapped in these trials, numbers were not significantly different than those in unbaited traps. Thus, the pheromone of *X. quadripes* must be considered as a sex pheromone, similar to those in other *Xylotrechus* species (Iwabuchi, 1982; Iwabuchi et al., 1987).

Lacey et al. (2004) recently reported production of an aggregation pheromone by males of *Neoclytus acuminatus acuminatus*, a Cerambycid belonging to the same subfamily and tribe as *X. quadripes*—Cerambycinae and Clytini, respectively. Lacey et al. (2004) did not test the natural pheromone, but the synthetic pheromone attracted significant numbers of both females and males, generally more of the former than the latter.

Trials are in progress in India to determine whether pheromone traps can cause any reduction in damage by *X. quadripes*, but, as suggested by Rhainds et al. (2001a), the chances of success at a cost-effective density would not seem to be high unless the effectiveness of the traps can be improved. Because adult *X. quadripes* do not feed (Visitpanich, 1994; Venkatesha et al., 1995 and observations of the authors), addition of feeding attractants as used with *A. subfasciatus* (Nakamuta et al., 1997) is probably not an option. Addition of oviposition attractants described by Rhainds et al. (2001b) would not be expected to have much effect in coffee plantations where these odors must be in abundance. However, the field studies reported here were limited in that only a single blend of the two EAG-active components was tested, and the effects of other potential pheromone components such as the 2-hydroxy-3-octanone and 2,3-decanedione were not evaluated. Thus, possibilities exist for optimizing the pheromone blend, the pheromone release rate, and the trap design in relation to mating behavior.

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(Z)-7-TRICOSENE AND MONOUNSATURATED KETONES
AS SEX PHEROMONE COMPONENTS OF THE
AUSTRALIAN GUAVA MOTH
Coscinoptycha improbana: IDENTIFICATION,
FIELD TRAPPING, AND PHENOLOGY

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Abstract—Pheromone gland extracts of the Australian guava moth *Coscinoptycha improbana* (Lepidoptera: Carposinidae), contained four compounds that elicited responses from male moth antennae in gas chromatography-electroantennogram detection (GC-EAD) analyses. These were identified by GC-mass spectrometry as (Z)-7-tricosene (Z7-23Hy), (Z)-7-octadecen-11-one (Z7-11-one-18Hy), (Z)-7-nonadecen-11-one (Z7-11-one-19Hy), and (Z)-7-tricosen-11-one (Z7-11-one-23Hy) at a ratio of 65:23.5:1.5:10, respectively. Z7-23Hy, Z7-11-one-18Hy, and Z7-11-one-23Hy have not previously been reported as lepidopteran sex pheromone components. Z7-11-one-18Hy was active as a single component, and was synergized by Z7-11-one-23Hy but not Z7-11-one-19Hy, although the latter compound was weakly attractive as a single component. Addition of Z7-23Hy further increased attraction. The amount of the major pheromone component, Z7-11-one-18Hy in female pheromone gland extracts was estimated to be 16.4 ng/female ($N = 8$).

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Phenological data gathered over a 12-mo period in 2002 and 2003 using the binary blend indicated that moths are active throughout the year. The pheromone has already been employed to monitor the spread of *C. improbana* in New Zealand and detect its presence in Queensland, Australia.

Key Words—Australian guava moth, *Coscinoptycha improbana*, carposiniidae, lepidoptera, (Z)-7-tricosene, (Z)-7-octadecen-11-one, (Z)-7-nonadecen-11-one, (Z)-7-tricosen-11-one, trapping trials, phenology.

INTRODUCTION

The Australian guava moth, *Coscinoptycha improbana* (Meyrick 1881), has recently invaded Northland, New Zealand, where it has rapidly developed significant pest status in several subtropical crops. The larvae are known to infest a range of fruits including mandarins and lemons (Rutaceae), plums, peach, nashi pears, and loquats (Rosaceae), guavas and feijoas (Myrtaceae), and macadamias (Proteaceae) (Jamieson et al., 2004). In its native Australia, the insect ranges from Eungella, Queensland, to Victoria and Tasmania, and also occurs on Norfolk Island (Common, 1990), but is not considered a significant pest. In New Zealand, *C. improbana* has been detected in commercial feijoa, macadamia, and plum orchards and is now a serious pest of macadamias and feijoas in Northland (Jamieson et al., 2004). Therefore, it was important to develop a system to monitor its phenology and likely rate of spread in New Zealand. The sex pheromone of *C. improbana* could be used for monitoring and possibly for pest management. The objective of this study was to characterize the pheromone of *C. improbana*.

Sex pheromones have been identified for only two carposinid moth species (El-Sayed, 2006). The pheromone of the peach fruit moth, *Carposina sasakii* (= *C. niponensis*), is either a mixture of (Z)-7-eicosen-11-one (Z7-11-one-20Hy) and (Z)-7-nonadecen-11-one (Z7-11-one-19Hy) (Tamaki et al., 1977; Honma et al., 1978) or the single component (Z)-7-eicosen-11-one (Z7-11-one-20Hy) (Shirasaki et al., 1979; Han et al., 2000). The pheromone of the New Zealand raspberry budmoth, *Heterocrossa rubophaga*, apparently consists of a single component, Z7-11-one-19Hy (Foster and Thomas, 2000). We report here the chemical identification of sex pheromone components for *C. improbana*, including the alkene (Z)-7-tricosene (Z7-23Hy) and three homologous mono-unsaturated ketones (Z)-7-octadecen-11-one (Z7-11-one-18Hy), Z7-11-one-19Hy, and (Z)-7-tricosen-11-one (Z7-11-one-23Hy).

METHODS AND MATERIALS

Insects. Ripening feijoa and macadamia infested with *C. improbana* larvae were collected from trees in Kaitaia and Mangonui in the Far North in April

2001 and from Kerikeri in 2002 and 2003. These were placed in 2-l containers on a layer of sawdust and kept at room temperature. Larvae emerged from the fruit and pupated in the sawdust. Pupae were shipped individually to quarantine facilities at Lincoln in sealed plastic containers (Lily[®] 35 ml portion cups with lids; Huhtamaki Henderson Limited, Auckland, N.Z.). Pupae were kept in the laboratory at room temperature in 6-cm plastic Petri dishes until emergence when they were sexed. Females had their pheromone glands excised and extracted, whereas males were used for coupled gas chromatography-electroantennogram analysis (GC-EAD). Male *C. improbana* have a raised tuft of silky white scales toward the base of the forewing (Hoare, 2001), which aided sex determination.

Pheromone Gland Extracts. All *C. improbana* gland extracts used for GC-EAD and GC-MS analysis were taken from 24- to 48-hr-old females at 1–2 hr into the scotophase. For quantitative analysis, the pheromone glands of eight females were extracted with a 50- μ l aliquot of *n*-hexane containing 50 ng of octadecane as an internal standard. This extract was held at 4°C for 12 hr before analysis.

Chemicals. Z7-11-One-18Hy, Z7-11-one-19Hy (both >97% isomerically pure), and Z7-11-one-23Hy (83% isomerically pure by gas chromatography) were purchased from Pherobank (Wageningen, The Netherlands). Z7-11-One-20Hy (97% isomerically pure) was provided by Shin-Etsu Chemical Co. Ltd. (Tokyo, Japan). Z7-23Hy and E7-23Hy (both >98% isomerically pure) were synthesized at HortResearch, Palmerston North (see below). (Z)-9-Tricosene (Z9-23Hy) (97%), octadecane (99%), and a straight chain hydrocarbon standard mixture for GC were purchased from Sigma-Aldrich.

Synthesis of (Z)-7-Tricosene (Z7-23Hy). *n*-Butyl lithium (4.7 ml, 7.5 mmol, 1.6 M in hexane) was added, under N₂, to a solution of 1-octyne (0.75 g, 6.8 mmol) in dry THF (50 ml) at –70°C. The solution was allowed to warm to 0°C, and bromopentadecane (2.0 g, 6.8 mmol) was added, followed by Bu₄NI (0.25 g, 0.68 mmol). The reaction was refluxed gently for 3 hr. Saturated aqueous NH₄Cl (30 ml) was added, and the organic phase was separated. The aqueous layer was extracted with diethyl ether (2 × 30 ml). After drying over MgSO₄, the solvent was removed under reduced pressure. The crude product was purified by using silica gel chromatography (petroleum ether) yielding 873 mg, (38%) of 7-tricosyne. This was reduced in MeOH (25 ml) using 10% palladium on charcoal (30 mg) and a balloon of H₂, for 24 hr at room temperature. After filtration, the product was purified by using silica gel chromatography (petroleum ether) yielding (Z)-7-tricosene as an oil (296 mg, 35%). ¹H NMR (CDCl₃, 400 MHz): δ 5.35 ppm (2H, m), 2.02 (4H, m), 1.26 (34H, m), 0.89 (6H, m) ppm. ¹³C NMR (CDCl₃, 125 MHz): δ 129.89, 129.88, 31.95, 31.81, 29.79, 29.76, 29.73 (6 carbons), 29.69 (2 carbons), 29.59, 29.39, 29.34, 29.02, 27.22, 22.72, 22.69, 14.13, 14.12 ppm.

Synthesis of (E)-7-Tricosene (E7-23Hy). Synthesis of (*E*)-7-tricosene was carried out using the Wittig reaction (Wittig and Schoellkopf, 1973). Hexadecylphosphonium bromide was prepared by refluxing bromohexadecane (10 g, 32.8 mmol) and triphenyl phosphine (7.87 g, 30 mmol) in benzene (45 ml) for 48 hr. The solvent was removed *in vacuo* to give a pale yellow oil that was refrigerated for 24 hr. The resulting solid was triturated with diethyl ether (2 × 50 ml) to give a white solid (8.8 g, 15.5 mmol, 52%). *n*-Butyl lithium (4.3 ml, 6.88 mmol, 1.6 M in hexane) was added to a suspension of hexadecylphosphonium bromide (3.6 g, 6.34 mmol) in diethyl ether (30 ml), and the resultant solution was stirred for 2 hr before adding heptanal (1.39 ml, 9.96 mmol) and refluxing 1 hr. The mixture was cooled to room temperature, filtered through celite, and rinsed with ether (2 × 50 ml). The filtrate was washed with water until the pH tested neutral, then dried over magnesium sulfate, and concentrated. The crude product was purified by column chromatography on silica gel (petroleum ether) to give a mixture of both isomers as a colorless oil (1.33 g, 4.43 mmol, 70%, 4:1 *Z/E*). The isomers were separated on silica gel impregnated with 30% silver nitrate eluting with 0.5% diethylether/petroleum ether, yielding each of the isomers in >98% isomeric purity. (*E*)-7-Tricosene ¹H NMR (CDCl₃, 400 MHz): δ 5.39 (2H, m), 1.97 (4H, m), 1.26 (34H, m), 0.89 (6H, m). ¹³C NMR (CDCl₃, 125 MHz): δ 130.30, 130.28, 32.32, 32.18, 30.16, 30.14, 30.10 (8 carbons), 30.06, 29.96, 29.77, 29.71, 29.39, 23.09, 23.06, 14.53, 14.52 ppm.

Gas Chromatography-Electroantennographic Detection. Pheromone gland extracts of *C. improbana* females were analyzed by GC-EAD using a Varian 3800 gas chromatograph with a splitless injector, coupled to an EAD Recording Unit (Syntech, Hilversum, The Netherlands). The injector and detector temperatures were 220 and 300°C, respectively. Samples were run on a ZB-5 (Phenomenex, Torrance, CA, USA) capillary column (30 m × 0.25 mm ID × 0.5 μm film) and a polar BPX70 (SGE, Victoria, AU) column (30 m × 0.25 mm ID × 0.25 μm film) provided with 1:1 split outlets with helium carrier gas (1 ml/min). The GC oven was programmed from 80°C/1 min to 240°C at 10°C/min and held for 15 min. An excised male *C. improbana* antenna was positioned between two glass electrodes, containing BE Ringer's solution with 10% polyvinylpyrrolidone (molecular weight = 360,000) (Sigma). Each glass electrode held a length of 1 mm silver wire to connect the preparation to the recording unit. The EAD exit port temperature was maintained at 200°C and the antennal preparation was placed in a charcoal filtered and humidified airstream (500 ml/min).

Gas Chromatography-Mass Spectrometry. Pheromone gland extracts were analyzed by GC-MS using three instruments. Initial analyses of gland extracts and comparison of these with synthetic compounds were carried out on a Hewlett-Packard (HP) 5890 GC, coupled to a VG-70SE magnetic sector mass spectrometer (VG Instruments, Manchester, UK). Later analyses were performed on an HP 5890 Series II Plus GC interfaced to an HP 5792

quadrupole mass spectrometer. Either an HP-Innowax column (30 m × 0.25 mm i.d. × 0.25 µm film) (Agilent, Palo Alto, CA, USA) or a BPX-70 column (30 m × 0.25 mm i.d. × 0.25 µm film) (SGE, Victoria, AU), was used in the first instrument (both columns, 80°C/2 min to 200°C at 20°C/min, then from 200 to 220°C at 10°C/min). The second instrument was fitted with a 30 m × 0.25 mm i.d. × 0.25 µm film ZB-Wax column (Phenomenex), and was used to compare retention times of *Z* and *E* isomers of the synthetic ketones with those of the natural pheromone components. Temperature programs of 80°C/2 min to 220°C at 5°C/min (for *Z7-11-one-18Hy* and *Z7-11-one-19Hy*) and 80°C/2 min to 230°C at 5°C/min (for *Z7-11-one-23Hy*) were used for this GC-MS. Both instruments were operated in EI mode (70 eV). Injections were splitless, using He carrier gas with a constant linear velocity of 30 cm/sec.

The third instrument (a Varian 3800 GC in splitless injection mode interfaced to a Varian 2200 MS with a VF-5ms column (30 m × 0.25 mm i.d. × 0.25 µm film) (Varian) was used for analyzing the adducts formed during dimethyldisulfide (DMDS) derivatizations of *Z7-23Hy* and *E7-23Hy* (both 10 ng in 20 µl *n*-hexane), and comparing their retention times and spectra with the adduct formed by DMDS derivatization of a 17 female pheromone gland extract of *C. improbana*. In addition, a 10 female pheromone gland extract of *C. improbana*, was compared with standards of *Z9-23Hy*, *Z7-23Hy*, and *E7-23Hy*. Injector 220°C, program 80°C/1 min to 200°C at 5°C/min and held for 3 min, then increased to 240°C at 2°C/min and held for 5 min. For the DMDS derivative analyses, the injector temperature was set at 220°C, and the GC oven was programmed from 100°C/1 min to 260°C at 10°C/min and held for 33 min.

Dimethyldisulfide Derivatizations. For DMDS derivatizations, we followed the procedure described by Buser et al. (1983) and Leonhardt and DeVilbiss (1985). Approximately 50 µl DMDS and 5 µl iodine solution (60 mg of I₂ in 1 ml of diethyl ether) were added to the solution of interest in a 1.8-ml glass vial, sealed with a Teflon-lined cap, and held at 40°C for 15 hr. The reaction was quenched with 50 µl 5% aqueous sodium thiosulfate, and the organic layer was dried with anhydrous sodium sulfate, and transferred to a clean 1.5-ml tapered-bottom vial, and blown down with a stream of argon to approximately 10 µl. A 1-µl aliquot [ca. 1.7 female equivalents (FE)] was immediately analyzed by GC-MS.

Isomerization of Synthetic Z7-11-one-18Hy. This was achieved by modifying the methods described by Ferreri et al. (2001) and Sgoutas and Kummerow (1969). Four µl of a 0.04 M solution of *Z7-11-one-18Hy* in *n*-heptane were added to 8 µl of 2-mercaptoethanol and 1 µl of azobisisobutyronitrile (AIBN) in a 2-ml vial flushed with N₂. This was capped and heated to 74°C for 48 hr, then diluted in 1 ml of *n*-heptane for GC-MS analysis.

Trapping Trials. Five trapping trials were carried out at Kerikeri (lat. 35.23°S, long. 173.95°E), Northland, NZ, during 2003 and 2004. Trial 1 was undertaken in

an organic macadamia orchard, whereas trials 2–5 were carried out in both a macadamia orchard and a feijoa orchard. In all trials, delta traps were attached to individual trees at a height of 1.5 m, with 10 m spacing between treatments and ca. 15 m spacing between replicates. Test compounds were loaded onto red rubber septa (Thomas Scientific Inc., Philadelphia, PA, USA), lures were replaced every 6 wk, and sticky trap inserts were replaced every week. Trap positions within each replicate were randomized weekly when trap catches were recorded. Pheromone blend ratios tested were based upon ratios found in pheromone gland extracts.

An initial trapping trial (April 10–June 20, 2003) tested Z7-11-one-18Hy and Z7-11-one-19Hy alone or in various combinations, including the 95:5 ratio present in pheromone gland extracts. Treatments were as follows: (1) a blank control, (2) 1000:0, (3) 990:10, (4) 950:50, (5) 900:100, (6) 500:500, and (7) 0:1000 μg loadings of Z7-11-one-18Hy and Z7-11-one-19Hy ($N = 5$).

A second trial (October 6–November 4, 2003, $N = 6$) tested the effects of addition of varying amounts of Z7-11-one-23Hy, to a 300- μg lure of Z7-11-one-18Hy and Z7-11-one-19Hy (95:5). Treatments were as follows: (1) a blank control, (2) 285:15:0, (3) 285:15:13.3, (4) 285:15:40, and (5) 285:15:120 μg loadings of Z7-11-one-18Hy, Z7-11-one-19Hy, and Z7-11-one-23Hy.

A third trial (November 14, 2003–January 9, 2004, $N = 6$) compared the best two-component lure from trial 1 (treatment 4) with various combinations of the three monounsaturated ketones. Treatments were as follows: (1) a blank trap as the control, (2) 950:50:0, (3) 670:40:290, (4) 700:0:300, (5) 0:120:880, and (6) 0:0:1000 μg loadings of Z7-11-one-18Hy, Z7-11-one-19Hy, and Z7-11-one-23Hy.

A fourth trial (February 11–May 7, 2004, $N = 6$) tested different doses of the best ternary blend (treatment 3 from trial 3). Treatments were as follows: (1) a blank control, (2) 20.1:1.2:8.7, (3) 67:4:29, (4) 201:12:87, (5) 670:40:290, and (6) 2010:120:870 μg doses of Z7-11-one-18Hy, Z7-11-one-19Hy, and Z7-11-one-23Hy.

A fifth trial (October 1–November 19, 2004, $N = 6$) tested the effect of addition of Z7-23Hy to the ternary blend. Treatments were as follows: (1) a blank control, (2) 670:40:290:0, (3) 950:50:0:0, (4) 250:0:0:750, and (5) 235:15:100:650 μg doses of Z7-11-one-18Hy, Z7-11-one-19Hy, Z7-11-one-23Hy, and Z7-23Hy.

Phenology. Three delta traps baited with septa loaded with 300 μg of a 95:5 ratio of Z7-11-one-18Hy and Z7-11-one-19Hy, were deployed in two macadamia orchards (HortResearch's orchard and Welch's organic orchard), from January 2002 to January 2003, to monitor the weekly catch of *C. improbana* male moths. Pheromone lures were replaced every 6 wk.

Statistics. All trapping data were analyzed by ANOVA (SAS Institute Inc., 1998). Residual plots were used to check the validity of the ANOVA assumptions. Square root transformations were undertaken when necessary. Means were compared by Fisher's Protected Least Significant Differences test (SAS Institute Inc., 1998).

RESULTS

Identification of Pheromone Components. GC-EAD analysis of *C. improbana* pheromone extracts (Figure 1) identified four compounds that repeatedly elicited responses from antennae of male moths. The greatest responses were elicited by compound **2**. Antennae of males also responded well to Z7-11-one-19Hy (the pheromone for *H. rubophaga*). This ketone had the same retention time as that of active compound **3** in the gland extracts on both ZB-5 and BPX-70 columns. Whereas male antennae gave a small response to Z7-11-one-20Hy (the major pheromone component for *C. sasakii*), no antennal response or peak was observed from gland extracts at the expected retention time for this compound. The ratio of compounds **1–4** in gland extracts was 65:23.5:1.5:10, respectively, with the amount of the major active compound **2** calculated to be 16.4 ng/female ($N = 8$).

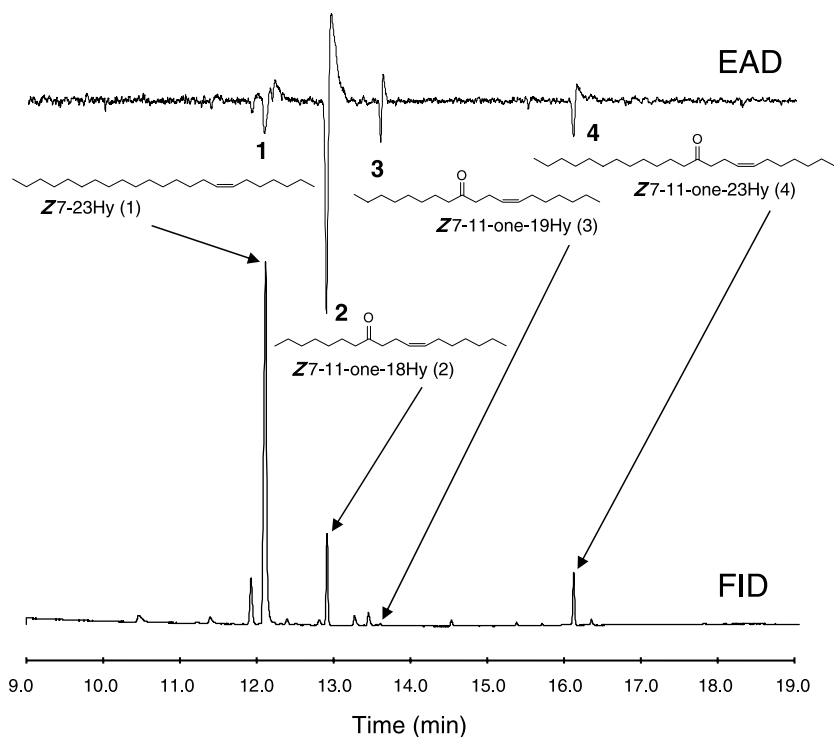


FIG. 1. Male *C. improbana* antennal responses (EAD) to compounds in female moth pheromone gland extracts, eluting from a BPX-70 GC column (FID).

GC-MS analysis of a 2 FE aliquot of *C. improbana* pheromone gland extract showed several peaks for straight chain alkanes and alkenes including heneicosane, tricosane, and a tricosene (largest peak in total ion chromatogram; m/z (relative intensity) 322 (15, M^+), 294 (2), 125 (21), 111 (42), 97 (82), 83 (83), 69 (78), 55 (100), 43 (95), HP 5792 spectrometer, 70 eV), the latter corresponding to EAD-active compound **1**. Whereas the spectrum of compound **1** in the 10 FE extract was similar to that of Z9-23Hy, its retention time was longer on the VF-5ms column [Kováts retention indices (KI) of 2277 and 2270, respectively]. Analysis of a DMDS derivatized aliquot of the 17 FE extract on the VF-5ms column showed a large peak with a parent ion at m/z 416 and diagnostic ions at m/z 145 and 271 (base peak), indicating the addition of DMDS to a tricosene with a double bond at position 7. In addition, the retention time and spectrum of the tricosene in the 10 FE extract was identical with that of synthetic Z7-23Hy rather than E7-23Hy (KI = 2279 and 2284, respectively). Comparison of retention times of the adducts formed from DMDS-derivatized synthetic tricosenes, with those of the derivatized tricosene in the gland extract, corroborated that **1** was Z7-23Hy rather than E7-23Hy (KI = 2931 and 2935, respectively).

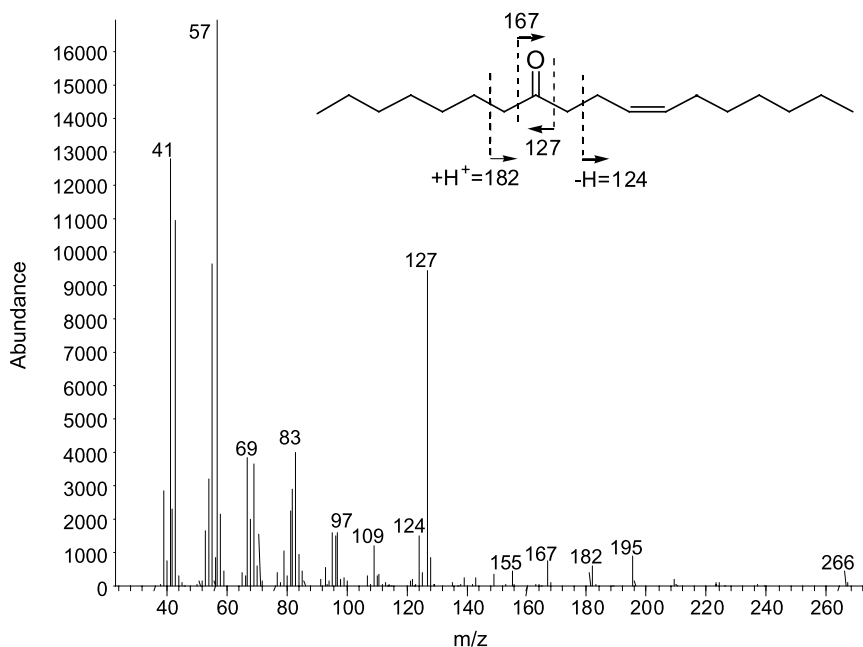


FIG. 2. Electron impact ionization mass spectrum (HP 5792 spectrometer at 70 eV) of Z7-11-one-18Hy, compound **2** in Figure 1.

There was also a major peak in the 2 FE gland extract with a mass spectrum containing a parent ion at m/z 266, and ions at m/z 195, 182, 167, 127, and 124 (Figure 2), which corresponded to EAD-active compound **2**. A relatively small peak corresponding to EAD-active compound **3** had a parent ion at m/z 280, and ions at m/z 141 (base peak) and 167, similar to the mass spectrum for *Z*7-11-one-19Hy reported by Foster and Thomas (2000) and Tamaki et al. (1977). Comparison with the synthetic standard of *Z*7-11-one-19Hy showed that they had identical mass spectra and retention times on HP-Innowax and BPX-70 columns.

A third major peak in the TIC of the 2 FE gland extract, with a retention time equivalent to EAD-active compound **4**, had mass spectral fragments at: m/z 336 (M^+), 197, 195, 182, 167, and 124 (Figure 3). Similarities in the spectra of **2**, **3**, and **4** suggested that they were homologous ketones, with differing chain lengths on the saturated side of the carbonyl group, i.e., Δ 7-11-one-18Hy for **2** and Δ 7-11-one-23Hy for **4**. This was confirmed by comparing the mass spectra of **2** and **4** with those of synthetic *Z*7-11-one-18Hy and *Z*7-11-one-23Hy, respectively, and with published data (Tamaki et al., 1977). The retention times of insect produced **2** and **4** and synthetic *Z*7-11-one-18Hy and *Z*7-11-one-23Hy were identical on the HP-Innowax (for **2**) or ZB-Wax (for **4**), and BPX-70 columns.

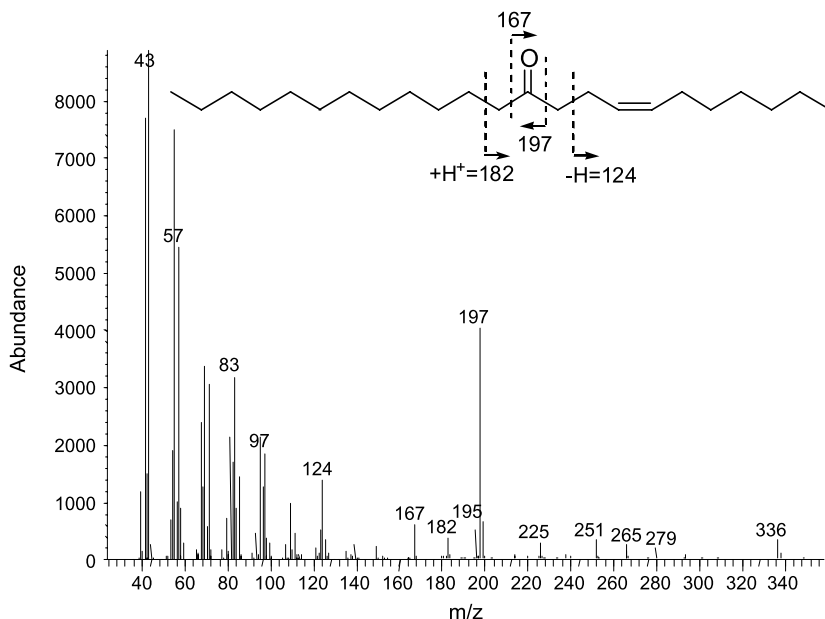


FIG. 3. Electron impact ionization mass spectrum (HP 5792 spectrometer at 70 eV) of *Z*7-11-one-23Hy, compound **4** in Figure 1.

To determine whether pheromone components **2–4** were *Z* or *E* isomers (or a mixture), the synthetic samples and gland extract were analyzed by GC-MS using a ZB-Wax column, with a slower temperature program. The *Z*7-11-one-19Hy standard contained approximately 3% of the *E* isomer (KI = 2367 and 2373, respectively), apparent as two peaks in the TIC and the selected ion chromatogram for *m/z* 141. Synthetic *Z*7-11-one-23Hy similarly contained 17% *E*7-11-one-23Hy (KI = 2765 and 2771, respectively). However, the synthetic *Z*7-11-one-18Hy was >99% pure, and any *E* isomer was poorly resolved from *Z*. Therefore, the sample was isomerized using 2-mercaptoethanol and AIBN to give a 2:1 mixture of *Z* and *E*7-11-one-18Hy (KI = 2261 and 2267, respectively). All three of the insect-produced ketones **2–4** had identical retention times to their respective synthetic *Z* isomers on the ZB-Wax column, with no peaks for *E* isomers apparent in the gland extract.

Field Trials. In all trapping trials, residual plots indicated that the analysis of variance assumptions were adequate. Results from trial 1 (Figure 4) established that *Z*7-11-one-18Hy alone was sufficient for attraction of male *C. improbana*, with a total of 84 moths trapped in treatment 2 during the course of the trial. The addition of 1% or 5% of *Z*7-11-one-19Hy did not enhance the trap

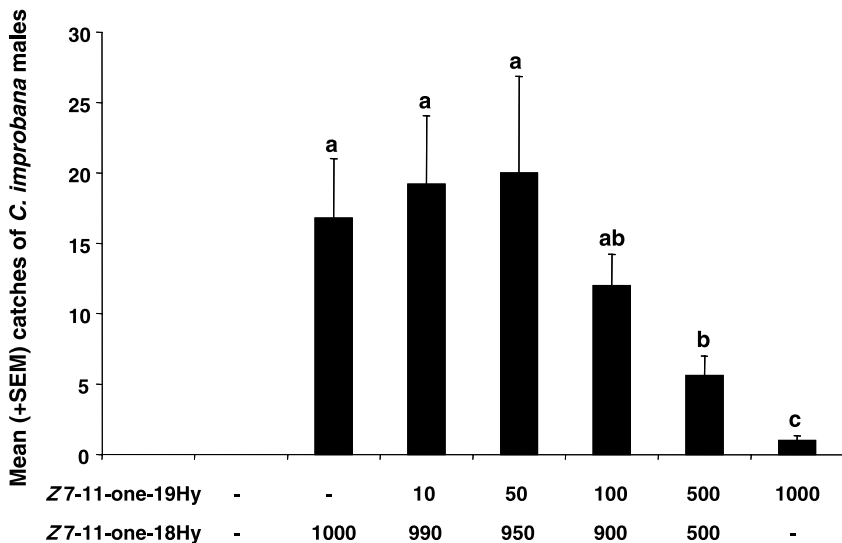


FIG. 4. Mean trap catches ($N = 5$) of *C. improbana* male moths with various ratios of *Z*7-11-one-18Hy and *Z*7-11-one-19Hy (1000 μg doses). Trial 1, macadamia orchard, Kerikeri, Northland, New Zealand, April 10–June 20, 2003. Treatments labeled with the same letter are not significantly different ($P > 0.05$).

catch. A 1:1 blend of *Z7-11-one-18Hy* and *Z7-11-one-19Hy* caught fewer moths, and *Z7-11-one-19Hy* alone trapped only a few moths ($N=5$).

In trial 2 (Figure 5), the addition of *Z7-11-one-23Hy* to a 300- μg lure of *Z7-11-one-18Hy* and *Z7-11-one-19Hy*, at a 95:5 ratio (corresponding to the ratio from gland extracts), increased trap catch in both the macadamia and feijoa orchards.

In trial 3 (Figure 6), no moths were trapped in traps baited with only *Z7-11-one-23Hy* (treatment 6), whereas one moth was attracted to the binary blend of *Z7-11-one-23Hy* and *Z7-11-one-19Hy* (treatment 5) in the macadamia orchard. The majority of moths at the macadamia orchard (316 out of 317 moths) and all the moths at the feijoa orchard (128 moths) were caught in traps baited with lures containing *Z7-11-one-18Hy* as one of the components, reinforcing the importance of this compound. The other blends in this trial (*Z7-11-one-18Hy*, *Z7-11-one-19Hy*, *Z7-11-one-23Hy* in ratios of 950:50:0, 670:40:290, and 700:0:300) were equivalent to each other (Figure 6).

In trial 4 (Figure 7), 300-, 1000-, and 3000- μg doses of the ternary ketone blend attracted equivalent numbers of moths in both orchards, whereas doses of 100 and 10 μg trapped fewer males.

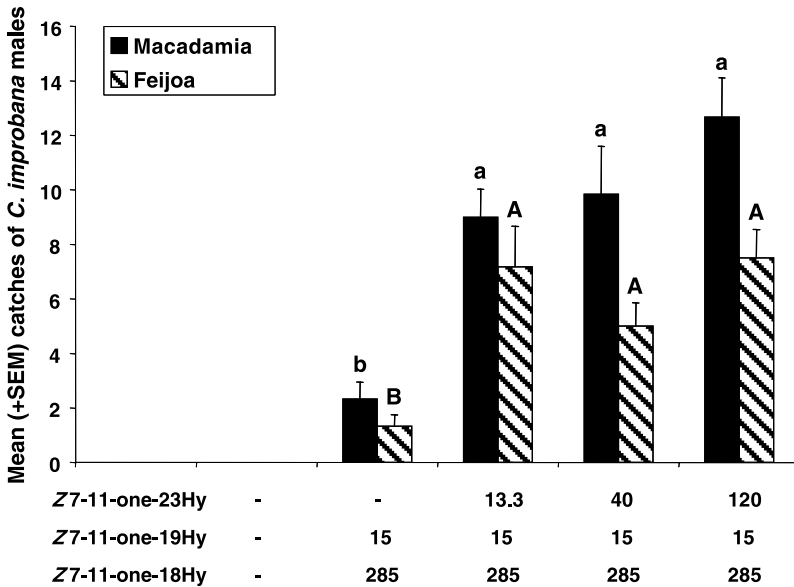


FIG. 5. Mean trap catches ($N=6$) of *C. improbana* male moths with 300 μg doses of *Z7-11-one-18Hy* and *Z7-11-one-19Hy*, with and without *Z7-11-one-23Hy*. Trial 2, macadamia and feijoa orchards, Kerikeri, Northland, New Zealand, October 6–November 4, 2003. Treatments labeled with the same letter are not significantly different ($P > 0.05$).

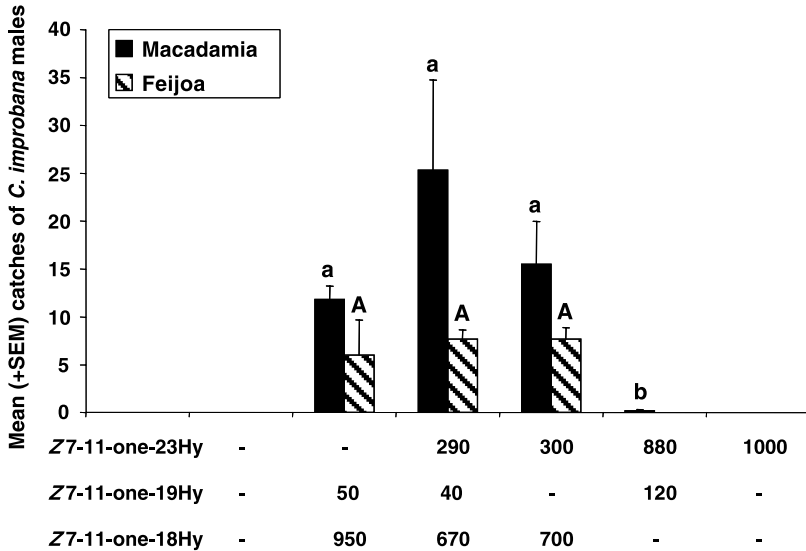


FIG. 6. Mean trap catches ($N = 6$) of *C. improbana* male moths with various combinations of Z7-11-one-18Hy, Z7-11-one-19Hy, and Z7-11-one-23Hy (1000 μg doses). Trial 3, Macadamia and Feijoa Orchards, Kerikeri, Northland, New Zealand, November 14, 2003–January 9, 2004. Treatments labeled with the same letter are not significantly different ($P > 0.05$).

In trial 5 (Figure 8), addition of Z7-23Hy to Z7-11-one-18Hy (treatment 4) appeared to increase trap catch in one orchard but not the other. However, addition of Z7-23Hy to the ternary blend of ketones (treatment 5) increased the numbers of moths trapped in comparison to a higher dose of the ternary ketones blend (treatment 2).

Phenology. Phenological data gathered from January 2002 to January 2003, using the two-component lure of Z7-11-one-18Hy and Z7-11-one-19Hy at a ratio of 95:5, indicated that the adult moth was active throughout the year in an organic macadamia orchard (Figure 9). However, essentially no moths were caught from July to December at the research orchard in Kerikeri.

DISCUSSION

In this paper, we report the identification of four possible pheromone components for the Australian guava moth *C. improbana*. Z7-11-One-18Hy was active as a single component, and in appropriate ratios it was synergized by Z7-11-one-23Hy and Z7-23Hy. Z7-11-One-23Hy was not active alone. Z7-11-One-19Hy, although present in pheromone gland extracts, did not synergize the

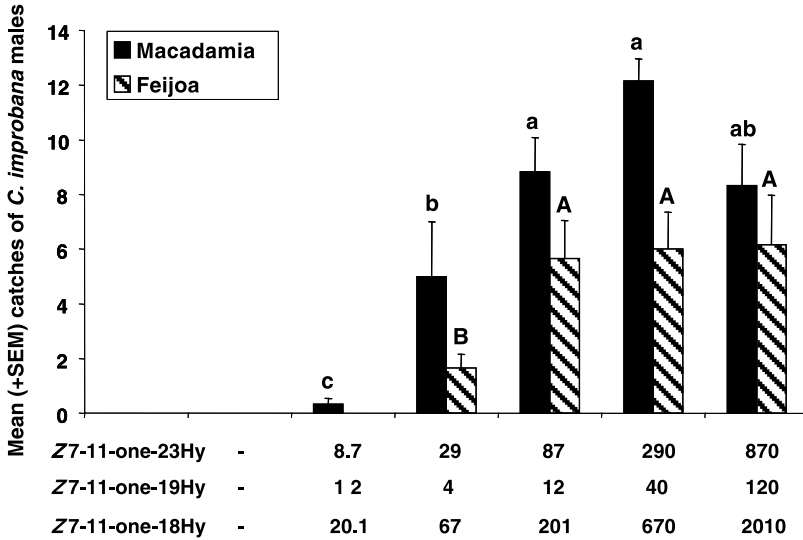


FIG. 7. Mean trap catches ($N = 6$) of *C. improbana* male moths to different doses of Z7-11-one-18Hy, Z7-11-one-19Hy, and Z7-11-one-23Hy. Trial 4, macadamia and feijoa orchards, Kerikeri, Northland, New Zealand, February 11–May 7, 2004. Treatments labeled with the same letter are not significantly different ($P > 0.05$).

attraction, and was only slightly attractive as a single component. With the identification of Z7-11-one-18Hy from *C. improbana*, all three carposinids investigated to date have been found to use homologous unsaturated ketones as their sex pheromones, with the major component for each species differing in chain length by one carbon (El-Sayed, 2006). The attractive blends of all three species overlap to some extent. Furthermore, all three species have more than one active component present in the female pheromone gland, but the major component in all cases appears to be adequate for attraction.

Although Z7-11-one-18Hy had not been previously identified from moth pheromone gland extracts, Tamaki et al. (1977) found weak attraction of *C. sasakii* (*C. niponensis*) to synthetic Z7-11-one-18Hy. Removal of Z7-11-one-18Hy from the blend in our study decreased attraction for male guava moths, and it consistently produced the greatest response from male guava moth antennae in GC-EAD studies, indicating that it is the major pheromone component of *C. improbana*.

There was a relatively large amount of Z7-11-one-18Hy in *C. improbana* gland extracts (16.4 ng/female) compared to the major components for the two other carposinids with known pheromones. Foster and Thomas (2000) found an average of 2.5 ng Z7-11-one-19Hy/female in *H. rubophaga*, whereas Tamaki et

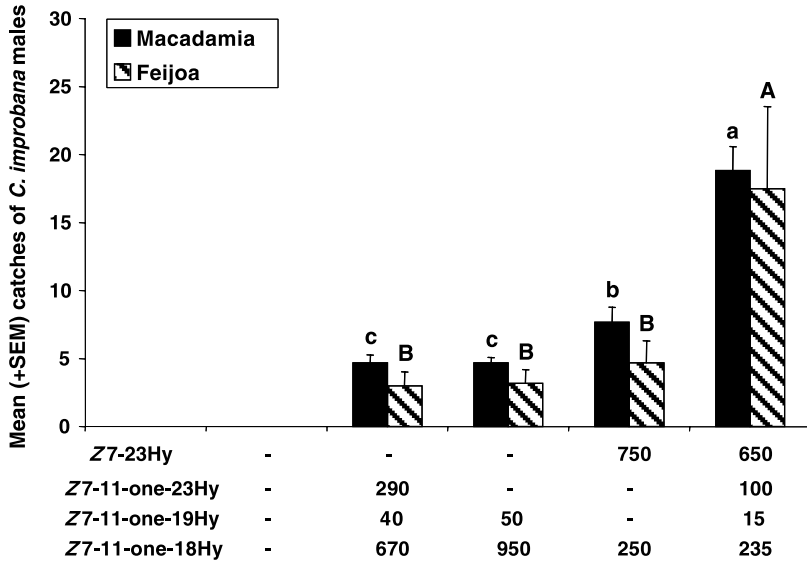


FIG. 8. Mean trap catches ($N = 6$) of *C. improbana* male moths to 1-mg doses of binary, ternary, and quaternary combinations of the four possible pheromone compounds identified from female pheromone gland extracts. Trial 5, macadamia and feijoa orchards, Kerikeri, Northland, New Zealand, October 1–November 19, 2004. Treatments labeled with the same letter are not significantly different ($P > 0.05$).

al. (1977) estimated the amount of Z7-11-one-20Hy in the gland extracts of 12,000 *C. sasakii* (*C. niponensis*) to be 18 μg (1.5 ng/female).

Both Z7-11-one-19Hy and Z7-11-one-23Hy alone attracted either few or no male *C. improbana* in our study. Han et al. (2000) found that addition of Z7-11-one-19Hy to Z7-11-one-20Hy had no effect or inhibited attraction of a Korean population of *C. sasakii*, whereas Tamaki et al. (1977) and Honma et al. (1978) reported synergism in a Japanese population of *C. sasakii* (*C. niponensis*) when ca. 5% of Z7-11-one-19Hy was added to a lure containing Z7-11-one-20Hy. However, Shirasaki et al. (1979) was not able to reproduce these results. Similarly, Foster and Thomas (2000) tentatively identified Z7-11-one-20Hy in a New Zealand population of *H. rubophaga* as a minor component, but addition of this compound to the major component (Z7-11-one-19Hy) did not increase trap catch at the ratio tested. In our study, addition of Z7-11-one-19Hy to Z7-11-one-18Hy at various ratios also did not enhance capture of male *C. improbana*.

Tamaki et al. (1977) isolated Z7-11-one-23Hy from female *C. sasakii* (*C. niponensis*) pheromone glands, but were not able to demonstrate any biological activity. In our study, addition of Z7-11-one-23Hy to the binary lure at a dose of

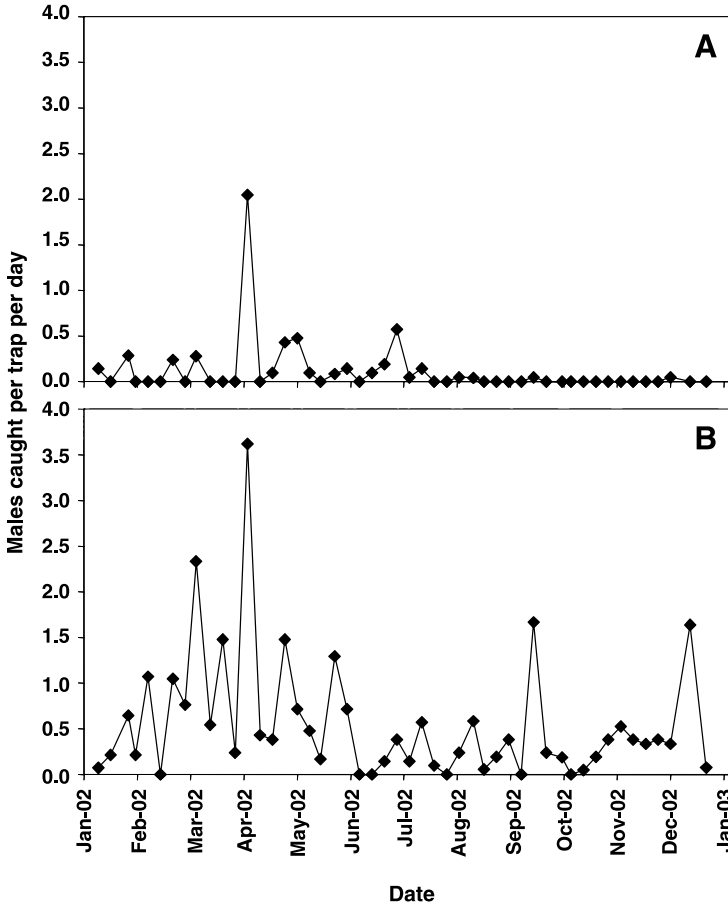


FIG. 9. Flight phenology of male *C. improbana* monitored with 300- μ g doses of a 95:5 blend of Z7-11-one-18Hy and Z7-11-one-19Hy deployed in delta traps from January 2002 to January 2003, in two macadamia orchards [(A) HortResearch Research Station and (B) Welch's organic macadamia orchard] in Kerikeri, Northland, New Zealand.

300 μ g increased the trap catch. However, in a subsequent trial with 1-mg doses, the ternary blend of ketones was no better than the binary blends of either Z7-11-one-18Hy and Z7-11-one-19Hy, or Z7-11-one-18Hy and Z7-11-one-23Hy.

Finally, addition of Z7-23Hy to Z7-11-one-18Hy alone marginally improved attraction, but it had a much larger effect when added to the ternary ketone blend. Whereas Z7-23Hy has previously been reported as a sex pheromone for the rove beetle *Aleochara curtala* (Peschke and Metzler,

1982), this is the first report of this compound as a sex pheromone component for a lepidopteran insect.

We have shown that the quaternary blend of Z7-23Hy, Z7-11-one-18Hy, Z7-11-one-19Hy, and Z7-11-one-23Hy at a ratio of 65:23.5:1.5:10, respectively, is an effective attractant for *C. improbana*, and we recommend the use of this lure for monitoring of *C. improbana*. The pheromone of *C. improbana* has already provided an effective tool for monitoring the phenology and range of this new arrival to New Zealand. Our trapping data, using a binary lure (Figure 9), indicate that the adult moth is active throughout the year. In 2003, 1-mg doses of the same binary lure were used to survey the guava moth's distribution in the Northland region (Jamieson et al., 2004). In addition, both the ternary ketone blend and the quaternary blend have been successfully used in Queensland, Australia, for detection of populations of *C. improbana* (J. Dymock, unpublished data).

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EFFECTIVENESS OF METAL–METAL
AND METAL–ORGANIC COMPOUND COMBINATIONS
AGAINST *Plutella xylostella*: IMPLICATIONS FOR PLANT
ELEMENTAL DEFENSE

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Abstract—Plants that contain elevated foliar metal concentrations can be categorized as accumulators or, if the accumulation is extreme, hyperaccumulators. The defense hypothesis suggests that these plants may be defended against folivore attack, and recent research has indicated that metal concentrations at or below the accumulator range may be defensively effective. This experiment explored the toxicity of four metals hyperaccumulated by plants (Cd, Ni, Pb, and Zn) and asked if combinations of metals, or metals and organic chemicals, might broaden the defensive effectiveness of metals. Metals were used alone and in certain metal + metal (Zn plus Ni, Pb, or Cd) and metal + organic defensive chemical (Ni plus tannic acid, atropine, or nicotine) combinations. Artificial diet amended with these treatments was fed to larvae of the crucifer specialist herbivore *Plutella xylostella*. Combinations of metals and metals + organic chemicals significantly decreased survival and pupation rates, compared to single treatments, for at least some concentrations in every experiment. Effects of combinations were additive rather than synergistic or antagonistic. Because Zn enhanced the toxicity of other metals and Ni enhanced the toxicity of organic defensive chemicals, our findings suggest that the defensive effects of metals are more widespread among plants than previously believed. They also support the hypothesis that herbivore defense may have led to the evolution of metal hyperaccumulation by increasing the preexisting defensive effects of metals at accumulator levels in plants.

Key Words—Accumulation, alkaloids, cadmium, elemental defenses, hyperaccumulation, herbivory, lead, metal, nickel, zinc.

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INTRODUCTION

Metals such as Ni, Zn, Pb, or Cd may reach high concentrations in soils because of geological processes (Reeves et al., 1981; Brooks, 1987) or industrial contamination (Reeves and Brooks, 1983; Baker and Brooks, 1989, 1994). Plants growing under natural conditions vary in tissue metal concentrations, and this variation can be used to classify them into three broad categories: normal, accumulator, and hyperaccumulator plants (Brooks et al., 1977; Baker, 1981; Baker et al., 2000; Reeves and Baker, 2000). These categories, summarized in Table 1, vary depending on the metal. In general, relatively small concentrations of Cd, Co, or Cr are unusual in plant tissues, whereas much larger concentrations of Mn or Zn are considered unusual. Other metals (Cu, Ni, and Pb) are elevated at levels between these extremes (Table 1).

Plants are defended by a variety of mechanisms, including organic chemicals (Gatehouse, 2002). Tannins, alkaloids, and glucosinolates are examples of organic defensive chemicals (Feeny, 1976; Rhoades and Cates, 1976; Schultz, 1988; Clausen et al., 1992; Agrawal, 2000). Certain elements, such as Si, F, Ni, Zn, Se, and Ca, also may have defensive functions for plants (McNaughton and Tarrants, 1983; Twigg and King, 1991; Boyd, 2004) or algae (Hay et al., 1994). Hyperaccumulated elements can defend against herbivores (Pollard and Baker, 1997; Boyd and Moar, 1999; Hanson et al., 2004) by reducing feeding (Pollard and Baker, 1997; Jhee et al., 1999; Hanson et al., 2004) or survival (Boyd and Martens, 1994; Boyd and Moar, 1999; Hanson et al., 2004). Termed “elemental defenses” by Martens and Boyd (1994), these

TABLE 1. NORMAL RANGE, MINIMUM ACCUMULATOR LEVEL, AND MINIMUM HYPERACCUMULATOR LEVEL FOR METALS OFTEN ACCUMULATED BY PLANTS^a

Metal	Normal range ($\mu\text{g g}^{-1}$)	Minimum accumulator level ($\mu\text{g g}^{-1}$)	Minimum hyperaccumulator level ($\mu\text{g g}^{-1}$)
Cd	0.1–3	20	100
Co	0.03–2	20	1000
Cr	0.2–5	50	1000
Cu	5–25	100	1000
Mn	20–400	2,000	10,000
Ni	1–10	100	1000
Pb	0.1–5	100	1000
Zn	20–400	2,000	10,000

^aNormal range, minimum accumulator level, and minimum hyperaccumulator level refer to tissue concentrations in field-collected plants and follow Reeves and Baker (2000). All values are expressed as $\mu\text{g metal g}^{-1}$ dry mass.

are based on elements taken from soil and sequestered rather than produced by metabolic pathways. Elemental defenses may be advantageous because they cannot be detoxified or degraded by herbivores (Martens and Boyd, 1994), unlike many organic chemicals (e.g., furanocoumarins, Weimin et al., 2003). They also may defend against specialist herbivores that have circumvented an organic chemical defense (e.g., Jhee et al., 2005).

Hyperaccumulator plants occur in many locations and on many soil types (Baker et al., 2000; Reeves and Baker, 2000; Macnair, 2003). Hyperaccumulators of Ni are typically found on ultramafic soils (Brooks et al., 1977; Reeves and Baker, 2000; Iturralde, 2004). Hyperaccumulators of Zn, Pb, and Cd are often found on Pb/Zn mineralized soils or metal polluted soils near mine sites or smelters (Reeves and Brooks, 1983; Baker et al., 2000). At least 418 hyperaccumulator taxa have been discovered, and most of these (318, or 76%) hyperaccumulate Ni (Reeves and Baker, 2000). Some species can hyperaccumulate multiple metals, such as Zn and Cd (Meerts and van Isacker, 1997; Escarré et al., 2000), Zn and Ni (Reeves and Baker, 1984, 2000), Zn and Pb (Meerts and van Isacker, 1997; Baker et al., 2000), Co and Cd (Reeves and Baker, 2000), or Co and Cu (Reeves and Baker, 2000).

Combinations of defensive chemicals can increase plant resistance in two ways. An additive effect of two chemicals can result in a larger combined effect on an herbivore. Compounds may also interact, so that their effects in combination differ from those predicted by adding their individual effects (Nelson and Kursar, 1999). Nelson and Kursar (1999) pointed out that interactions may increase toxicity of compounds in combination (synergism) or may decrease toxicity (antagonism). Most studies of synergism among plant defenses have focused on synergism between organic defenses (e.g., Dyer et al., 2003). Boyd (1998) suggested that multiple elemental defenses, or an elemental defense and an organic defense, may act together to provide greater herbivore resistance than each defense alone. Many Ni hyperaccumulating species belong to families (such as Lauraceae, Rutaceae, Verbenaceae, and Lamiaceae) that possess aromatic substances that may provide resistance to herbivores (Baker et al., 2000; Borhidi, 2001). Many other Ni hyperaccumulators belong to the Brassicaceae, known for production of glucosinolates (Louda and Mole, 1991), or the Rubiaceae, known for alkaloids (Borhidi, 2001). Thus, elemental and organic defenses co-occur in plant species, and together they may be more effective than each alone. Pioneering investigations of defensive effects of combinations of elemental and organic chemicals have been conducted for marine algae (e.g., Hay et al., 1994) but, to our knowledge, the possibility that combination effects contribute to the defensive ecology of metal accumulating or hyperaccumulating plants has yet to be formally addressed. Testing these questions is potentially significant for two reasons. First, combination effects may allow elemental defenses to contribute to plant fitness at concentrations

less than expected based on studies of the defensive effects of a metal alone. This could extend the defensive effects of metals to more plant species than otherwise expected. Second, combination effects (either additive effects or synergy) may have contributed to the evolution of metal accumulation and hyperaccumulation by plants (Boyd, 2004). In this scenario, a plant capable of taking up and tolerating elevated levels of a metal could derive a defensive benefit from that ability at a relatively low metal concentration in its tissues. Further stepwise increases in uptake and tolerance abilities could then be selected for in the population, as a result of differential damage from natural enemies, resulting in still greater levels of elemental defenses. By lowering the level of metal at which a defensive benefit first accrues to a plant, combination effects may help explain the evolution of metal accumulation, and later hyperaccumulation, by plants.

Recently, Coleman et al. (2005) used the diamondback moth (DBM), *Plutella xylostella*, to explore the boundaries of defensive effects of eight metals (Cd, Co, Cr, Cu, Mn, Ni, Pb, and Zn) often accumulated by plants (Reeves and Baker, 2000). They found that all metals were toxic to DBM larvae at hyperaccumulator levels. However, all also were toxic to larvae at accumulator concentrations. Five metals (Cd, Mn, Ni, Pb, and Zn) were toxic below accumulator levels, Cd and Pb were toxic near the concentration ranges of normal plants, and Zn was toxic at a concentration within the normal range. Their results indicated that single metals may be effective at concentrations far lower than previously hypothesized.

This study tests the hypothesis that combinations of metals, or combinations of metals and organic defense chemicals, have greater defensive effects than each compound alone. If this is the case, then defensive effects of metals in plants may be more extensive than previously proposed (Coleman et al., 2005). We used DBM as a bioassay herbivore because of its relative ease of colony maintenance and short generation time. We used Zn combined with Cd, Ni, and Pb to represent metals often found in combination in metal accumulator or hyperaccumulator plants. We also used several representative organic defensive compounds to test for combination effects with Ni. Nickel was chosen because it is the metal most often hyperaccumulated by plants (Reeves and Baker, 2000). Tannic acid was used to represent the “digestibility” reducing quantitative defense commonly found in apparent plants (Feeny, 1976; Rhoades and Cates, 1976; Behmer et al., 2002). Nicotine and atropine, both alkaloids, represented qualitative toxins that can defend against generalist herbivores (Feeny, 1976; Rhoades and Cates, 1976; Rhoades, 1979; Gómez et al., 2003). The toxic effects of both these alkaloids on herbivores have been well studied (e.g., Muller, 1998; Yildiz, 2004). Our experiments consisted of a series of artificial diet feeding trials using DBM larvae. In each trial, larvae were reared on one of four diet treatments: two treatments consisting of each chemical

added to diet separately, a third diet consisting of both chemicals in combination, and a fourth containing no added chemicals (control). Specifically, we asked the following questions:

- 1) Does a combination of two metals decrease survival or pupation of DBM larvae compared to each metal alone?
- 2) Does a combination of Ni and an organic chemical decrease larval survival or pupation compared to Ni alone or to the organic chemical alone?

METHODS AND MATERIALS

Experimental Colony. The diamondback moth, *P. xylostella* (L.) (Lepidoptera: Plutellidae), is an oligophagous herbivore of the Brassicaceae (Talekar and Shelton, 1993). This family contains a large percentage (about 25%) of the known hyperaccumulator species (Reeves and Baker, 2000). Although DBM has not been reported as attacking hyperaccumulators in the wild, surveys of herbivores on hyperaccumulator species are few (for exceptions, see Mesjasz-Przybyłowicz and Przybyłowicz, 2001; Wall and Boyd, 2002). The laboratory colony (Department of Entomology and Plant Pathology, Auburn University, Auburn, AL, USA) was maintained on an artificial DBM diet (BioServe, Frenchtown, NJ, USA). The diet's exact ingredients are proprietary information, but wheat germ and cabbage leaf powder are two of the main ingredients (Carpenter and Bloem, 2002). Colony maintenance procedures generally followed those of Harvey (2002). Eggs were collected on grooved aluminum foil sheets that had been coated with sterilized collard juice as an oviposition stimulant (Harvey, 2002). A 10% bleach solution was used to soak aluminum foil sheets of DBM eggs for 20 sec. Foil sheets were rinsed with deionized (DI) water for 1 min and allowed to dry. Dried egg sheets were cut into strips containing approximately 300–400 eggs per strip. Each strip was placed into a 250-ml paper cup (Solo, Twin Falls, ID, USA) with about 1 cm of congealed artificial diet covering its bottom. Cups were incubated at 37°C and approximately 30–50% humidity until eggs hatched and the instars first began to feed (~60 hr from egg collection). Larvae were allowed to feed for approximately 10–12 d after hatching. Pupae were placed in screen cages kept at room temperature where eclosed adults could mate and lay eggs on the aluminum foil sheets.

Metal + Metal Combination Experiments. Artificial diet was amended with metals to examine the effect of single, combination, and control treatments on DBM larvae. Each combination experiment compared DBM survival on diet containing each metal alone, diet containing both metals, and a control of unamended diet. Three combination experiments were conducted, using Zn

paired with Cd, Ni, or Pb. Metals were obtained as chloride salts from Sigma (St. Louis, MO, USA). Metal salts were dissolved in DI water to form stock solutions of 0.02 M Ni, 0.2 M Zn, 2 mM Pb, or 0.4 mM Cd. For each combination experiment, 100 ml of diet were amended with stock solutions to yield the concentrations of metal listed in Table 2. Experimental concentrations of metals were selected based on preliminary results that determined approximate lethal concentrations. Approximately 2–3 ml of diet were distributed into each 30-ml plastic rearing cup to give 12 replicates of each treatment within a combination experiment. A separate cup of 30 ml of diet from each treatment was saved for later metal concentration analysis.

DBM eggs were collected from a single cage containing adults that were 2–4 d posteclosion. Eggs were collected and sterilized in a 10% bleach solution and rinsed with DI water for 1 min. After egg sheets were dried, they were cut into strips containing approximately 80–100 eggs. Strips of eggs were arbitrarily distributed into diet cups by adding a strip to each cup in the first replicate (i.e.,

TABLE 2. METAL AND CHEMICAL CONCENTRATIONS IN ARTIFICIAL DIET^a

Experiment	Chemical	Units	Unamended diet		Amended diets		
Zn + Ni	Zn	$\mu\text{M Zn g}^{-1}$	0	12	18	24	140
		$\mu\text{g Zn g}^{-1}$	22 ^b	760	1200	1500	8900
	Ni	$\mu\text{M Ni g}^{-1}$	0	1.2	1.9	2.9	3.7
		$\mu\text{g Ni g}^{-1}$	3.4 ^b	73	110	170	220
Zn + Pb	Zn	$\mu\text{M Zn g}^{-1}$	0	11	19	25	150
		$\mu\text{g Zn g}^{-1}$	21 ^b	740	1200	1600	10,000
		$\mu\text{M Pb g}^{-1}$	0	4.6	83	120	170
	Pb	$\mu\text{g Pb g}^{-1}$	0.088 ^b	9.5	17	26	36
		$\mu\text{M Zn g}^{-1}$	0	12	18	23	150
		$\mu\text{g Zn g}^{-1}$	22 ^b	750	1200	1500	10,000
Zn + Cd	Cd	$\mu\text{M Cd g}^{-1}$	0	19	58.1	118	189
		$\mu\text{g Cd g}^{-1}$	0.063 ^b	2.1	6.5	13	21
		$\mu\text{M Ni g}^{-1}$	0	1.3	1.9	2.9	4.0
Ni + Tannic acid	Ni	$\mu\text{g Ni g}^{-1}$	4.3 ^b	77	110	170	230
		mg ml^{-1}	0	0.5	1	1.5	2
Ni + Atropine	Ni	$\mu\text{M Ni g}^{-1}$	0	1.3	1.9	2.6	3.4
		$\mu\text{g Ni g}^{-1}$	3.6 ^b	74	110	150	200
	Atropine	mg ml^{-1}	0	0.04	0.05	0.08	0.1
		$\mu\text{M Ni g}^{-1}$	0	1.3	1.8	2.7	3.8
Ni + Nicotine	Ni	$\mu\text{g Ni g}^{-1}$	3.4 ^b	77	110	160	220
		mg ml^{-1}	0	0.02	0.03	0.035	0.04

^aMolar concentrations are amounts added by dilution of stock solutions during diet preparation. Data for metals in $\mu\text{g g}^{-1}$ are from dry mass elemental analyses of diet samples.

^bAmounts of these elements were present in unamended (control) diets.

metal 1, metal 2, combination, control), then a strip to each cup in the second replicate, etc., until the cups in all 12 replicates had received an egg sheet.

Diet cups were placed into an incubator at 37°C and approximately 30–50% humidity. Egg sheets were removed from cups after larvae hatched and had begun to feed (approximately 60 hr after the sheets were collected). When egg sheets were removed, we counted the number of first instars in each cup. The number of live larvae was counted every 2–3 d thereafter. Once pupation began, numbers of live pupae also were counted, and each pupa was recorded as a surviving individual. Counting for all cups within a combination experiment ended when adults began to eclose from the control treatment (approximately 14–17 d after eggs were collected).

Nickel + Organic Chemical Experiments. Artificial diet was amended with combinations of Ni and an organic defense chemical. Each combination experiment compared DBM survival on diet containing each chemical alone, diet containing both chemicals, and a control of unamended diet. Nickel was paired with tannic acid, atropine, or nicotine. Organic chemicals were obtained from Sigma. To create a stock solution of tannic acid, we dissolved 400 mg of powdered tannic acid in 4 ml of ethanol and then diluted with DI water to form a 5-mg ml⁻¹ concentration stock solution. Atropine stock solutions were formed by dissolving 200 mg of atropine, in powdered form, in 4 ml of methanol and diluting with DI water to create a 1-mg ml⁻¹ solution. The stock solution of nicotine was made by dissolving 50 mg of powdered nicotine into 1 ml of ethanol and diluting with water to create a 0.25-mg ml⁻¹ solution. For each combination experiment, 100 ml of diet were amended with the Ni and organic chemical concentrations listed in Table 2. Experimental concentrations of metals and organic chemicals were selected based on preliminary results that determined approximate lethal concentrations to DBM. Diet was distributed into 30-ml plastic cups as with the metal–metal experiments, and a separate cup of 30 ml of diet from each treatment was saved for later metal concentration analysis. Addition of eggs, incubation, and counting of larvae and pupae followed the procedures of the metal–metal combination experiments described.

Elemental Analysis. Plant metal concentrations in the literature on accumulation and hyperaccumulation are expressed in µg g⁻¹ dry mass (e.g., Brooks, 1987; Reeves and Baker, 2000). We performed diet analyses to provide comparable data on metal concentration in each treatment expressed as µg g⁻¹ dry mass of diet. The 30-ml sample of diet retained from each metal concentration was dried at 60°C for 5 d and ground to a fine powder. Four 0.5-g dry mass subsamples from each metal concentration were wet digested using 10 ml of acid mix (700 ml concentrated HNO₃ + 300 ml concentrated HClO₄) within 250-ml glass digestion tubes for 24 hr. The next day, tubes were heated on a block digester within a perchloric acid fume hood at 190°C until digestion was complete. Once the tubes cooled, 2.5 ml of 1 M HCl were added

to each tube, and contents were transferred to 25-ml volumetric flasks. Contents of the volumetric flasks were brought to 25 ml by adding DI water and transferred to 100-ml plastic storage bottles (Nalgene, Rochester, NY, USA). Metal concentrations were determined using an inductively coupled argon plasma (ICP-AE) spectrophotometer (SPECTRO CIROS CCD, Cleves, Germany).

Statistical Analysis. Percent survival of DBM larvae for all treatments was calculated based on the final number of survivors (larvae plus pupae) on d 16 for all treatments (d 15 for the Ni + tannic acid experiment). Because our experiment was not a complete factorial design, we compared percent survival among metal + metal experiment treatments and Ni + organic chemical experiment treatments by using pairwise orthogonal contrasts after one-way analysis of variance (ANOVA) with JMP IN 5.1 (SAS Institute, 2005). Prior to analysis, percent survival data were arcsine square root-transformed to better fit the assumptions of ANOVA (Zar, 1996). The orthogonal contrasts allowed us to answer two questions for each combination experiment:

- 1) Did chemicals singly or in combination decrease survival relative to the control?
- 2) Did the combination of chemicals result in decreased survival compared to each chemical singly?

Differences in survival were considered significant at $\alpha \leq 0.05$.

We further examined the data to subdivide combination effects into additive or interactive (synergistic or antagonistic) effects. We converted DBM survival data into mortality data and, by using the technique of Salama et al. (1984), calculated an expected mortality for each combination treatment using the mean mortality from each chemical in a combination. These expected mortalities were compared to the actual combination mortality by using a chi-square test at $\alpha \leq 0.05$ (Salama et al., 1984). Significant deviation from expected values would indicate either synergy (if actual mortality were greater than expected) or antagonism (if actual mortality were lesser than expected).

To detect sublethal effects of treatments on DBM larvae, we compared pupation rates between treatments at the time pupae were first observed in the control treatment for each experiment. Thus, this measure included both mortality effects and decreased development time due to treatments. Pupae counts used for each experiment were taken from the data at 14 d for Zn + Ni, 10 d for Zn + Pb, 10 d for Zn + Cd, 9 d for Ni + tannic acid, 12 d for Ni + atropine, and 11 d for Ni + nicotine. We calculated % pupation by dividing the number of pupae in each cup by the maximum number of larvae that had been counted for that cup. Percent pupation values were arcsine square root-transformed to better fit the assumptions of ANOVA (Zar, 1996) and analyzed by one-way ANOVA to determine if treatments significantly affected pupation,

and pairwise orthogonal contrasts were used to compare means using JMP IN 5.1 (SAS Institute, 2005).

As with survival data, we further examined pupation data in cases where combinations had greater effects than single compounds to determine if this was caused by an additive or by an interactive (synergistic or antagonistic) effect. We calculated an expected percentage of larvae that did not pupate for each combination treatment by using the mean “failure to pupate” value from each chemical in a combination. As above, comparison of actual and expected values showed if synergy or antagonism had occurred.

RESULTS

Data analysis revealed statistically significant treatment effects for all experiments (ANOVA, $\alpha < 0.05$ in all cases). Here, we focus on the results from the pairwise orthogonal contrasts for each experiment. We use these to determine the concentrations of chemicals at which a combination effect was observed, defining a combination effect as when the survival or pupation rate for the combination treatment differed from that of both chemical treatments alone. We then ask if each combination effect is an additive or an interactive (synergy or antagonism) effect.

Metal + Metal Combination Experiments. For Zn + Ni, combination effects on survival were found at the two highest concentrations used: $1500 \mu\text{g Zn g}^{-1} + 170 \mu\text{g Ni g}^{-1}$ (Figure 1C) and $8900 \mu\text{g Zn g}^{-1} + 220 \mu\text{g Ni g}^{-1}$ (Figure 1D). Both these combination effects were additive. A combination effect for pupation (sublethal effect) occurred for the $1200 \mu\text{g Zn g}^{-1} + 110 \mu\text{g Ni g}^{-1}$ treatment, for which a combination effect was not detected from survival data. This combination effect was additive. Greater concentrations of Zn + Ni did not show combination effects for pupation because the Zn treatment produced almost no pupation in those cases.

For Zn + Pb, combination effects on survival were detected at $1200 \mu\text{g Zn g}^{-1} + 17 \mu\text{g Pb g}^{-1}$ and $10,000 \mu\text{g Zn g}^{-1} + 36 \mu\text{g Pb g}^{-1}$ (Figure 2B and D). These effects were additive. For pupation data, combination effects were observed for all but the lowest concentrations examined (Figure 2E–H). These effects also were additive.

Combination effects on larval survival for Zn + Cd treatments were detected for all concentrations except $1200 \mu\text{g Zn g}^{-1} + 6.5 \mu\text{g Cd g}^{-1}$ (Figure 3B). The effects were additive in all three cases (Figure 3A, C, and D). Pupation data revealed combination effects in all four trials (Figure 3E–H). These effects were striking, as pupation was zero for the combination treatments of all four trials, whereas at least some larvae pupated in all single metal treatments. The effects for the pupation data were additive in all cases.

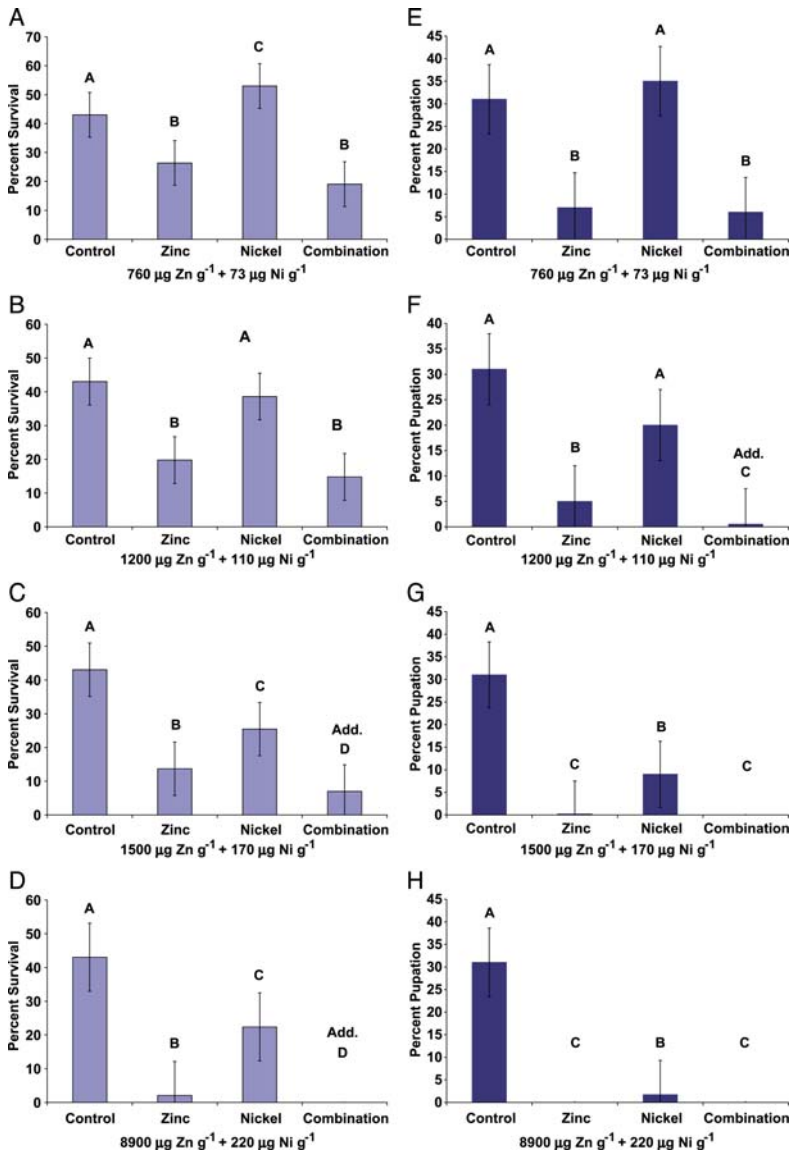


FIG. 1. Effects of Zn and Ni on diamondback moth (DBM) survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation “Add.” denotes those experiments for which a significant additive combination effect was detected.

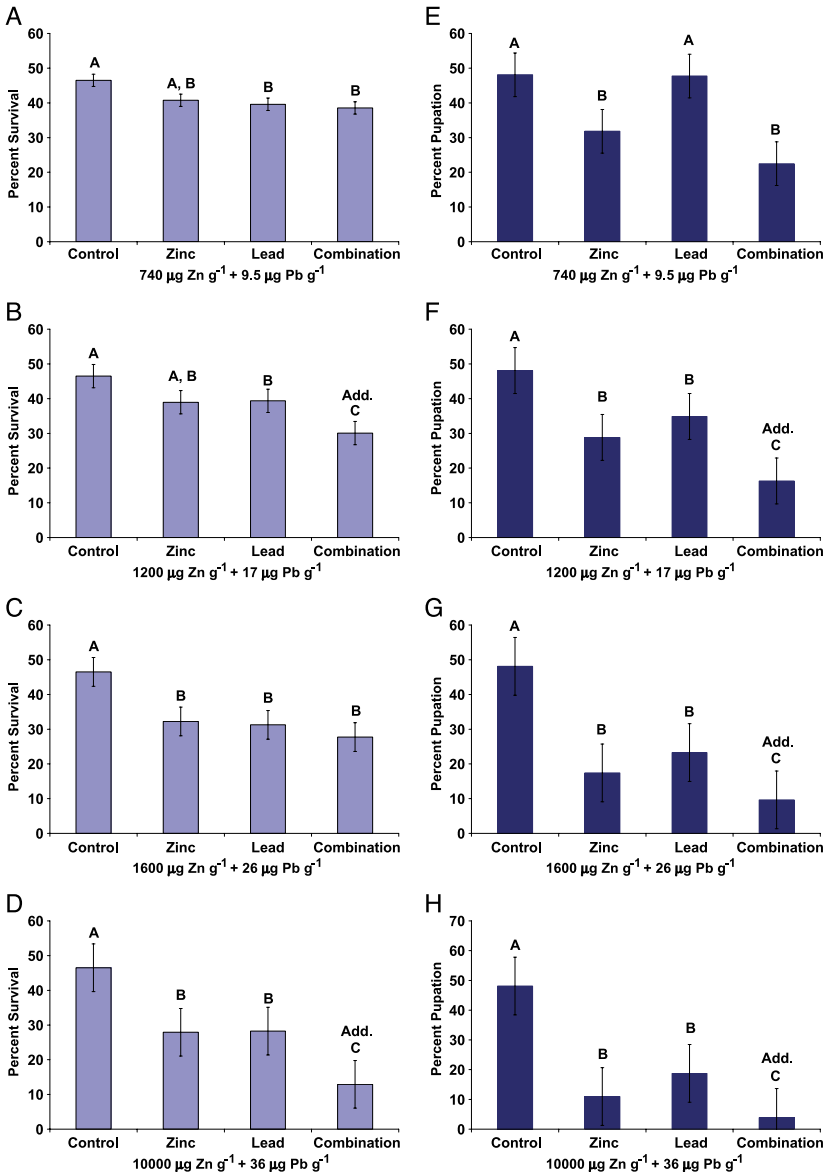


FIG. 2. Effects of Zn and Pb on DBM survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation “Add.” denotes those experiments for which a significant additive combination effect was detected.

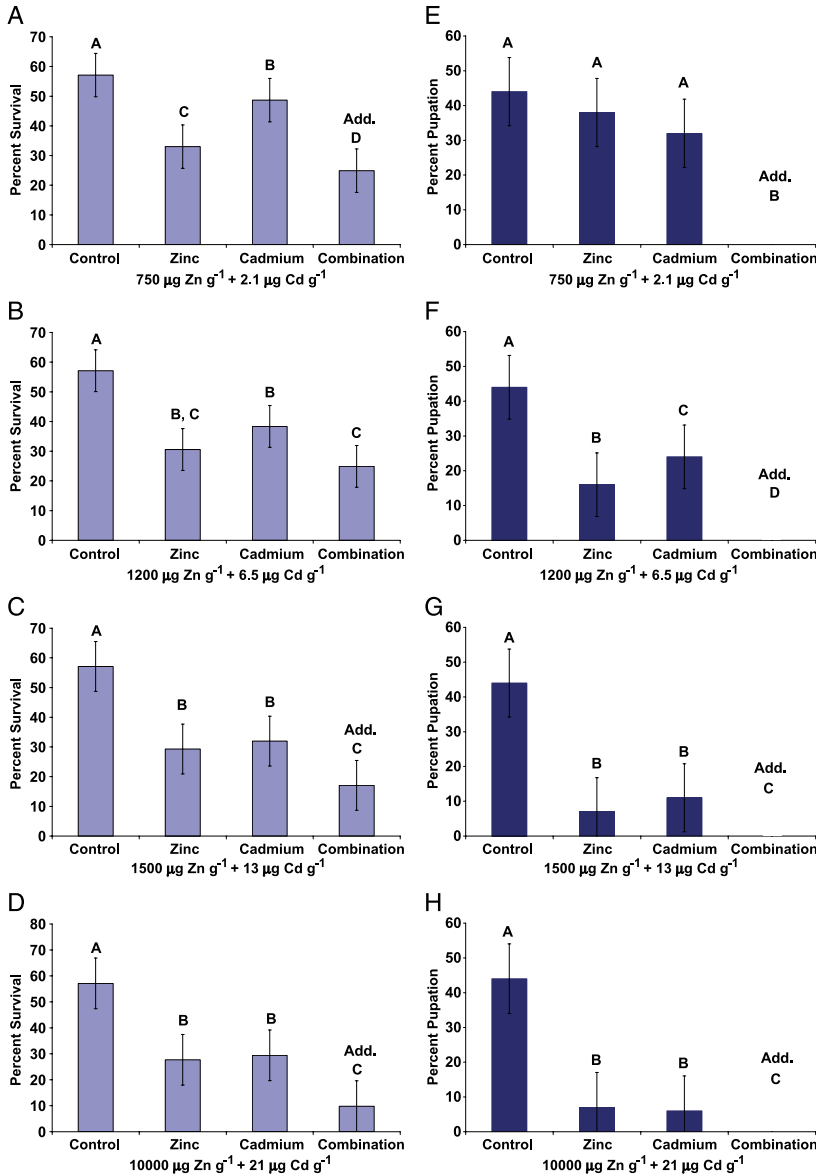


FIG. 3. Effects of Zn and Cd on DBM survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation “Add.” denotes those experiments for which a significant additive combination effect was detected.

Nickel + Organic Chemical Combination Experiments. Combination effects for Ni + tannic acid were detected for survival data for all but the lowest concentrations used ($77 \mu\text{g Ni g}^{-1} + 0.5 \text{ mg tannic acid ml}^{-1}$; Figure 4A). All were additive. Pupation data showed effects for all concentrations (Figure 4E–H), and all were additive.

For Ni + atropine, combination effects for survival data were detected for the two greatest concentrations used ($150 \mu\text{g Ni g}^{-1} + 0.08 \text{ mg atropine ml}^{-1}$ and $200 \mu\text{g Ni g}^{-1} + 0.1 \text{ mg atropine ml}^{-1}$; Figure 5C and D). Both the combination effects were additive. Pupation data showed effects for all trials (Figure 5E–H), and all were additive.

Combination effects on DBM survival in the Ni + nicotine experiment were detected for the two greatest concentrations ($160 \mu\text{g Ni g}^{-1} + 0.035 \text{ mg nicotine ml}^{-1}$ and $220 \mu\text{g Ni g}^{-1} + 0.04 \text{ mg nicotine ml}^{-1}$; Figure 6C and D). These were additive. For pupation data, effects were detected for all trials except that using the least concentrations ($77 \mu\text{g Ni g}^{-1} + 0.02 \text{ mg nicotine ml}^{-1}$; Figure 6E), and these effects were additive.

DISCUSSION

Our experiments provide a first test against an herbivore of the effects of metals in combination with other metals and with organic compounds. Defining a combination effect as a significantly greater impact in combination than for either chemical alone, we detected a combination effect for each pair of metals and for each Ni + organic chemical pairing (Figures 1–6). Furthermore, combination effects were found for both survival and pupation data. Results for the pupation data extended combination effects to still lower concentrations than were detected by the survival data for all experiments except Ni + tannic acid (in which all treatments showed significant combination effects; Figure 4) and Zn + Pb (Figure 2). Thus, we show that combination effects can magnify the protective effects of metals, and that metals may provide protection against herbivores at lower concentrations than previously believed. We note that combination effects were detected only at concentrations for which at least one of the chemicals alone had a significant negative effect on DBM relative to the control treatment (Figures 1–6). This indicated that the toxicity of one chemical made DBM more susceptible to the negative effects of the second chemical. Further tests will be needed to explore other metal + metal and metal + organic chemical combinations, but our results suggest that combination effects are common for the metals accumulated by plants.

All the combination effects we detected were additive. Boyd (1998) speculated that synergy between metals and organic defenses might magnify the effectiveness of each type of defense. We found no cases of synergy. We also

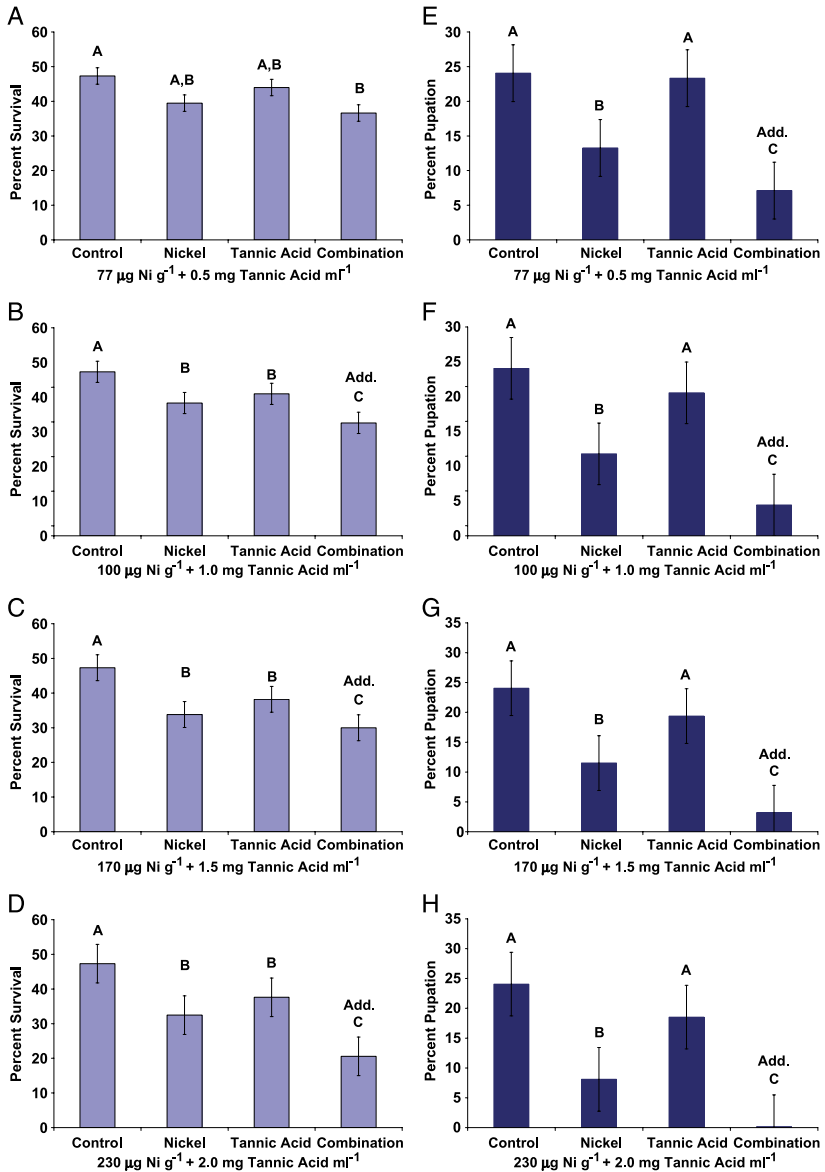


FIG. 4. Effects of Ni and tannic acid on DBM survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation “Add.” denotes those experiments for which a significant additive combination effect was detected.

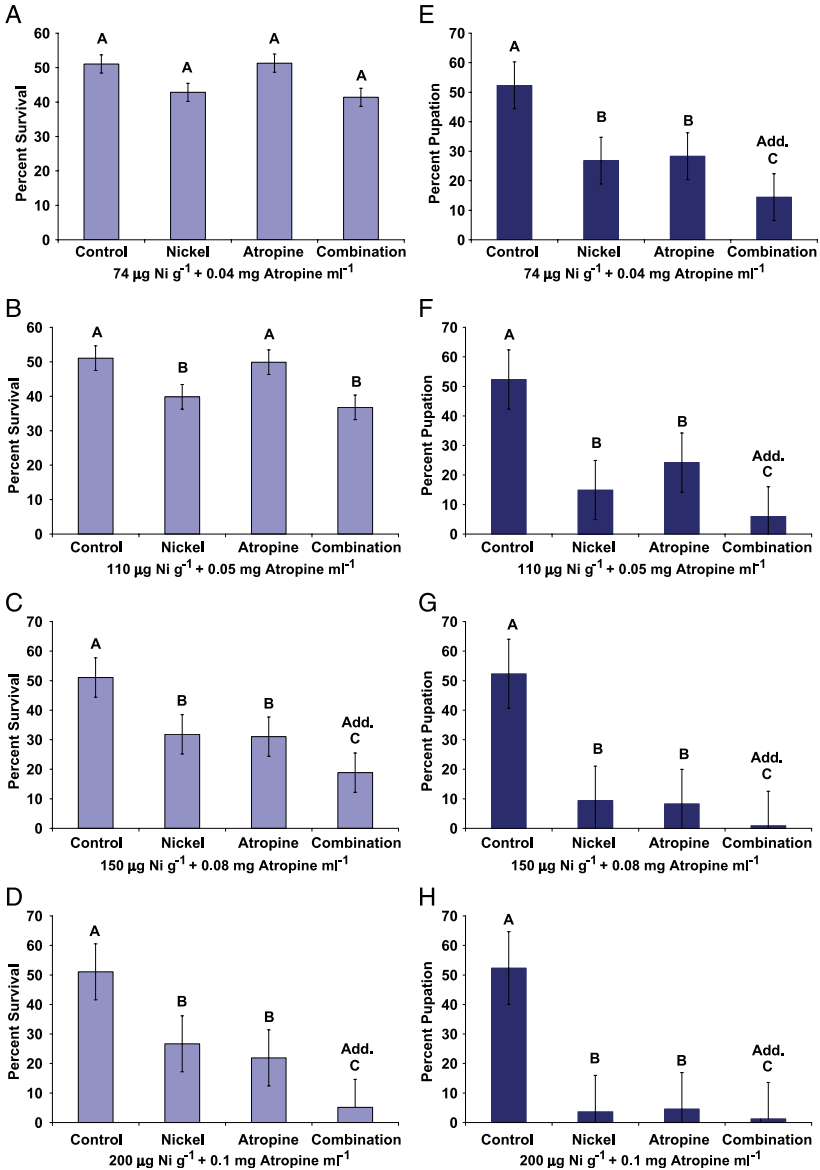


FIG. 5. Effects of Ni and atropine on DBM survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation “Add.” denotes those experiments for which a significant additive combination effect was detected.

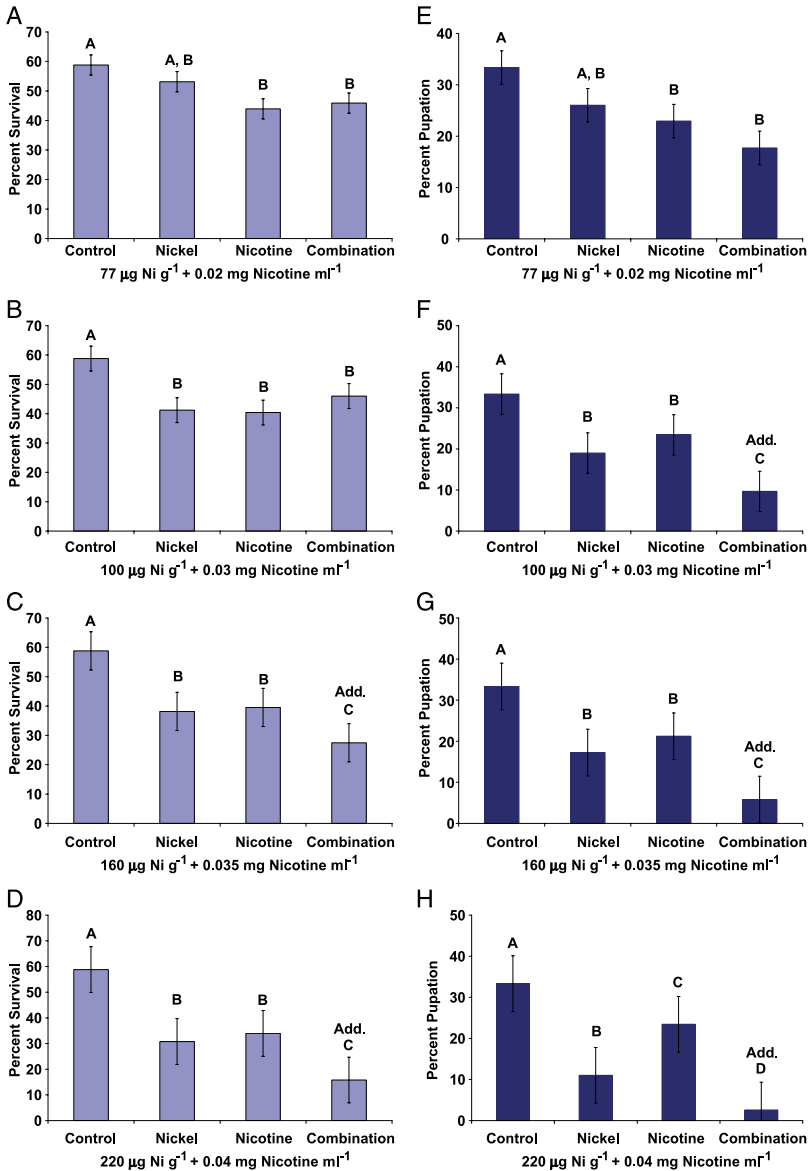


FIG. 6. Effects of Ni and nicotine on DBM survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation “Add.” denotes those experiments for which a significant additive combination effect was detected.

found no cases of antagonism, which would decrease the effectiveness of combinations of defensive chemicals. Our ability to detect interactive effects was limited because of the relatively high mortality rates of DBM larvae in our experimental trials (Hay, 1996; Pennings, 1996). This mortality rate was not unusual for a DBM colony (Shelton and Collins, 2000), but further explorations should be made for synergy/antagonism between metals, and between metals and organic chemicals, using another experimental system.

The demonstration of combination effects between metals and organic compounds (Figures 4–6) suggests that plants may be able to reduce production of organic defenses by sequestering metals. Martens and Boyd (1994) suggested that a trade-off between elemental and organic defenses allows hyperaccumulators to invest less carbon in the construction of organic defenses and yet remain defended. Tolrà et al. (2001), studying the Zn hyperaccumulator *Thlaspi caerulescens* J. & C. Presl. (Brassicaceae), demonstrated that hyperaccumulating plants possessed significantly lower concentrations of glucosinolates than low-Zn plants. Our study shows that additive effects between Ni and organic compounds enable Ni combined with a low concentration of organic compounds to provide the same defensive effect as higher levels of those organic compounds alone. Thus, plant uptake of metals to relatively low but defensively effective concentrations can allow reduced organic chemical production. Because organic chemical production must have a physiological cost (Agrawal, 2005), then the cost reduction may be significant.

Accumulation or hyperaccumulation of multiple metals may have selective value because plant enemies differ in susceptibility to different elemental defenses. For example, Boyd and Shaw (2004) showed that a plant pathogenic bacterium (*Xanthomonas campestris*) was particularly sensitive to Cu but not to Co. Therefore, an accumulator of both Cu and Co might be protected against this pathogen by its Cu concentration but not its Co concentration. However, Coleman et al. (2005) showed that low levels of Co ($40 \mu\text{g g}^{-1}$) are defensively effective against DBM, so that Co would protect a Co/Cu accumulator against DBM. Similar considerations regarding multiple natural enemies may explain contrasting results of tests of the defense hypothesis for single metals. For example, Zn hyperaccumulation in *T. caerulescens* is effective against some pathogenic fungi and some herbivores (lepidopteran larvae and grasshoppers; Pollard and Baker, 1997; Jhee et al., 1999) but ineffective against other herbivores (snails; Huitson and Macnair, 2003; Noret et al., 2005).

The evolution of hyperaccumulation is likely complex and may have occurred multiple times for each hyperaccumulated element (Borhidi, 2001) and under different selection pressures. However, Boyd (1998, 2004) suggested that defensive effects of elemental accumulation at levels below the hyperaccumulation threshold might have led to the evolution of hyperaccumulation. Coleman et al. (2005) showed that low concentrations (accumulator concentrations or less)

of most metals increase the mortality of DBM larvae. Our current work shows that relatively low concentrations of metals can be effective defenses because metal + metal and metal + organic chemical combinations increase the effectiveness of these metals. This suggests that low concentrations of metals in plants, even lower than those documented by Coleman et al. (2005), can contribute to plant defense. If these lower concentrations result in increased plant fitness, then plant traits responsible for relatively low levels of metal uptake and sequestration will be favored in plant populations faced with natural enemies. Metal accumulation ability may spread in a population and be enhanced in a stepwise process driven by increased defensive benefits of increased metal accumulation. These defensive effects, therefore, provide a likely pathway for the evolution of accumulation and hyperaccumulation of metals by plant populations.

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PHENOTYPIC PLASTICITY OF CYANOGENESIS IN LIMA BEAN *Phaseolus lunatus*—ACTIVITY AND ACTIVATION OF β -GLUCOSIDASE

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Abstract—Cyanogenesis, the release of toxic HCN from damaged plant tissues, is generally considered as a constitutive plant defense. We found phenotypic plasticity of cyanogenesis in young leaves of lima bean *Phaseolus lunatus* based on increased activity of the β -glucosidase in response to herbivore attack. Two aspects of plant cyanogenesis have to be considered in ecological analyses: (1) the cyanogenic potential (HCNp), which indicates the total amount of cyanide-containing compounds present in a given tissue, and (2) the cyanogenic capacity (HCNc), representing the release of HCN per unit time. This release is catalyzed by specific β -glucosidases, whose activity is a crucial parameter determining overall toxicity. Enzymatic activity of β -glucosidase—and, in consequence, the rate of HCN release—was increased significantly after 72 hr of incubation with spider mites as compared to noninfested leaves. Feeding by L1 larvae of Mexican bean beetles also led to enhanced enzymatic activity, whereas mechanical damage of leaf tissue had no effect on β -glucosidase activity and the release of HCN. The results place plant cyanogenesis in the group of induced resistance traits, whose degree of activity depends on the feeding by a particular herbivore.

Key Words—Cyanogenesis, β -glucosidase, chemical defense, constitutive defense, phenotypic plasticity, *Phaseolus lunatus*, *Tetranychus urticae*, *Epilachna varivestis*, plant–herbivore interactions.

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INTRODUCTION

Plant cyanogenesis, the release of hydrogen cyanide from cyanogenic precursors as response to cell or tissue disruption, is considered a constitutive plant defense against generalist herbivore attack (Nahrstedt, 1985; Belotti and Riss, 1994; Gleadow and Woodrow, 2002). Most studies focus on the variation of the content of cyanogenic plant compounds; however, cyanogenesis, if defined as the liberation of hydrogen cyanide per unit time, also has a kinetic character. Therefore, three different components have to be quantified to fully describe this phenomenon. These components are (1) the cyanogenic potential (HCNp), which gives the total amount of cyanide-containing compounds present in a given tissue (Loyd and Gray, 1970), (2) the activity of specific β -glucosidases, and (3) the resulting cyanogenic capacity (HCNc), defined as the release of hydrogen cyanide per unit time (Lieberei, 1988). In contrast to the widely studied HCNp, the capacity of plants or particular plant organs for HCN release following tissue damage is rarely studied, but data available on *Phaseolus lunatus* indicate a substantial role of such kinetics in herbivore deterrence (Ballhorn et al., 2005). In this context, two modes of defense have to be considered. The function of deterrence depends on the plants' ability for fast release of high amounts of HCN during the feeding process of the herbivore. In contrast, extensive consumption of plant material with lower cyanogenic features in the long term leads to an intoxication of the herbivore. This intoxication results from the endogenous release of hydrogen cyanide within the gastrointestinal tract, which appears to be more harmful to the herbivore than the development of gaseous HCN during the feeding process (Ballhorn et al., 2005; Miguel and Alberto, 2005).

The activity of specific β -glucosidases represents the most important parameter determining the kinetics of hydrogen cyanide release (Swain et al., 1992). However, studies on possible changes of specific β -glucosidase activity and resulting changes of the cyanogenic capacity in response to herbivore or fungal attack are lacking. Only a study on the cyanogenic rubber tree (*Hevea brasiliensis*) reported a distinct increase of β -glucosidase activity in response to local, mechanical damage of leaf tissue (Voß, 2001). For many cyanogenic plants such as rubber tree, cassava (*Manihot esculenta* Crantz), sorghum (*Sorghum bicolor* (L.) Moench), clover (*Trifolium repens* L. and *Lotus corniculatus* L.), bracken fern (*Pteridium aquilinum* (L.) Kuhn), and lima bean (*P. lunatus* L.) there is evidence for the protective effect of plant cyanogenesis (Jones, 1962; Cooper-Driver and Swain, 1976; Hruska, 1988; Bernays, 1991). Furthermore, experiments with genetically modified plants have underlined the effectiveness of cyanogenesis as an antiherbivore defense system. Transferring the complete pathway for synthesis of cyanogenic glycosides and a corre-

sponding, specific β -glucosidase into *Arabidopsis thaliana* provided resistance against *Phyllotreta nemorum*, a chrysomelid beetle that is specialized on cruciferous plants (Tattersal et al., 2001).

However, a broad range of factors on both sides of the plant–herbivore interaction affects the effectiveness of cyanogenesis as an herbivore deterrent. Focusing on the herbivores, the mode of feeding, the availability of alternative foods, as well as mechanisms of adaptation may affect the defensive potential (Walling, 2000; reviewed by Gleadow and Woodrow, 2002).

In the last decades, cyanogenesis has received considerable interest in analyses of ecological costs of plant defense (Kakes, 1989; Blaise et al., 1991; Lieberei et al., 1996; Hayden and Parker, 2002). A number of authors have described genetically based variation in cyanogenesis among populations (Lieberei, 1988; Shore and Obrist, 1992; Caradus and Forde, 1996). In addition to genotypic variability, the state of cyanogenesis may be correlated with the ontogenetic stage of the particular plant or specific plant organs (Lloyd and Gray, 1960; Till, 1987; Coley, 1980, 1988; Thayer and Conn, 1981; Schappert and Shore, 2000).

There seem to be some counteractive forces selecting against cyanogenesis because populations of some potentially cyanogenic plants are partly or even almost entirely composed of acyanogenic genotypes (Hughes, 1991; Schappert and Shore, 1995, 1999). Distribution of cyanogenesis within and among populations may depend on the presence or absence of herbivores, which lowers or increases the net fitness effects of this constitutive plant defense trait, respectively (Abbott, 1977; Ellis et al., 1977; Bokanga et al., 1994; Gleadow and Woodrow, 2000a,b). In addition, abiotic conditions, such as temperature and humidity, and their seasonal variation, as well as nutrient availability may have an impact directly on the status of cyanogenesis within a given plant and, therefore, can have the potential to affect the distribution of plant cyanogenesis at the level of populations (Jones, 1962, 1966, 1972; Cooper-Driver et al., 1977; Kakes, 1989; Calatayud et al., 1994; Calatayud and Le Rü, 1996). Thus, general problems of estimating the relevance of cyanogenesis in an ecological context arise from the highly dynamic nature of this trait that also comprises the putatively different responses of its separate parameters, i.e., HCNp, β -glucosidase activity, and HCNc.

In the present study, we use lima bean (*P. lunatus*) as a presumably obligate cyanogenic plant with high intraspecific and ontogenetic variability in its cyanogenic features (Baudoin et al., 1991; Debouck, 1991; Ballhorn et al., 2005). Spider mites (a generalist cell-content feeder) and larvae of the Mexican bean beetle (a specialist herbivore with chewing mouthparts) were used for analysis of phenotypic plasticity of β -glucosidase activity and the corresponding HCNc.

METHODS AND MATERIALS

Plants. The experiments were carried out with 12 accessions of *P. lunatus* including 11 domesticated lines and a wild type. For further characterization of the accessions see (Table 1). Trifoliolate leaves of these accessions were characterized by different concentrations of cyanide-containing compounds (Ballhorn et al., 2005). The cyanogenic potential (HCNp) of young leaves ranged from 7.4 ± 2.1 (accession 8071) to 65.3 ± 13.4 (accession 2357) $\mu\text{mol HCN per gram leaf fresh weight (fw; mean} \pm \text{SD; } N = 11 \text{ clonal plants per accession)}$. Seed material was provided by the Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany.

Growing and Treatment of Plants. Single plants of the different accessions were vegetatively propagated (28 plants per accession) to reduce genetic variability of the experimental plants. These plants were used for the experiments when they were 8 wk old and had developed two fully expanded leaves. Clonal plant material was obtained by preparing one-node cuttings from the mother plant. Cuttings were rooted in water supplemented with Rhizopon AA 1%® (Rhizopon bv, Hazerwoude, Holland). The mother plants obtained from seeds as well as the cuttings were cultivated under green house conditions at a

TABLE 1. CHARACTERIZATION OF *Phaseolus lunatus* GENOTYPES

Labeling and origin of genotypes		Physiological and morphological parameter		
Accession-Number	Country of origin	Cyanogenic potential of young leaves ($\mu\text{mol HCN/g leaf fw}$) ^b		
		Leaf surface	Habit	
2233 (WT ^a)	Cuba	63.7 ± 11.6	glabrous	tendrillar
8078	China	61.8 ± 10.8	hairy	tendrillar
2357	Spain	65.3 ± 13.4	hairy	tendrillar
8073	–	28.9 ± 6.6	glabrous	tendrillar
1738	Cuba	37.1 ± 5.8	glabrous	tendrillar
1315	Peru	54.2 ± 8.5	hairy	tendrillar
8067	–	18.6 ± 5.2	glabrous	tendrillar
8151	–	36.9 ± 2.9	glabrous	low tendrillar
8071	–	7.4 ± 2.1	glabrous	tendrillar
8079	–	8.6 ± 1.7	glabrous	low tendrillar
2441	Bulgaria	19.1 ± 3.9	glabrous	low tendrillar
2116	Italy	78.8 ± 10.4	hairy	tendrillar

^aAccession 2233 represents lima bean, *P. lunatus*, wild type (WT) with small seeds and small, relatively hard textured leaves.

^bYoung leaves of accessions were screened for their general cyanogenic potential ($N = 11$ individual plants per accession, values are means \pm SD) according to the method described in (Ballhorn et al., 2005).

light regime of 16:8 hr light–dark period by a photon flux density of $400 \mu\text{mol sec}^{-1} \text{m}^{-2}$ at the plant container and $900 \mu\text{mol sec}^{-1} \text{m}^{-2}$ on the top of the plants. Supplementary light was provided by 400-W high-pressure sodium lamps with plant grow broad-spectrum fluorescent bulbs (Son-Targo 400, Philips®). Temperature in the chamber was 30:20°C, and the ambient relative air humidity ranged between 60 and 70%. Plants were fertilized with a nitrogen–phosphate fertilizer (Blaukorn®–Nitrophoska®–Perfekt, Compo GmbH & Co. KG, Münster, Germany) twice a week and rooted in standard substrate (TKS®–1-Instant, Floragard®, Oldenburg, Germany) that was mixed with two thirds of washed sand with different grain size (0.3–0.7 and 1–2 mm). Plant containers with a diam of 18 cm were used for cultivation.

Selection of Leaf Material. Young leaves were selected for the experiments. Leaves of this developmental stage revealed the highest homogeneity of structural parameters among the accessions and the highest constancy of physiological traits (HCNp and general β -glucosidase activity) within the lines. This leaf developmental stage was classified by the position of leaf insertion at the stem as well as by leaf morphological parameters. By definition, “young leaves” are inserted near the top of the stem or a side stem. They were fully unfolded for at least 4 d, but no longer than 6 d, and the midrib of the central leaflet of these trifoliate leaves was about 4 cm long. Leaves of this ontogenetic stage were characterized by a bright green color and a soft texture.

Arthropods. Two-spotted spider mites (Acari: Tetranychidae: *Tetranychus urticae* Koch) and Mexican bean beetles (Coleoptera: Coccinellidae: *Epilachna varivestis* Mulsant) were used as activating agents. Both the maintenance culture of the spider mites as well as of the Mexican bean beetles were kept on *Phaseolus vulgaris* cv. “Saxa” under identical ambient conditions as experimental plants. The progenitors of the Mexican bean beetle culture were provided by C.P.W. Zebitz, Department of Applied Entomology, University of Hohenheim, Otto-Sander Str. 5, Germany.

Infestation of Leaves with Spider Mites. Fourteen clonal plants were used per accession. The terminal leaflet of one trifoliate leaf per plant was infested with 10 adult females that had been kept on *P. vulgaris* before. Experiments were carried out in the green house under identical ambient conditions as adjusted for plant cultivation. For analysis of plant responses to spider mite attack, the infested leaflets of selected trifoliate leaves were bagged with perforated, transparent polyethylene foil (approx. 9 holes/cm², each with a diam of 0.5 mm), whereas the other two leaflets of the leaf were bagged but remained uninfested. In addition, leaves of control plants were bagged in the same way. These plants were cultivated in a separate chamber under identical ambient conditions. In parallel experimental settings, mites were left on the leaflets for 24, 48, and 72 hr, respectively. The β -glucosidase activity of the infested leaflets was quantitatively compared to the two untreated leaflets of

the same leaf, which were pooled for analysis, as well as to leaves of the control plants characterized by the same ontogenetic stage. Leaflets of these control leaves were pooled for analysis of the β -glucosidase activity.

Leaf Damage by Mexican Bean Beetles. In experiments using the Mexican bean beetle as attacking herbivore, three freshly hatched L1 larvae were placed on the terminal leaflet of a trifoliolate leaf. Running time of the feeding trials and bagging of leaflets were consistent with the experiments using spider mites as herbivores. Noninfested control plants were used in the same way as mentioned above.

Effect of Artificial Damage. Artificial damage was applied to the leaflets using a plastic (PE) pin with a diam of 1.5 mm. Initially, 15 injuries were set per leaflet. In parallel experimental settings, additional 15 injuries were set every 24 hr to achieve an increasing damage of leaf tissue visually similar to the leaf area damaged by the Mexican bean beetles. The damaged leaflets were bagged in the same way, and untreated but bagged control plants were used as described for the plants wounded by herbivores.

Harvest of Leaves for Analysis. Leaves were cut off with a razor blade after the respective period of incubation. The three leaflets per leaf were separated by the criteria "treated" and "untreated" and were immediately prepared for analysis of β -glucosidase activity. Leaf material of control plants was prepared in the same way.

Extraction and Purification of β -Glucosidase. Whole leaflets were weighed and homogenized in a fourfold volume of 67 mmol l^{-1} phosphate buffer adjusted to pH 6.4. The extract was filtered through cotton fabric and centrifuged at $20,000 \times g$ and 4°C (RC5C, Sorvall). The protein-containing supernatant was concentrated by ammonium sulfate fractionation and filtered through membrane caps with a pore size $<10,000 \text{ kD}$ (Schleicher & Schuell BioScience GmbH, Dassel, Germany).

Constitutive β -Glucosidase Activity. The quantification of β -glucosidase activity in the extracts was based on the detection of *p*-nitrophenol released by hydrolysis of the chromogenic artificial substrate *p*-NP-glucoside (Merck; Hösel and Nahrstedt, 1975; Selmar, 1981, 1986). The standard incubation mixture for analysis of the enzymatic activity contained 1-ml substrate solution (2 mmol) in citrate buffer adjusted to pH 5.6. The incubation mixture was made up by citrate buffer (pH 5.6) to a volume of 4.9 ml, and finally 0.1 ml extract was added. After 10 min of incubation at 30°C , the reaction was stopped by adding 1 ml ice-cold sodium carbonate solution (1 mol/l), and the released *p*-nitrophenol was quantified spectrophotometrically at 400 nm (Pharmacia Biotech, Ultraspec 3000). The β -glucosidase activity was calculated per gram leaf dry weight as katal (kat). An enzyme activity of 1 kat is defined as a substrate conversion rate of 1 mol substrate per second under standard temperature and pressure. The calculation of the enzyme activity was carried out by use of a coefficient of extinction for *p*-nitrophenol ($\epsilon_{400 \text{ nm}} = 16,159 \text{ l/mol cm}$; Voß, 2001).

HCN Detection System. The kinetic analysis of HCN release from experimentally treated lima bean leaves was carried out using an airflow system (Ballhorn et al., 2005). This vessel system was passed by a constant airflow adjusted at 7.0 l/hr. Infested and noninfested leaflets of the experimental plants as well as terminal leaflets of the control plants were placed in the equipment, respectively. The leaflets were treated with chloroform (250 μ l/leaflet) to achieve complete tissue disintegration and, in consequence, the release of gaseous hydrogen cyanide from the accumulated cyanogenic precursors. At the discharge opening of the equipment, the air together with the transported HCN was led into a test tube containing 0.1 mol/l NaOH solution. Thus, cyanide was fixed as NaCN and then was spectrophotometrically quantified at 585 nm as a polymethine dye that was formed by use of Spectroquant® cyanide test (Merck). We used a coefficient of extinction of ($\epsilon_{585 \text{ nm}} = 131,600 \text{ l/mol cm}$) for calculation of the cyanide concentration in the samples following the product description.

Statistics. Statistical analyses were carried out with Statistica 6.0 (Statistica System Reference, 2001).

RESULTS

Variability of β -Glucosidase Activity. Substantial interaccession variability of β -glucosidase activity in young leaves was found among the 12 accessions of *P. lunatus* (Figure 1A). Among these lines, the enzymatic activity ranged from 0.016 to 3.765 μ kat per gram leaf dry weight ($N=9$ per accession) and varied significantly depending on the genotype (ANOVA: $F=21.861$, $df=11$, $P < 0.001$).

Attacking Period. In experimental settings that used spider mites as well as Mexican bean beetles as damaging agents, substantial increase of β -glucosidase activity was found after 72 hr of incubation, whereas no distinct increase of the enzymatic activity was observed after an incubation period of 24 and 48 hr. Thus, results presented hereafter refer to an incubation period of 72 hr (Figure 1B–D).

Effect of Spider Mite Infestation on β -Glucosidase Activity. A factor of β -glucosidase activity increase was calculated by dividing the enzyme activity (μ kat) of the infested leaflets by the values of activity of the noninfested leaflets belonging to the same leaves. Enzyme activity in untreated leaflets of treated leaves was not significantly different from activity in completely untreated leaves of control plants (ANOVA: $F = 0.012$, $df = 1$, $P = 0.917$). Relative increases in β -glucosidase activity are therefore expressed as a factor of β -glucosidase activity increase comparing the damaged and undamaged leaflets of single leaves. These factors generally gave values of 1 or more and indicated an increase of β -glucosidase activity following spider mite attack. Only the accession 1315 showed a slight decrease of enzymatic activity after infes-

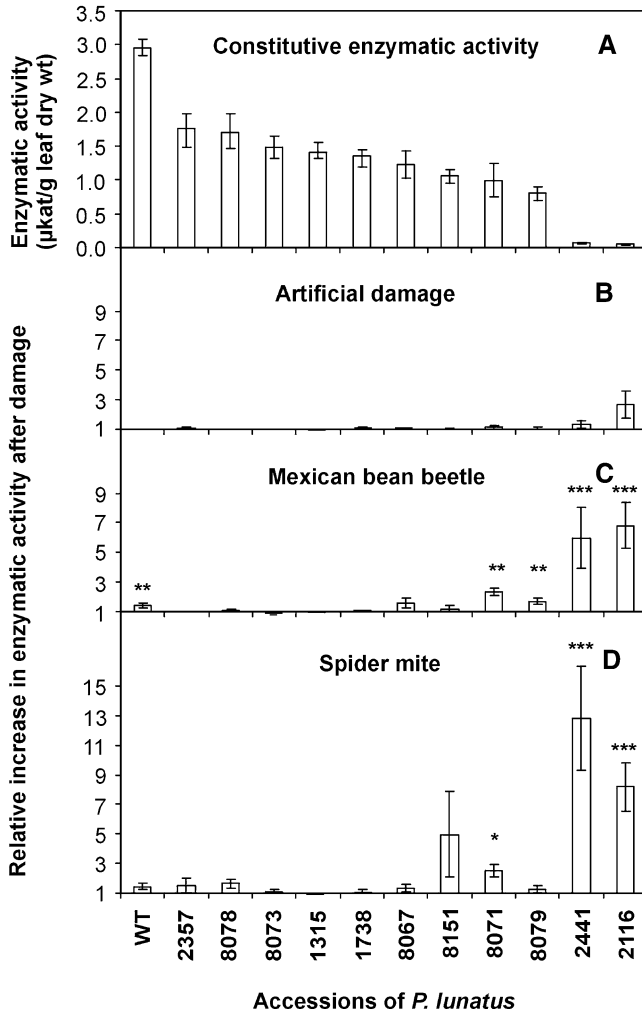


FIG. 1. Increase in β -glucosidase activity following leaf damage. Presented is the constitutive β -glucosidase activity in young *Phaseolus lunatus* leaves (A) and relative increases in enzyme activity in response to attack from artificial damage (B), larvae of Mexican bean beetles (C), and spider mites (D). Values are means \pm SE [($N = 9$ leaves per accession (A) and $N = 7$ leaflets of different plant individuals per accession (B–D))]. Asterisks represent significant increase in enzymatic activity (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$).

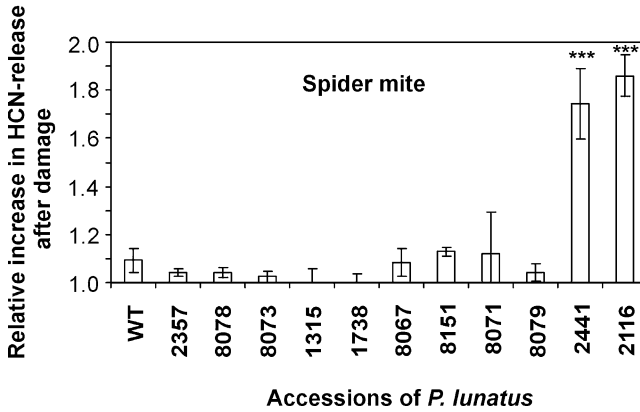


FIG. 2. Increase in HCN liberation by enhanced β -glucosidase activity. The relative increase of gaseous hydrogen cyanide release per unit time was accelerated in response to increased β -glucosidase activity provoked by spider mite attack. Values are means \pm SE ($N = 7$ leaflets of different plant individuals per accession). Asterisks represent significant increases in HCN liberation (***) $P < 0.001$.

tation with spider mites (Figure 1B). The accessions 2441 and 2116 showed a substantial increase of enzymatic activity by the factor 12.8 ± 3.6 and 8.2 ± 1.6 (mean \pm SE), respectively. For these accessions, as well as for the accession 8071, the enzymatic activity in infested leaflets increased significantly in response to spider mite attack (ANOVA; 2441: $F = 130.614$, $df = 1$, $P < 0.001$; 2116: $F = 317.091$, $df = 1$, $P < 0.001$; and 8071: $F = 5477$, $df = 1$, $P < 0.05$). However, none of the other accessions showed significantly enhanced enzymatic activity following spider mite attack.

Effect of Damage by Mexican Bean Beetle Larvae on β -Glucosidase Activity. The β -glucosidase activity of selected *P. lunatus* accessions was increased by feeding of Mexican bean beetle larvae (Figure 1B). Exceptions are only the accessions 2357, 1315, and 8073, which showed a slight decrease of the enzymatic activity. Leaflets of the accessions 2116, 2441, 8071, and 8079 exhibited significantly increased β -glucosidase activity in response to leaf damage by these insects as compared to the nondamaged leaflets of the same leaf (2116: $F = 31.131$, $df = 1$, $P < 0.001$; 2441: $F = 23.968$, $df = 1$, $P < 0.001$; 8071: $F = 13.320$, $df = 1$, $P < 0.01$; 8079: $F = 14.052$, $df = 1$, $P < 0.01$; WT: $F = 10.397$, $df = 1$, $P < 0.01$). These nondamaged leaflets, in turn, never showed significant differences of β -glucosidase activity when compared to leaves of the control plants ($F = 0.006$, $df = 11$, $P = 0.994$).

Artificial Damage of Leaves. Among all accessions, injuries of leaflets set with a plastic pin had no effect on β -glucosidase activity in these leaflets as

compared to the remaining leaflets ($F = 0.055$, $df = 1$, $P = 0.815$). However, 8 among 12 accessions of *P. lunatus* showed factors of β -glucosidase activity increase higher than 1.

Effect of Increased β -Glucosidase Activity on HCNc. The increase in β -glucosidase activity in spider-mite-infested leaflets compared to untreated leaflets of the same leaves was correlated with increased HCN release from these leaflets as reaction to breakdown of cell integrity (Figure 2). Similar to the relative increase of enzymatic activity, we calculated a factor of relative increase of HCN release per unit time by dividing the amount of released HCN of the infested leaflets by the values of HCN release of noninfested leaflets belonging to the same leaves. The cyanogenic capacity of spider-mite-infested leaflets of the accessions 2116 and 2441 was accelerated compared to noninfested leaflets of these *P. lunatus* lines (2116: $F = 41.850$, $df = 1$, $P < 0.001$ and 2441: $F = 45.984$, $df = 1$, $P < 0.001$). The other accessions showed no increase of HCN release in response to spider mite attack. However, the increase in HCN release of the accession 2357 was close to statistical significance ($F = 4.52$, $df = 1$, $P = 0.051$).

DISCUSSION

Plant cyanogenesis represents a complex trait in herbivore defense because of high variability of the cyanogenic system. Besides the heterogeneous distribution of cyanogenesis among or within plant populations, the cyanogenic status of an individual plant is not static (Till, 1987). Therefore, analyses testing only for the presence or absence of cyanogenic glycosides and the general ability for HCN release do not address the variability of this constitutive herbivore defense.

In this study, enzymatic activity of β -glucosidase, a crucial factor limiting the rate of HCN release, increased in response to herbivore attack. Cyanogenesis thus comprises an inducible component, a finding placing it in the group of induced plant responses to herbivores. We found substantial interaccession variability because significant increases in β -glucosidase activity were observed only in some of the accessions tested. Furthermore, the responses of these accessions depended on the type of herbivores causing the damage. Spider mite attack generally led to a higher increase in β -glucosidase activity than leaf damage caused by larvae of the Mexican bean beetle (Figure 1C and D). This result most likely is a consequence of the different modes of feeding of these herbivores. Spider mites are cell-content feeders and damage single cells or limited areas of epidermal and mesophyll cells per feeding incident (Raven, 1983), whereas Mexican bean beetles remove larger areas. Furthermore, elicitors may be present in the herbivores' saliva, which can have varying

effects on resistance-related plant enzymes such as pectinases, cellulases, amylases, proteases, lipases, alkaline and acidic phosphatases, and peroxidases (Miles, 1999). Support for this interpretation comes from the observation that pure mechanical damage of similar leaf areas as damaged by the herbivores did not significantly induce β -glucosidase activity.

To test whether increased β -glucosidase activity, in fact, leads to increased HCN release, we used spider mite-infested leaves. The highly significant increases in β -glucosidase activity of the accessions 2116 and 2441 indeed were correlated with significant increases in HCN release (Figure 2). This finding underlined the impact of β -glucosidase activity on kinetic patterns of cyanogenesis. However, the increase in HCN release was quantitatively lower than the increase in β -glucosidase activity. Accessions 2116 and 2441 showed increases in β -glucosidase activity by factors greater than 8 and 12 (Figure 1D), whereas the magnitude in the relative increases in HCN release was less than twofold (Figure 2). This result can be explained when we assume that increased β -glucosidase activity occurred only in limited areas directly adjacent to the cells affected by spider mite feeding. In experiments with rubber tree, areas of enzymatic activation following leaf damage appeared to be local (Voß, 2001). Thus, increased enzymatic activity and enhanced HCN liberation because of herbivory might not affect the whole leaf. Another explanation for the non-proportional increase of β -glucosidase activity and HCN release could be the activity of hydroxynitrile lyases, which are catalyzing the dissociation of hydroxynitriles as the second step of cyanogenesis and, therefore, have the potential to limit the release of HCN (Selmar et al., 1989).

A further point to be regarded in this context is the multifunctional character of β -glucosidases as pointed out by Selmar et al. (1987), particularly the putative involvement of β -glucosidases in mechanisms belonging to systemic acquired resistance against pathogens (SAR). SAR represents a systemic and multigenic plant response to a local infection that leads to a broad-spectrum resistance to subsequent infections (Lamb and Dixon, 1997). SAR is primarily induced by pathogens, but tissue damage by piercing and sucking insects and mites induces similar responses (Russo et al., 1997; Bostock, 1999; Walling, 2000; Grinberg et al., 2005). Two important aspects of SAR are the accumulation of phytoalexins and enhanced local lignification of cell walls (Hammerschmidt, 1999a,b; Bruxelles and Roberts, 2001), and β -glucosidases are involved in both processes. Garcia et al. (1995) reported an essential function of β -glucosidases for the release of the phytoalexin scopoletin from its glycoside scopolin in leaves of the cyanogenic rubber tree (*H. brasiliensis*). The role of β -glucosidases for lignin biosynthesis in the course of defense reactions of *Triticum aestivum* against the fungal pathogen *Puccinia graminis* was demonstrated by Kogel et al. (1991). The induction of β -glucosidase reported is likely to have functions beyond the increase in HCN release.

Taken together, the results of our study underline the plastic character of plant cyanogenesis caused by phenotypic plasticity of β -glucosidase activity. This enzyme is an essential agent for the kinetics of HCN release, but further functions of the β -glucosidase present in *P. lunatus* cannot be excluded. Nevertheless, inducibility of β -glucosidase activity further adds to the ecological complexity in the interaction of cyanogenic plants with their biotic environment.

Mechanisms involved in the activation of specific β -glucosidases remain elusive. Voß (2001) reported immediate activation of specific β -glucosidase of the cyanogenic rubber tree (*H. brasiliensis*) as response to mechanical injury of leaves. This activation takes place within seconds. Hence, mechanisms of gene activation and mRNA synthesis could be excluded for this HCN-releasing plant, and posttranslational processes affecting the β -glucosidase activity have to be assumed. In *P. lunatus*, a significant increase of the enzymatic activity was measured at the earliest after 72 hr of incubation. This time lag of activation may be a result of mRNA synthesis in the course of gene activation.

The plastic character of enzymatic activity in *P. lunatus* (and therefore the plasticity of cyanogenesis) appeared to be local because feeding of spider mites and L1 larvae of the Mexican bean beetle on one single leaflet had no inductive effect on β -glucosidase activity in the other two leaflets of the same leaf. This was verified by comparing β -glucosidase activity of these untreated leaflets with leaves of entirely untreated clonal control plants, which were not significantly different. Thus, the increase in β -glucosidase activity of leaflets damaged by herbivores was only detected in the damaged leaflets. The lima bean represents an experimental plant that is well established for analyses of plant responses to herbivore damage. The focus of research lies on the mechanisms of VOC emission and the processes induced on the molecular level (Arimura et al., 2000) and, more recently, on the activation of extrafloral nectar in the field of indirect defenses (Heil, 2004). Cyanogenesis of the lima bean as a direct defense with high intraspecific and ontogenetic variability, as well as a certain state of phenotypic plasticity, represents an aspect that has to be considered in ecological analysis and evaluation of ecological costs of plant defense in this model system. Plant cyanogenesis of *P. lunatus* appears not to be a constitutive defense in the classical sense.

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LUTEIN SEQUESTRATION AND FURANOCOUMARIN METABOLISM IN PARSNIP WEBWORMS UNDER DIFFERENT ULTRAVIOLET LIGHT REGIMES IN THE MONTANE WEST

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Abstract—Both biotic and abiotic selection pressures can contribute to geographic variation in allelochemical production in plants. We examined furanocoumarin production in western North American populations of *Heraclium lanatum* and *Pastinaca sativa* that, at different latitudes and altitudes, experience different ultraviolet (UV) light regimes. Total furanocoumarins and linear furanocoumarins of fruits were negatively correlated with UV irradiance, whereas amounts of angular furanocoumarins, which are generally less phototoxic, were not. Another factor potentially influencing furanocoumarin production is the presence of the parsnip webworm *Depressaria pastinacella*, (Lepidoptera: Oecophoridae), an herbivore that feeds on reproductive structures of both plant species. These insects sequester lutein from their host plants; this carotenoid acts to ameliorate furanocoumarin toxicity. Although the concentration of lutein in fruits did not vary with UV irradiance, lutein sequestration by sixth instars was positively correlated with UV irradiance. Webworm populations are variably infested with the polyembryonic webworm parasitoid *Copidosoma sosares* Walker (Hymenoptera: Encyrtidae). *H. lanatum* fruits from populations with webworms parasitized by *C. sosares* had lower concentrations of furanocoumarins, with the exception of sphondin, than fruits from plants infested with webworms free from parasitism. Lower levels of these furanocoumarins may reduce negative effects on the fitness of this parasitoid. In contrast with the variation

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in furanocoumarin content, the ability of webworms to metabolize furanocoumarins by cytochrome P450 did not differ significantly among populations from New Mexico to Alberta.

Key Words—Furanocoumarin, cytochrome P450, ultraviolet light, carotenoid, lutein, altitude, tritrophic interaction, geographic mosaic, *Depressaria pastinacella*, *Heracleum lanatum*, *Pastinaca sativa*, *Copidosoma sosares*.

INTRODUCTION

Chemical defenses in plants against biotic stress agents function in environments that influence their efficacy and, therefore, their adaptive value to the plant (Baldwin and Preston, 1999). Among plant allelochemicals that are particularly dependent on abiotic conditions are phototoxins, chemicals whose toxicity is enhanced by exposure to light (Arnason et al., 1992). Many phototoxic plants grow preferentially in sunlit habitats and maximize exposure of herbivores to photoactivating wavelengths (Berenbaum, 1981; Arnason et al., 1992). In that light itself can cause damage to plants, many phototoxic plants also produce antioxidant allelochemicals that ameliorate light-induced oxidative damage; the content of these antioxidant compounds in plants varies in response to oxidative stress created by high light exposure (Frankel and Berenbaum, 1999; Polle et al., 1999; Hansen et al., 2002). Because variation in light intensity can exist across the host range of a phototoxic plant and its herbivores, the relative benefit of a phototoxic allelochemical as a herbivore defense may vary on a landscape scale.

Specialist herbivores of phototoxic plants have behavioral and physiological adaptations that limit the effects of photoactivation (Aucoin et al., 1990, 1995; Arnason et al., 1992). Among these counteradaptations is sequestration of plant antioxidants that when ingested along with phototoxins can reduce toxicity (Ahmad and Pardini, 1990; Aucoin et al., 1995). Chief among these sequestered plant antioxidants are carotenoids and xanthophylls (Valadon and Mummery, 1978; Rothschild et al., 1986; Aucoin et al., 1990; Fields et al., 1990). These tetraterpenoids function as accessory pigments, photoprotectants, and antioxidants in photosynthetic tissues (Britton, 1993); as singlet oxygen quenchers and free radical scavengers (Burton and Ingold, 1984; Krinsky, 1989), carotenoids allow plants to cope with oxidative stress (Larson, 1988). Because light exposure presents oxidative challenges to herbivores as well as plants, carotenoids and xanthophylls also reduce phototoxicity in insects (Robertson and Beatson, 1985; Aucoin et al., 1990; Green and Berenbaum, 1994).

Lepidoptera do not synthesize carotenoids but rather acquire them directly from host tissues with little modification (Feltwell, 1978; Kayser, 1985). Among lepidoptera that consume phototoxic plants, most sequester lutein, the

predominant carotenoid in angiosperm photosynthetic tissues (Ahmad and Pardini, 1990; Britton, 1993). This selective sequestration allows lepidopteran specialists on phototoxic plants to reduce effects of phototoxins before and during photoactivation (Rothschild et al., 1986; Ahmad and Pardini, 1990).

One herbivore that encounters host plant phototoxins under a variety of light conditions is the parsnip webworm *Depressaria pastinacella*, an oligophagous caterpillar that feeds on the reproductive tissues of plants in the genera *Heraclium* and *Pastinaca* (Hodges, 1974; McKenna and Berenbaum, 2003). The interaction of the parsnip webworm with its host plants is mediated by furanocoumarins, phototoxic compounds activated by ultraviolet A (UVA) light (320–400 nm). Furanocoumarins cause damage in the presence of UVA by directly binding to DNA and proteins, and by forming reactive oxyradicals and free radicals that damage biologically sensitive molecules (Berenbaum, 1991). The parsnip webworm tolerates high concentrations of host plant furanocoumarins through rapid detoxification by cytochrome P450 monooxygenases (Nitao, 1989). In populations of the parsnip webworm and the wild parsnip *Pastinaca sativa*, its predominant host plant in the midwestern United States, furanocoumarin production by the plant and detoxification capabilities of the webworms are genetically variable traits subject to reciprocal selection pressures (Berenbaum et al., 1986; Berenbaum and Zangerl, 1992; Zangerl and Berenbaum, 1997). Parsnip webworms also cope with furanocoumarin phototoxicity by sequestering lutein and other xanthophylls from host plant tissues (Carroll et al., 1997). This selective sequestration of dietary lutein is associated with decreased behavioral avoidance of UVA light, presumably because of decreased oxidative stress from furanocoumarins (Carroll et al., 1997).

Although examined most intensively in the Midwest, the interaction between the parsnip webworm and its apiaceous host plants occurs throughout much of North America. The parsnip webworm was introduced to eastern North America about 150 yr ago and spread to disturbed habitats throughout eastern and northern North America (Riley, 1889; Berenbaum and Zangerl, 1991). It has since spread west and become established throughout the Pacific Northwest and the intermontane region of the western United States and southwestern Canada, although it occurs much more sporadically in the southern part of its range (Hodges, 1974; McKenna and Berenbaum, 2003).

Throughout much of its range, the parsnip webworm has acquired another host plant, a native cow parsnip, *Heraclium lanatum* (Berenbaum and Zangerl, 1991). In many western locales, the webworm utilizes the more abundant *H. lanatum* exclusively. Webworms feeding on *H. lanatum* fruits encounter concentrations of phototoxic furanocoumarins significantly different from webworms feeding on *P. sativa* fruits (Berenbaum and Zangerl, 1991; Zangerl and Berenbaum, 2003). Relative to eastern and midwestern congeners, western webworms encounter high intensities of photoactivating UVA light through the

southern half of their introduced range because of the occurrence of host plants at higher altitudes in lower latitudes (Young, 1985; Blumthaler et al., 1997). Through the combined effects of altitude and latitude on UVA light intensity, populations of *D. pastinacella* in western North America may vary more than twofold in experience of maximum UVA exposure along a UVA gradient from British Columbia to New Mexico.

The ability of webworms to act as selective agents on host plant chemistry may be affected not only by abiotic factors, such as UV intensity, but also by biotic factors, such as the presence of a third trophic level, e.g., a specialist parasitoid. Webworms in New Mexico, Utah, and southeastern Idaho are heavily attacked by the polyembryonic egg-larval parasitoid *Copidosoma sosares* Walker, an encyrtid wasp native to Europe that specializes on *Depressaria* spp. (Ode et al., 2004). *C. sosares* has not been previously observed in webworm populations in other regions of North America that lack webworm parasitoids of comparable impact (Ode et al., 2004). Significant rates of parasitism can lead to decreased selection pressure on host plant chemical defenses because of relaxation of herbivory (Price et al., 1980; but see Coleman et al., 1999). At the same time, host plant chemical defenses may negatively affect parasitoid fitness, especially if the parasitoid is more sensitive to the allelochemicals than its herbivore host (Reitz and Trumble, 1996; Ode et al., 2004; Ode, 2006). Dietary furanocoumarins can limit the size of polyembryonic parasitoid broods as well as the growth and development of individual parasitoid larvae in the host (Reitz and Trumble, 1996). Thus, host plants may experience selection for reduced chemical defense to increase parasitoid fitness (Turlings and Benrey, 1998; Ode et al., 2004).

Throughout its North American host range, the parsnip webworm has been introduced into a mosaic of different UVA light intensities, host plant furanocoumarin levels, and parasitoid infestation levels. In this study, we characterized the effects of geographically variable abiotic (UV) and biotic (parasitism) factors on the interaction between parsnip webworms and their apiaceous host plants. To quantify photochemical stresses encountered by webworms in these populations, we measured host plant furanocoumarin content. Because furanocoumarins are metabolically expensive to synthesize (Zangerl and Berenbaum, 1997) and because their toxicity is enhanced by photoactivating UVA, furanocoumarin content should be lower in populations that experience high UVA light intensities. We quantified individual linear and angular furanocoumarins separately in fruits because individual furanocoumarins differ in their contributions to resistance against the parsnip webworm (Berenbaum et al., 1986). Given the importance of metabolism in resistance to furanocoumarins (Nitao, 1989), we also compared the cytochrome-P450-mediated metabolism of furanocoumarins in webworms from different populations (Berenbaum et al., 1986; Berenbaum and Zangerl, 1998). Webworms experience higher mortality on host plant

TABLE 1. POPULATIONS OF *Depressaria pastinacella* COLLECTED IN WESTERN NORTH AMERICA, ARRANGED FROM NORTH TO SOUTH, WITH LATITUDE (N) AND LONGITUDE (W) LISTED BELOW EACH LOCATION

Population	Latitude/longitude	Host plant	No. of plants	Percentage attacked	Percentage parasitized
Hixon, BC (HXBC)	53°15.760/122°28.129	<i>H. lanatum</i>	52	77	0
Cypress Hills, AB (CHAB)	49°36.279/110°15.554	<i>H. lanatum</i>	132	42	0
Birch Bay, WA (BBWA)	48°87.807/122°46.232	<i>H. lanatum</i>	76	24	0
Lost Airport, WA (AIWA)	48°60.608/120°44.488	<i>H. lanatum</i>	34	100	0
Bumblebee Creek, ID (BUID)	47°69.439/116°14.182	<i>H. lanatum</i>	56	13	0
Route 152, ID (RTID)	47°48.525/115°58.799	<i>H. lanatum</i>	82	73	0
Shoshone Creek, ID (SHID)	47°45.303/115°58.706	<i>H. lanatum</i>	150+ est.	45 est.	0
Montpelier Canyon, ID (MCID)	42°19.851/111°14.003	<i>H. lanatum</i>	250+ est.	25 est.	97 (2000) 48 (2001)
Lower Paris, ID (LPID)	42°09.853/111°23.939	<i>P. sativa</i>	10,000+	40 est.	88 est. (2001)
Payson Lakes, UT (PLUT)	39°56.185/111°38.415	<i>H. lanatum</i>	1500 est.	40 est. 60 est.	60 est. (2001) 90 est. (2000)
Dalton Canyon, NM (DCNM)	35°46.519/105°41.622	<i>H. lanatum</i>	45	9	60 (2001)
Little Tesuque, NM (LTNM)	35°43.727/105°50.342	<i>H. lanatum</i>	134	69	3 (2001)
Big Tesuque, NM (BTNM)	35°39.523/105°48.641	<i>H. lanatum</i>	168	9	20 (2001)

Percent of plants attacked by *D. pastinacella* and webworms parasitized by the encyrtid *Copidosoma sosares* are given (for small populations) or estimated (for populations > 100). All the populations except for BBWA and SHID represent new locales for the parsnip webworm.

chemotypes that do not match their metabolic phenotype, presumably because of inefficient detoxification and subsequent accumulation of unmetabolized furanocoumarins (Berenbaum and Zangerl, 1998; Zangerl and Berenbaum, 2003). By examining populations with mismatched profiles, factors that vary predictably on a landscape scale can be identified that alter the efficacy of traits mediating the interaction (Thompson, 1999; Zangerl and Berenbaum, 2003).

Because UV irradiance also affects carotenoid production in plants (Jahnke, 1999), we measured lutein content of host plants across the

altitudinal/latitudinal gradient as well as lutein sequestration in sixth instar webworms from western populations experiencing different UV light regimes as a result of latitude and altitude. Arthropods can increase antioxidants and pigmentation in response to high UVA intensities (Vega and Pizarro, 2000; Borgeraas and Hessen, 2002). If parsnip webworms use dietary lutein to reduce oxidative stress associated with furanocoumarins, greater sequestration would be expected in western populations that experience high UVA radiation.

Finally, because parasitism by *C. sosares* constitutes a significant biotic factor that affects the chemical mediation of the interaction of western webworms and their host plants, we examined the effect of the presence of *C. sosares* on host plant furanocoumarin chemistry by comparing furanocoumarin content of fruits from populations with and without the parasitoid.

METHODS AND MATERIALS

Sampling of Insect and Plant Populations. We examined 13 populations of parsnip webworms and their host plants in western North America along a latitudinal and altitudinal gradient from 35.4°N to 53.2°N (Table 1; Figure 1). For one of these populations (LPID), *P. sativa* was the exclusive host, whereas *H. lanatum* was the exclusive host for the other 12 populations. In six populations in New Mexico, Utah, and southeastern Idaho, parsnip webworms were attacked by *C. sosares* (Table 1). These parasitoid populations represent a new record for *C. sosares* in North America (Ode, personal communication). Parasitism rates of webworms varied considerably among populations and years, ranging from 5% (Dalton Creek, NM) to over 90% (Payson Lakes, UT) of the sixth instars present, creating a mosaic of attacked and unattacked webworms across the North American range of this parasitoid (Table 1).

To minimize the degradation of lutein, samples for chemical analysis were collected directly onto powdered dry ice (Oliver and Palou, 2000). Half-filled fruits, which are regularly consumed by sixth instar webworms (Berenbaum et al., 1986), were sampled from host plants for analysis of furanocoumarins and lutein. Sixth instars collected for lutein quantification were separated from plant material and isolated for 2 hr to clear plant material from their guts before placement on powdered dry ice. Samples were kept in a -80°C freezer until extraction.

FIG. 1. Approximate locations of *Depressaria pastinacella* populations sampled in western North America. Populations are labeled by acronyms presented in Table 1 and the text. *Pastinaca sativa* is the only host plant present in the LPID population, whereas *Heracleum lanatum* is the only host plant found in all other populations.



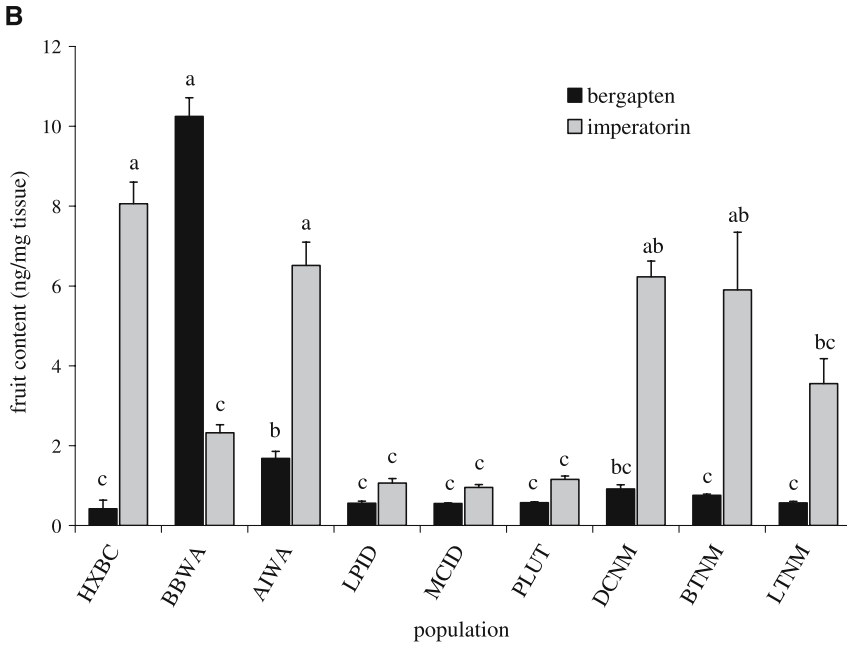
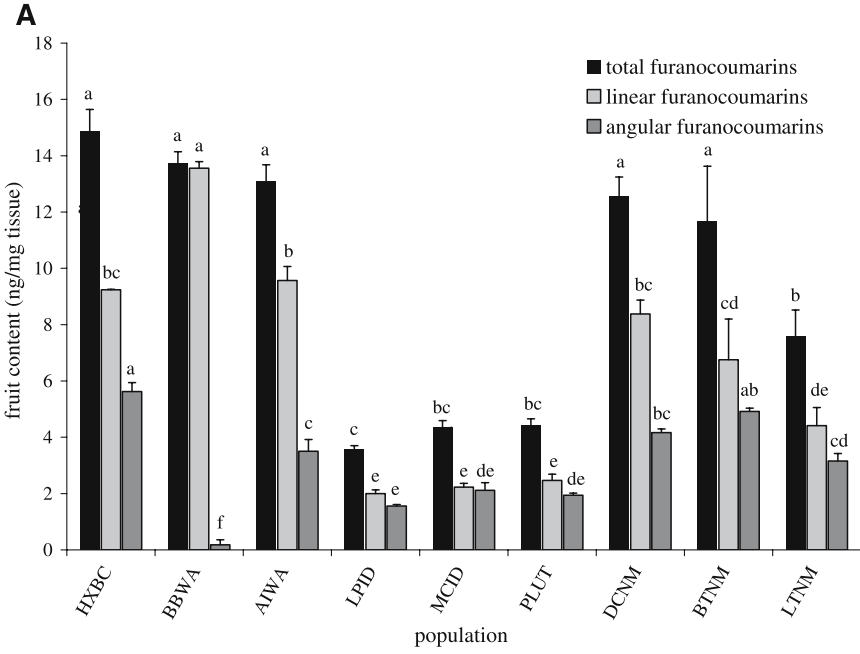
TABLE 2. ESTIMATION OF DAILY UV IRRADIANCE (DUV) FROM THE COMPARISON OF COLLECTION SITE CHARACTERISTICS (LATITUDE, ALTITUDE, COLLECTION DATE) WITH DATA FROM THE NATIONAL ULTRAVIOLET MONITORING CENTER (NUVMC) MONITORING STATION

Population	Latitude (N)	Altitude (m)	Date	Station	Station latitude	Δ Elevation (m)	DUV (J/cm^2)
HXBC	53°15.760	702	08/09/01	Olympic	48°51	196	4596
CHAB	49°36.279	1243	08/08/01	Glacier	48°51	267	4272
BBWA	48°87.807	2	07/02/00	Olympic	48°51	-6	4797
AIWA	48°60.608	805	07/02/00	Olympic	48°51	799	5218
SHID	47°45.303	1439	07/03/00	Glacier	48°51	463	6198
MCID	42°19.851	1911	07/18/00	RMNP	40°03	-985	6427
LPID	42°09.853	1829	07/18/00	RMNP	40°03	-1067	6363
PLUT	39°56.185	2404	07/14/01	Canyonlands	38°47	1106	8516
DCNM	35°46.519	2250	07/13/01	Albuquerque	35°09	635	8340
LTNM	35°43.727	2560	07/13/01	Albuquerque	35°09	945	8603
BTNM	35°39.523	3006	07/13/01	Albuquerque	35°09	1391	8983

The latitude and collection date from the population site are used to determine on which day the monitoring station has the same solar angle as the population site. The raw DUV value acquired for this day is corrected for the difference in elevation between the population and monitoring station sites by methods described in the text. The acronyms for population sites are described in Table 1. NUVMC monitoring stations: Glacier—Glacier National Park; Olympic—Olympic National Park; RMNP—Rocky Mountain National Park; Canyonlands—Canyonlands National Park; Albuquerque—Albuquerque.

Estimates of Daily UV Irradiance. To estimate daily UV exposure at a population site, we used measurements of total daily UV irradiance collected by the National Ultraviolet Monitoring Center (NUMC; University of Georgia and Environmental Protection Agency, 2003) at seven sites throughout North America (Table 2). At each site, the total daily UV irradiance was measured by a Brewer spectrophotometer as the total combined flux of solar UVA (320–360

FIG. 2. Tissue concentrations of (A) total furanocoumarins, linear furanocoumarins, and angular furanocoumarins; (B) the linear furanocoumarins bergapten and imperatorin; (C) the linear furanocoumarins isopimpinellin and xanthotoxin; and (D) the angular furanocoumarins angelicin and sphondin in half-filled fruits of *H. lanatum* and *P. sativa* from populations in western North America. Populations are labeled by acronyms presented in Table 1 and the text. *P. sativa* is the only host plant present in the LPID population, whereas *H. lanatum* is the only host plant found in all other populations. Content means for each class of furanocoumarin significantly differ across populations by one-way analysis of variance (ANOVA; $P < 0.05$). Populations that do not share a superscript letter have significantly different mean contents by Tukey's honestly significantly different (HSD) test ($P < 0.05$). Error bars indicate one standard error ($N = 18$ –20 for each population, 176 total).



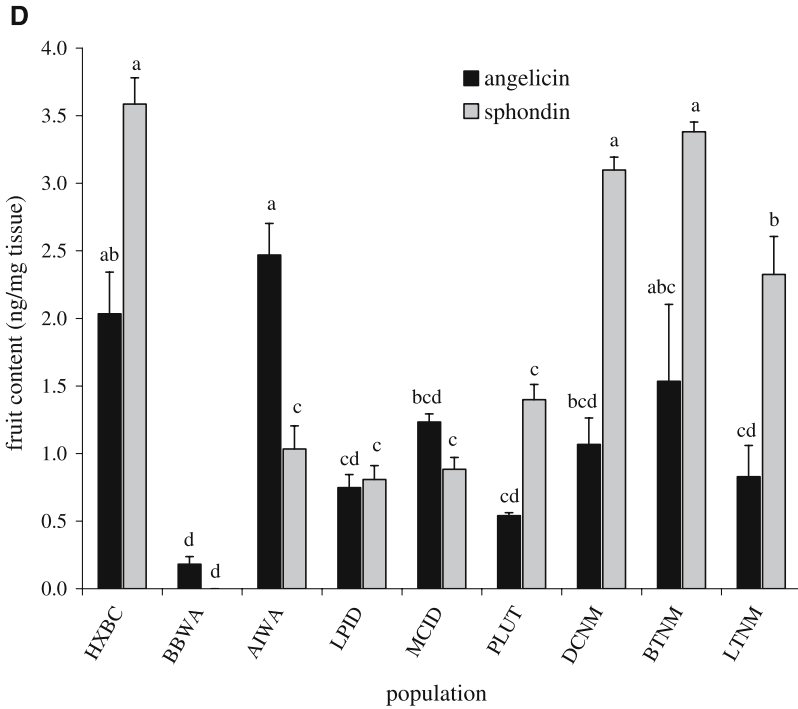
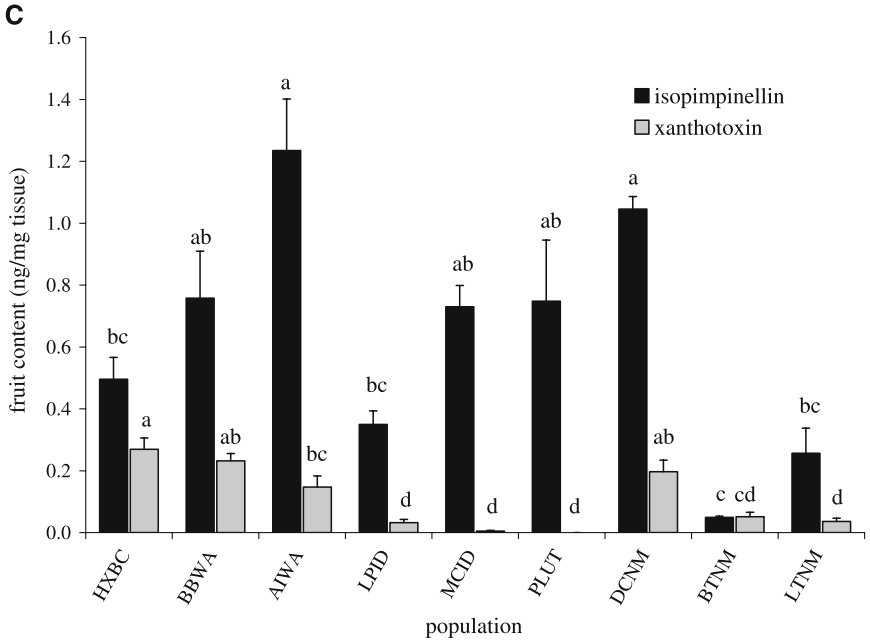


FIG. 2. (continued).

nm) and UVB irradiance (290–320 nm). To calculate daily UV irradiance by using the NUMC data, we had to compensate for differences in latitude and altitude between the sampling site and the monitoring station. The intensity of solar radiation varies with latitude on a given day, primarily because of differences in the angle of incidence between the sun and the horizon, as measured by the maximum solar angle at solar noon. However, sites at different latitudes may receive the same amount of UV irradiance on different days if they share the same maximum solar angle. To correct for differences in angle of incidence due to latitude, the maximum solar angle at solar noon was determined for the sampling site for the day of collection by using the site's latitude and longitude with a solar position calculator (NOAA, 2003). We then used the coordinates of the UV monitoring station and the solar position calculator to determine the precise day on which the UV monitoring station experienced the same maximum solar angle as the sampling site. Because UV irradiance is positively correlated with altitude due to a reduction in the amount of radiation intercepted or scattered by air molecules (Diffey, 1991; Schmucki and Philipona, 2002), the daily UV irradiance obtained from the NUMC data was corrected for differences in altitude as:

$$(\text{daily UV irradiance}) \times (1 + (\Delta \text{ altitude})(\text{altitude effect}))$$

where Δ altitude is [(altitude (sampling site) – altitude (UV monitoring site))] and the altitude effect, or change in the intensity of radiation with altitude, is 0.000107 per meter based on the studies conducted during the summer with recording stations over a wide range of elevation in midlatitude mountains (Blumthaler et al., 1997; Schmucki and Philipona, 2002).

Furanocoumarin Analysis of Plant Samples. *H. lanatum* and *P. sativa* furanocoumarins were quantified as in Berenbaum and Zangerl (1992). Freeze-dried fruits were weighed, crushed in an Eppendorf tube with a micropestle, and extracted with 1 ml ethyl acetate. Normal-phase liquid chromatography (HPLC) was performed on a Waters high-pressure liquid chromatograph with a Model 440 absorbance (254 nm) detector. Ten microliters of each diluted sample were auto-injected and separated on an Alltech Absorbosphere Silica 5U (150 × 4.6 mm) with a 55:32:3 cyclohexane/isopropyl ether/butanol eluent mixture. Furanocoumarins were quantified by comparison of the integrated peaks against a furanocoumarin standard containing known quantities of bergapten (Sigma Inc., St. Louis, MO, USA), xanthotoxin (Sigma Inc., St. Louis, MO, USA), imperatorin (Serva, Heidelberg, Germany), isopimpinellin (Indofine, Belle Mead, NJ, USA), angelicin (Sigma Inc., St. Louis, MO, USA), and sphondin (gift of Dr. W. Wulff, University of Chicago). Sample size (number of plants) varied from 18 to 20, depending on the number of intact half-filled fruits available from each population.

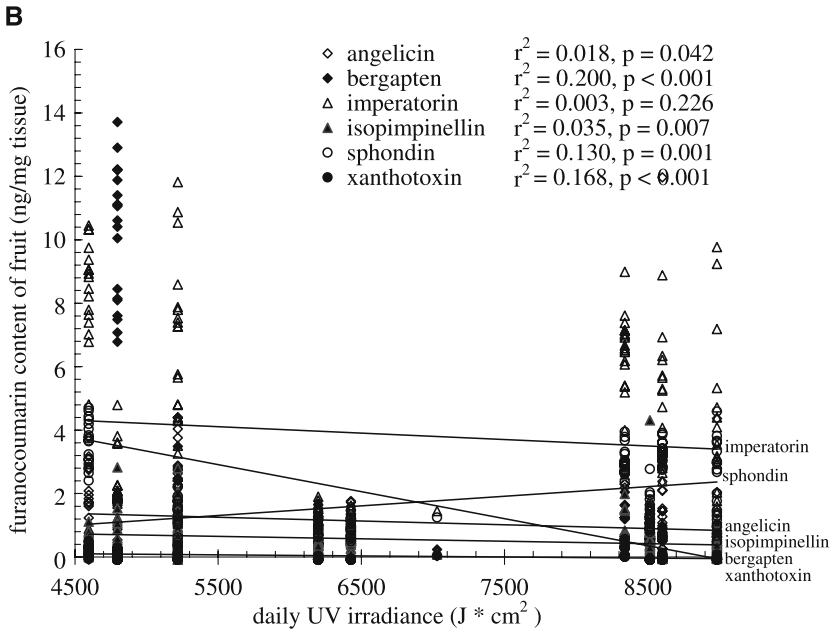
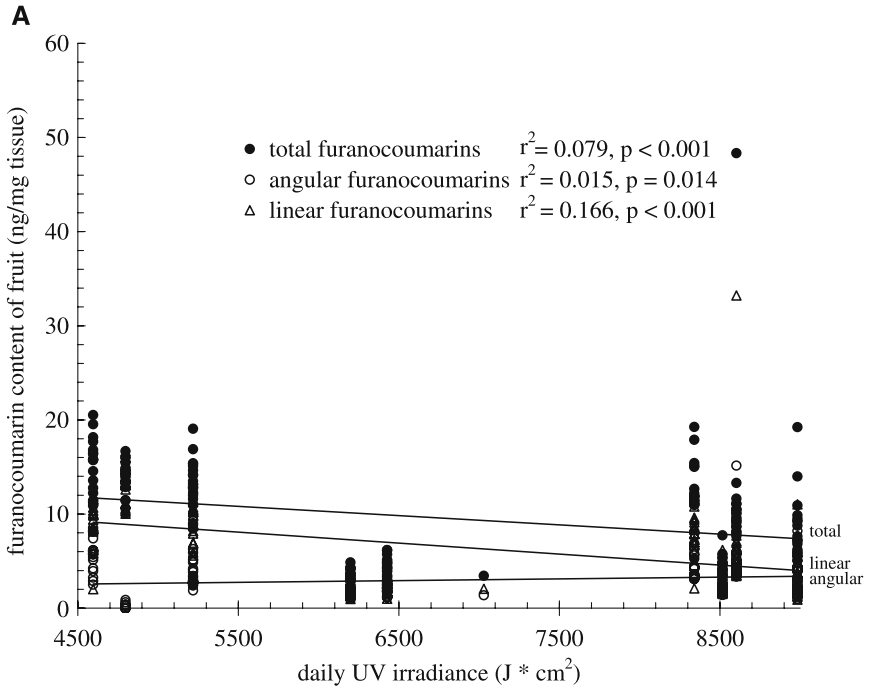


TABLE 3. DIFFERENCES IN FURANOCOUMARIN CONTENT OF *Heracleum lanatum* FRUITS FROM POPULATIONS WHERE THE WEBWORM PARASITOID *C. sosares* WAS PRESENT AND FRUITS FROM POPULATIONS WHERE THIS PARASITOID WAS ABSENT

Furanocoumarin	Concentration ($\mu\text{g}/\text{mg}$ dry mass)		<i>t</i> value	<i>df</i>	<i>P</i> value
	Population				
	<i>C. sosares</i> absent	<i>C. sosares</i> present			
Angelicin	1.546 (0.182)	1.043 (0.133)	2.235	114.906	0.027
Bergapten	4.243 (0.605)	0.670 (0.027)	5.898	57.223	<0.001
Imperatorin	5.549 (0.417)	3.531 (0.395)	3.512	139.221	0.001
Isopimpinellin	0.841 (0.089)	0.564 (0.629)	2.548	111.873	0.012
Sphondin	1.469 (0.213)	2.208 (0.117)	-3.032	91.751	0.003
Xanthotoxin	0.214 (0.020)	0.057 (0.011)	7.024	90.846	<0.001
Linear furanocoumarins	10.848 (0.382)	4.822 (0.411)	10.745	148.520	<0.001
Angular furanocoumarins	3.015 (0.335)	3.250 (0.192)	-0.608	94.684	0.544
Total furanocoumarins	13.863 (0.353)	8.072 (0.577)	8.563	148.024	<0.001

Furanocoumarin means are compared by an independent samples *t* test. Standard error is given in parentheses. Degrees of freedom (*df*) are corrected in each test because equal variances of samples are not assumed. Sample size is 58 from populations where *C. sosares* is present and 98 from populations where *C. sosares* is absent.

Furanocoumarin Metabolism in Parsnip Webworms from Different Populations. Because dietary exposure to plant components affects larval P450 enzyme activity, field-caught webworms were reared to adulthood and mated to obtain offspring reared under common environmental conditions. Parsnip webworms collected as late instar larvae or pupae were allowed to emerge as adults, then subjected to photoperiod and temperature parameters simulating winter conditions to break reproductive diapause (Nitao and Berenbaum, 1988). "Overwintered" adults were caged with wild parsnip leaves at 20°C and 16:8 light/dark photoperiod to allow for mating and oviposition. Neonates were reared to sixth instar on a semidefined artificial diet that lacks a significant carotenoid or furanocoumarin source (Carroll et al., 1997). Five of

FIG. 3. Two-dimensional plot of furanocoumarin concentrations of *H. lanatum* half-filled fruits against estimated daily ultraviolet (UV) irradiance for (A) linear, angular, and total furanocoumarins as well as (B) the individual furanocoumarins angelicin, bergapten, imperatorin, isopimpinellin, sphondin, and xanthotoxin. Univariate regression analysis was conducted for combined linear (bergapten, imperatorin, isopimpinellin, and xanthotoxin), combined angular (angelicin and sphondin), and total furanocoumarins (all six furanocoumarins) as well as the individual compounds. For each compound, a best-fit regression line has been added. The r^2 value and *P* value from the linear regression model are presented above each regression line ($N = 176$).

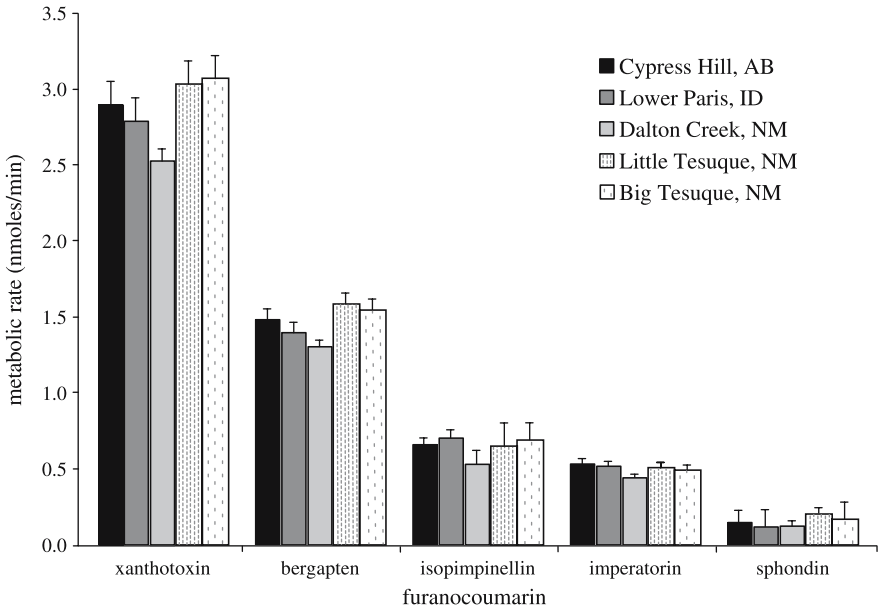


FIG. 4. Whole midgut metabolism rates for five furanocoumarins (xanthotoxin, bergapten, isopimpinellin, imperatorin, and sphondin) in sixth instar *D. pastinacella* from five populations in western North America. No significant differences were observed among population means for the metabolism of any furanocoumarin by a one-way ANOVA ($P < 0.05$). Error bars indicate one standard error. Sample size varied from 17 to 49, depending on the availability of sixth instars reared from parents collected in each population (LTNM—Little Tesuque, NM; DCNM—Dalton Creek Canyon, NM; BTNM—Big Tesuque, NM; LPID—Lower Paris, ID; CHAB—Cypress Hills, AB).

the 13 populations (Little Tesuque, NM; Big Tesuque, NM; Dalton Creek Canyon, NM; Lower Paris/Bear Lake, ID; Cypress Hills, Alberta) from which webworms were collected produced sufficient number of larvae to be used in metabolism bioassays. Sample size varied from 17 to 49, depending on the availability of sixth instars reared from parents collected in each population.

Midgut metabolism rates for five furanocoumarins (xanthotoxin, bergapten, isopimpinellin, imperatorin, and sphondin) in sixth instars were compared among populations according to Berenbaum and Zangerl (1992). Whole midguts were dissected, rinsed, and homogenized with a Model 985-370 Tissue Tearor (Biospec Products, Bartlesville, OK, USA). A portion of the homogenate (70 μ l) was added to 1 ml phosphate reaction buffer (pH 7.8) containing a mixture of xanthotoxin, bergapten, isopimpinellin, imperatorin, and sphondin. The reactions were run for 15 min in a 30°C water bath and terminated by exposure to an 85°C water bath for 5 min. Unmetabolized furanocoumarins were extracted

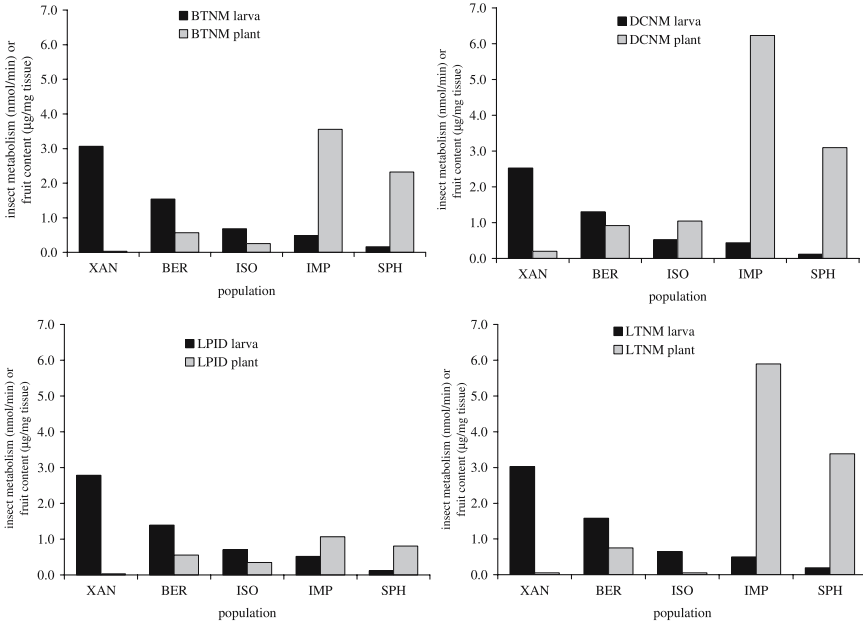
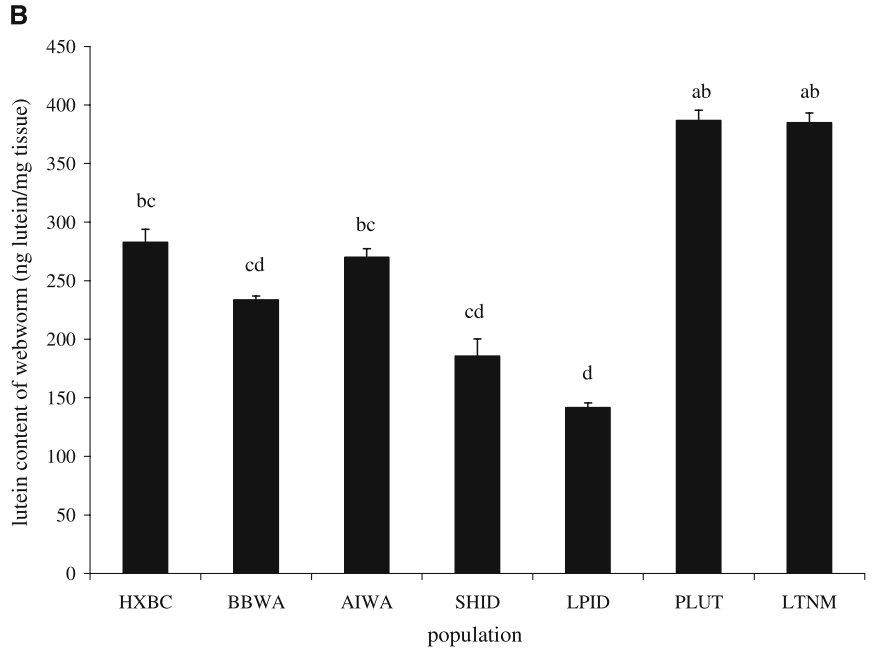
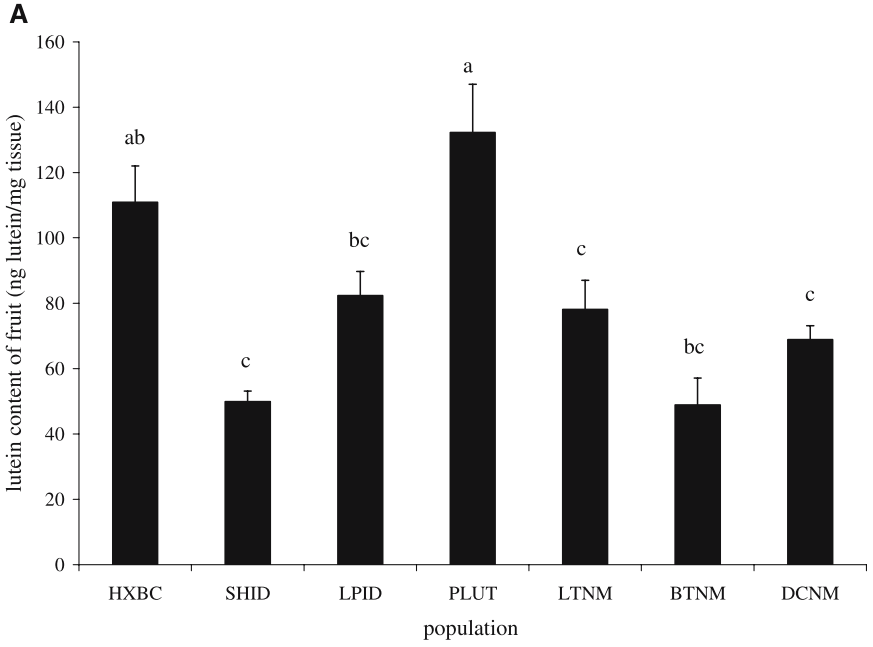


FIG. 5. Host plant furanocoumarin concentrations of half-filled fruits and whole midgut furanocoumarin metabolism rates of sixth instar *D. pastinacella* from the BTNM, DCNM, LTNM, and LPID populations in western North America. On the y-axis, the units for metabolism apply to the webworms, whereas the units for fruit content apply to the host plants. *P. sativa* is the only host plant present in the LPID population, whereas *H. lanatum* is the only host plant in all other populations. Furanocoumarin content for half-filled fruits and webworm midgut metabolic rates appear mismatched in all populations (LTNM—Little Tesuque, NM; DCNM—Dalton Creek Canyon, NM; BTNM—Big Tesuque, NM; LPID—Lower Paris, ID).

with ethyl acetate and analyzed by normal-phase HPLC (Berenbaum and Zangerl, 1992). The amount of each furanocoumarin metabolized in each reaction was determined by comparison against time-zero controls.

Lutein Analysis of Plant Samples. To quantify lutein in parsnips from different populations, plant material was extracted according to a protocol modified after Riso and Porrini (1997) and O’Neil and Schwartz (1992). All samples were kept frozen on dry ice and extracted under minimal light conditions to reduce degradation of lutein because of oxygen, light, and heat. Frozen plant material was weighed and crushed to a powder in liquid nitrogen with a mortar and pestle. Because of the sensitivity of plant material to lutein degradation, samples were not dried to obtain a direct measure of sample dry mass; instead, a subsample of the powdered plant sample was weighed,



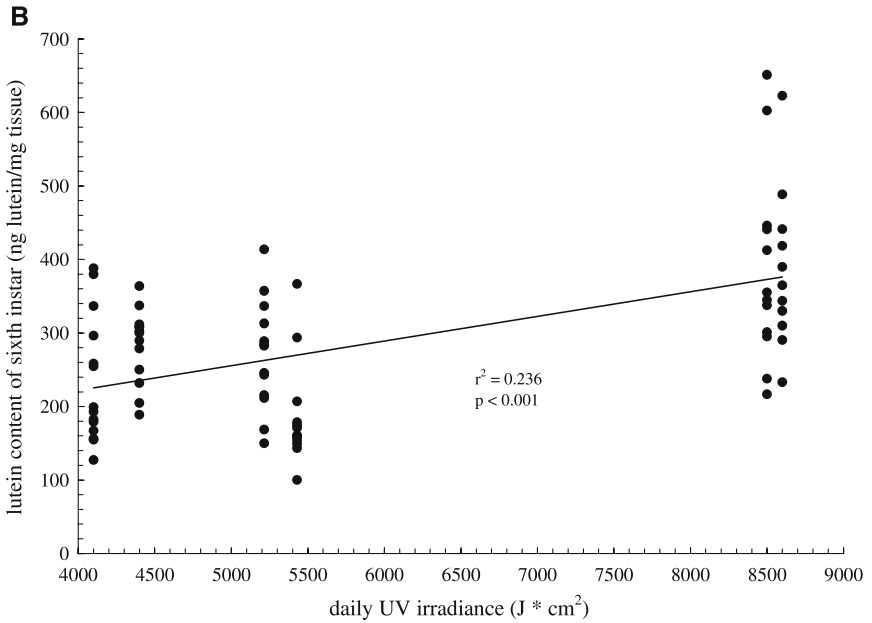
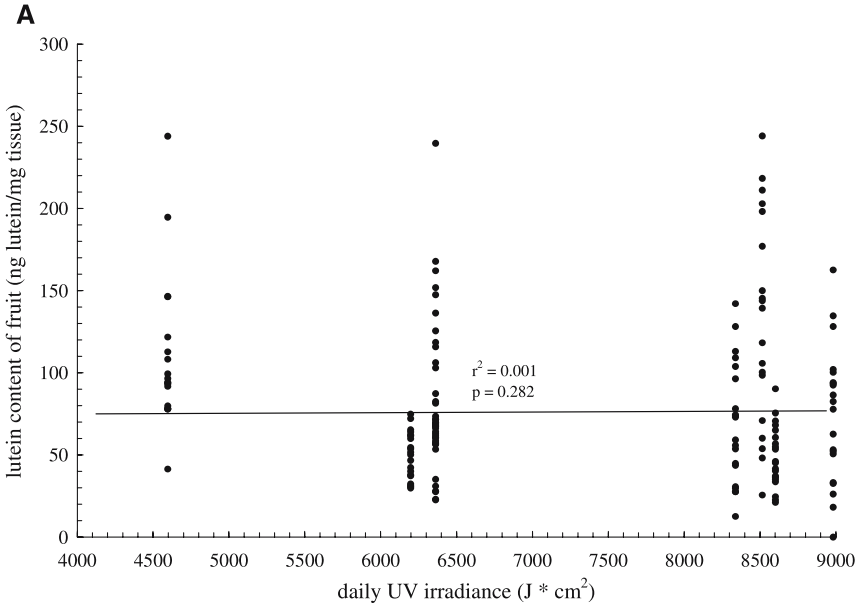
removed, and freeze-dried for later calculation of a wet mass–dry mass conversion factor. Approximately 200 mg of powdered material were placed in a preweighed Eppendorf tube, weighed, and extracted with 1 ml methanol containing 0.1% of the antioxidant butylhydroxytoluene (BHT) (Oliver and Palou, 2000). The samples were vortexed and sonicated in a Model 250 Ultrasonic Cleaner (RAI Research) for 2 min and then placed on ice for 30 min under a nitrogen atmosphere to maximize the extraction of lutein while minimizing oxidation. The sample mixture was then microcentrifuged for 5 min at 14,000 rpm at 5°C. Samples were stored in the dark at –80°C until separation by reverse-phase HPLC. Sample size varied from 19 to 39 for each population.

Reverse-phase HPLC was performed with a Waters 996 photodiode array (PDA) detector with detection of xanthophylls at 445 nm. Separation of lutein from other xanthophylls was improved by the use of a Vydac 201TP54 C₁₈ column (Yeum et al., 1996). Twenty microliters of each sample were auto-injected and separated with a 90:10 methanol/acetonitrile eluent mixture containing 0.01% BHT and 0.05% of the solvent modifier triethylamine (Oliver and Palou, 2000). Three-dimensional UV–VIS spectral data were captured, analyzed, and quantified with Millenium³² System software. Lutein was identified and quantified by comparison of an integrated peak against a purified lutein standard generously supplied by Kemin, Inc. (Des Moines, IA, USA).

Lutein Analysis of Insect Samples. To extract lutein from webworms, preweighed whole frozen webworms were crushed to a powder in liquid nitrogen in a 2.0-ml Eppendorf tube with a micropestle. The powdered remains were extracted with 1 ml methanol containing 0.1% BHT, vortexed, sonicated for 2 min, and placed on ice for 30 min under a nitrogen atmosphere. The sample mixture was then microcentrifuged for 8 min at 14,000 rpm at 5°C to separate solid remains and refractory lipids from the lutein extract. Lutein was isolated by HPLC as described. Sample size varied from 11 to 14, depending on the availability of sixth instars from each population.

Statistical Analysis. For the half-filled fruits, amounts of total furanocoumarin, total linear furanocoumarin, and total angular furanocoumarin, as well as individual furanocoumarins and lutein concentrations, were compared among

FIG. 6. Lutein content of (A) half-filled fruits of *P. sativa* (LPID) and *H. lanatum* (all other populations) and (B) sixth instar *D. pastinacella* from populations in western North America. Populations are labeled by acronyms presented in Table 1 and are arranged left to right from north to south. *P. sativa* is the only host plant present in the LPID population, whereas *H. lanatum* is the only host plant in all other populations. Lutein content differs significantly across populations by a one-way ANOVA ($P < 0.05$). Populations that do not share a superscript letter have significantly different mean contents by Tukey's HSD test ($P < 0.05$). Sample size for each population varied from 19 to 39 for fruits and 11 to 14. Error bars indicate one standard error.



populations by one-way analysis of variance (ANOVA). Insect lutein and whole midgut metabolic rates (for each furanocoumarin) were also compared among populations by one-way ANOVA. Treatment means were compared among individual populations by Tukey's honestly significantly different test (SPSS, 1999). Univariate regression analysis was conducted to determine the effect of daily UV irradiance on plant and insect chemistry (SPSS, 1999).

To determine the effect of the presence of the parasitoid *C. sosares* on host plant chemistry, we compared the furanocoumarin concentration of cow parsnips from populations where the parasitoid was present with cow parsnips from populations where the parasitoid was absent by independent sample *t* tests (SPSS, 1999). Separate tests were conducted for total furanocoumarins, linear furanocoumarins, and angular furanocoumarins, as well as each individual furanocoumarin.

To evaluate the match between host plant furanocoumarin production and webworm metabolic capabilities within a population, we examined the interaction between furanocoumarins (host plant furanocoumarins and midgut metabolism capability) and organism (wild parsnip or parsnip webworm) as compared by repeated-measures multivariate analysis of variance (MANOVA), with furanocoumarins as the between-subject factor and organism as the within-subject factor (SPSS, 1999). Mean levels of furanocoumarin production by plants and midgut metabolism by webworms were compared separately for each population.

RESULTS

Furanocoumarin Analysis of Plant Samples. Significant differences were observed among populations for all measures of half-filled fruit furanocoumarin concentration (Figure 2, one-way ANOVA). Both total furanocoumarins and total linear furanocoumarins of fruits were negatively correlated with daily UV irradiance ($r^2 = 0.079$ and 0.166 , respectively, $P < 0.001$; regression analysis), whereas total angular furanocoumarin content of fruits was not correlated with UV light exposure ($r^2 = 0.015$, $P = 0.054$; regression analysis; Figure 3). Among the individual furanocoumarins in fruits, angelicin ($r^2 = 0.018$, $P = 0.042$), bergapten ($r^2 = 0.200$, $P < 0.001$), isopimpinellin ($r^2 = 0.035$, $P = 0.007$), and

FIG. 7. Two-dimensional plot of lutein concentrations of (A) *H. lanatum* half-filled fruits and (B) sixth instar *D. pastinacella* against estimated daily UV irradiance for the host plant's population. A regression line has been added, with the r^2 value and P value from the linear regression model presented above the line. Linear regression models indicate that daily UV irradiance is a good predictor of webworm, but not fruit, lutein content ($N = 155$ for fruits and 75 for webworms).

xanthotoxin ($r^2 = 0.168$, $P < 0.001$) were negatively correlated with daily UV irradiance, whereas sphondin ($r^2 = 0.130$, $P < 0.001$) was positively correlated with daily UV irradiance (Figure 3). Imperatorin ($r^2 = 0.003$, $P = 0.226$) was not significantly correlated with daily UV irradiance (Figure 3).

The furanocoumarin concentration of fruits from populations with and without *C. sosares* differed significantly for all individual furanocoumarins tested (Table 3). Fruits from populations with *C. sosares* present had lower concentrations of total furanocoumarins and linear furanocoumarins than fruits from populations where *C. sosares* was absent ($P < 0.05$, independent samples *t* test; Table 3). Total angular furanocoumarins did not differ in the presence or absence of *C. sosares* ($P > 0.05$, independent samples *t* test; Table 3). Among the individual furanocoumarins, angelicin, bergapten, imperatorin, isopimpinellin, and xanthotoxin were lower and sphondin was higher in *H. lanatum* populations where *C. sosares* was present ($P < 0.05$, independent samples *t* test; Table 3).

Furanocoumarin Metabolism in Parsnip Webworms from Different Populations. Despite the disparate geographical origins of the five webworm populations, the midgut metabolic rates for all five furanocoumarins did not differ among sixth instars ($P < 0.05$; one-way ANOVA; Figure 4). For four of these populations, fruit concentrations and insect metabolism rates were compared for five individual furanocoumarins. In all four, a significant interaction between furanocoumarin and organism type occurred in a comparison of fruit concentration and midgut metabolism [Pillai's Trace = 0.901 (BTNM), 0.915 (DCNM), 0.936 (LPID), and 0.958 (LTNM); $P < 0.001$; MANOVA], indicating that the relative amount of individual furanocoumarins metabolized by insects differed from the plant furanocoumarin profile (Figure 5). In particular, webworm capacity for metabolizing xanthotoxin proportionally exceeded the relatively low production of xanthotoxin in fruits, whereas webworm capacity for metabolizing imperatorin and sphondin was disproportionately less than the relative amounts of these two furanocoumarins in fruits.

Lutein Analysis of Plant and Insect Samples. Although mean lutein concentration of fruits varied among populations ($P < 0.05$, one-way ANOVA; Figure 6), daily UV irradiance was not a good predictor of fruit lutein ($r^2 = 0.001$, $P = 0.282$; regression analysis; Figure 7). By contrast, webworms from populations exposed to more intense daily UV irradiance had higher lutein ($r^2 = 0.236$, $P < 0.001$; regression analysis; Figure 7), with significant differences observed across the populations ($P < 0.05$, one-way ANOVA; Figure 6).

DISCUSSION

At high altitudes, *H. lanatum* produces less total furanocoumarin and linear furanocoumarins but not angular furanocoumarins in half-filled fruits than

plants under less intense UV light regimes at lower altitudes. *H. lanatum* from populations in the central and southwestern United States (New Mexico, Utah, and southern Idaho) produced more sphondin (range 0.9–3.5 $\mu\text{g}/\text{mg}$) and less xanthotoxin (range 0.0–0.2 $\mu\text{g}/\text{mg}$) than cow parsnips from the Midwest (0.4 $\mu\text{g}/\text{mg}$ sphondin and 1.1 $\mu\text{g}/\text{mg}$ xanthotoxin; Zangerl and Berenbaum, 2003). A similar pattern pertains to *P. sativa*: western wild parsnip populations from Lower Paris, ID, have higher fruit content of sphondin (0.9 $\mu\text{g}/\text{mg}$) and lower content of bergapten (0.6 $\mu\text{g}/\text{mg}$) and xanthotoxin (0.03 $\mu\text{g}/\text{mg}$) than parsnips from the Midwest (0.3 $\mu\text{g}/\text{mg}$ sphondin, 1.9 $\mu\text{g}/\text{mg}$ bergapten, and 4.0 $\mu\text{g}/\text{mg}$ xanthotoxin; Zangerl and Berenbaum, 2003) or the Netherlands (0.5 $\mu\text{g}/\text{mg}$ sphondin, 2.2 $\mu\text{g}/\text{mg}$ bergapten, and 2.7 $\mu\text{g}/\text{mg}$ xanthotoxin; Ode et al., 2004).

The differences between linear and angular furanocoumarins under high UV intensities may be partially explained by the relative importance of photoactivation to overall toxicity. Because linear furanocoumarins form diadducts with pyrimidine bases in the presence of photoactivating UVA light, their phototoxicity is greatly enhanced relative to their dark toxicity (Berenbaum, 1991). Most of the toxic effects of linear furanocoumarins against insect herbivores are attributed to photogenotoxicity rather than photooxidation (Berenbaum, 1991). In contrast, angular furanocoumarins form only monoadducts with pyrimidine bases because of steric inhibition, resulting in a more modest increase in phototoxicity over their dark toxicity and a greater reliance on photooxidation overall. In addition, the efficacy of furanocoumarin photogenotoxicity increases under lower oxygen partial pressures (Bianchi et al., 1996), such as those experienced at high altitudes. Because enhancement of linear furanocoumarin toxicity by UVA is proportionally greater and more dependent on photogenotoxicity, increases in UVA intensity at high altitudes should improve the efficacy of linear furanocoumarins more than angular furanocoumarins. Under consistently greater UVA light intensities experienced, proportionately fewer linear furanocoumarins yield equivalent toxicity toward herbivores. In *P. sativa*, furanocoumarin biosynthesis has a significant cost in terms of reproductive fitness (Zangerl and Berenbaum, 1997). If similar costs occur in *H. lanatum*, a reduction in linear furanocoumarin production could allow plants to shift resources to more costly production of angular furanocoumarins (Berenbaum et al., 1986; Zangerl and Berenbaum, 1997), resulting in the increase in sphondin observed here.

Decreased production of phototoxic allelochemicals in plants under high light intensities has also been observed with benzyloisoquinoline alkaloids, compounds activated in the near UV spectrum. Larson et al. (1991) compared the production of phototoxic isoquinoline alkaloids in the alpine columbine *Aquilegia caerulea* and the low-elevation columbine *A. canadensis* under ambient and elevated UVB radiation. Alkaloid concentrations in *A. caerulea* under elevated UVB were significantly lower than *A. canadensis* or *A. caerulea*

under ambient UVB, an effect the authors attributed to increased UVB-mediated destruction of the alkaloids; the possibility that reduced production may represent an adaptation to increased efficacy of chemical defenses under higher light intensities was not considered. Although isoquinoline alkaloids are photoactivated by near UV rather than UVB radiation (Larson et al., 1991), increases in near UV and UVB occur concomitantly under natural light conditions.

Previous studies of allelochemical variation along altitudinal and latitudinal transects have found a general trend toward lower concentrations of allelochemicals at high altitudes and latitudes, which has usually been attributed to reduced herbivory (Salmore and Hunter, 2001, but see Koptur, 1985 and Preszler and Boecklen, 1996). It is unlikely that differences in insect herbivory account for the results obtained in our study; in our limited survey over 2 yr, rates of attack by *D. pastinacella* in infested populations of *H. lanatum* did not vary consistently with UV intensity, altitude, or latitude. However, differences in furanocoumarin concentrations across populations may have arisen in response to selection pressures from other herbivores, including deer, tule elk, moose, black bear, and grizzly bear, as well as domesticated cattle (McMillan, 1953; Hamer et al., 1991; Holcroft and Herrero, 1991; Ralph and Pfister, 1992; Gogan and Barrett, 1995; Ramcharita, 2000).

An alternative and not mutually exclusive explanation for the differences in host plant furanocoumarins is the impact of the parasitoid *C. sosares*, which occurred in populations that experienced high ambient UV intensity at high altitudes. Compared to populations from western North America that lack a major parasitoid, plant populations associated with webworms parasitized by *C. sosares* display furanocoumarin profiles consistent with increased fitness of the parasitoid (Ode et al., 2004). In the Netherlands, the likelihood of parasitoid attack was negatively correlated with host plant isopimpinellin content, and the survivorship of all male and mixed-sex broods was negatively correlated with xanthotoxin content. Parsnips from the Netherlands had lower content of all linear furanocoumarins, including isopimpinellin and xanthotoxin, relative to parsnips from the midwestern United States, where *C. sosares* is absent; lower furanocoumarin content may be a response to selection for greater parasitoid fitness. In our survey of western *H. lanatum*, concentrations of all furanocoumarins except sphondin were lower in fruits from populations where *C. sosares* was present than in fruits from populations attacked by webworms in the absence of parasitoids. The furanocoumarin chemistry of host plants from these populations appears more conducive to the survival of the parasitoid and may have facilitated its ability to adapt to the parsnip webworm's host plant switch to the novel North American host plant *H. lanatum*.

The impact of *C. sosares* on host plant chemistry due to increased webworm mortality may be limited, because the host completes larval feeding before it is killed by the parasitoid (Ode et al., 2004); rather, *C. sosares* could

indirectly exert strong selection pressure on the host plant through changes in host physiology and behavior (Turlings and Benrey, 1998; Coleman et al., 1999). Koinobiont parasitoids can alter the foraging behavior (Karban and Englishloeb, 1997), developmental time (Harvey et al., 1999), consumption rates (Rahman, 1970; Slansky, 1978; Coleman et al., 1999), larval performance (Rahman, 1970; Coleman et al., 1999), and metabolism (Rahbé et al., 2002) of their hosts, all of which can be components of herbivore selection pressures on host plant chemistry.

That furanocoumarin metabolism rates did not vary among webworms from different populations in western North America contrasts with previous findings (Berenbaum and Zangerl, 1998; Zangerl and Berenbaum, 2003) that metabolic rates of midwestern webworms on wild parsnip differ across distances as small as 1 km. In these midwestern populations, phenotype mismatching increased with the presence of an alternative host plant (Zangerl and Berenbaum, 2003). We found significant overall differences between metabolic capabilities and host plant furanocoumarins in four western webworm populations despite the fact that only one host species was present. If anything, western webworm metabolic profiles resemble the furanocoumarin profiles of wild parsnip plants from the Midwest more closely than the host plants actually utilized by these caterpillars (Berenbaum and Zangerl, 1998; Zangerl and Berenbaum, 2003). These western webworms display metabolism rates near the maximum rates reported for webworms in the Midwest (Table 4; Berenbaum and Zangerl, 1998; Zangerl and Berenbaum, 2003); these rates may represent the maximum given the genetic variation present in North America.

If limits on P450 metabolism as a means of resistance against furanocoumarins exist, webworms from the southwestern populations may have other mechanisms that reduce the efficacy of host plant furanocoumarin defenses. The patterns of lutein sequestration among populations of *D. pastinacella* from western North America are consistent with the idea that webworms offset the stress of furanocoumarin toxicity by greater incorporation of dietary lutein under high UVA intensities, despite an absence of differences in host plant lutein concentrations. By sequestering carotenoid pigments from their host plants, parsnip webworms may extend the amount of time they remain exposed to UV light. Although exposure to photoactivating light can damage a specialist herbivore on phototoxic plants, complete light avoidance during foraging has costs in terms of reduced internal temperatures and increased developmental time (Rawlins and Lederhouse, 1981; Ali et al., 1990). In addition, increased pigmentation in itself can significantly improve absorption of solar radiation and raise internal body temperatures during foraging, resulting in faster development (Price et al., 1980; Goulson, 1994).

Increased pigmentation is a common characteristic in montane populations, where stress from high UV intensities, suboptimal foraging temperatures, and

TABLE 4. COMPARISON OF MIDGUT METABOLISM RATES OF FURANOCOUMARINS BY SIXTH INSTARS FROM POPULATIONS IN THE WESTERN AND MIDWESTERN UNITED STATES

Host plants present	Furanocoumarin metabolism rate (nmol/min)				
	Carroll and Berenbaum (2006)		Zangerl and Berenbaum (2003)		Berenbaum and Zangerl (1998)
	Geographic location (Population number)		<i>P. sativa</i> and <i>H. lanatum</i>		<i>P. sativa</i>
	<i>P. sativa</i>	<i>H. lanatum</i>	<i>P. sativa</i>	<i>H. lanatum</i>	<i>P. sativa</i>
	ID (1)	ID, NM, UT (3)	IL, WI (16)	WI (4)	IL, MN (4)
Furanocoumarin					
Bergapten	1.40	1.31–1.58	0.75–1.07	0.93–1.29	0.31–0.96
Imperatorin	0.52	0.44–0.53	0.27–0.43	0.20–0.44	n/a
Isopimpinellin	0.70	0.53–0.68	0.31–0.49	0.47–0.72	0.05–0.13
Sphondin	0.12	0.12–0.20	0.07–0.12	0.02–0.19	0.12–0.17
Xanthotoxin	2.79	2.53–3.07	1.28–2.43	1.68–2.13	0.57–1.72

The average furanocoumarin metabolism rates for each study are reported along with the geographic location (by state) and number of the populations covered by the study. If metabolism rates were quantified for more than one population in the study, a range of population means for furanocoumarin metabolism rates is presented. Because the presence of alternative host plants has a significant effect on the average metabolism rates in a population, metabolism rates are subdivided into categories by the webworm host plants (*H. lanatum* or *Pastinaca sativa*) present in or near the source populations.

the risk of oxidative damage from freezing are generally higher than at lower elevations (Bidigare et al., 1993; Jung et al., 1998). Conversely, pigmentation can increase convective cooling losses in microhabitats that experience wind and reduced irradiation, thereby reducing the chance of overheating in direct sunlight (Willemsen and Hailey, 1999). In summer, the montane habitat of the parsnip webworm in the Southwest is dominated by the North American monsoon, which results in frequent formation of cloud cover and thunderstorms in foothills and mountains (Adams and Comrie, 1997). Increased pigmentation by lutein sequestration may allow for thermal heating during sunny intervals while reducing stress related to furanocoumarin phototoxicity.

The higher rates of lutein sequestration observed in the montane populations may also be a result of the enhancement of carotenoid antioxidant and photoprotectant activities in the low oxygen partial pressures (pO_2) experienced at these altitudes. The ability of carotenoids to ameliorate both photogenotoxicity and photooxidation mechanisms is dependent on the ambient pO_2 , with loss of function occurring in heavily oxygenated tissues (Burton and Ingold, 1984; Palozza et al., 1997; Bianchi et al., 1996; Eichler et al., 2002). Because of the high efficiency of the insect tracheal respiratory system, where pO_2 declines

only slightly from the external atmosphere to the smaller tracheoles that penetrate tissues (Tenney, 1985; Timmins et al., 1999), the efficacy of lutein as an antioxidant in webworm tissues is largely dependent on the pO_2 of the surrounding atmosphere. At the altitudes of the New Mexico and Utah populations (above 2200 m), the partial pressure of oxygen is at least 25% lower than at sea level. The pO_2 of anoxic internal cavities that encounter high concentrations of furanocoumarins, such as the midgut lumen (Johnson and Barbehenn, 2000), would be even lower in these high altitude populations and, therefore, even more amenable to antioxidant and antigenotoxicity activities. Enhanced sequestration of lutein by the webworm at high altitudes under intense UV light is consistent with such physiological functions.

If dietary lutein serves as a means of reducing furanocoumarin toxicity, sequestration represents an exploitation of primary plant compounds that cannot be readily altered because of their critical roles in photosynthesis (Demmig-Adams and Adams, 1996). Costs associated with the loss of photosynthetic functions in lutein-deficient tissues may outweigh potential costs incurred by webworm herbivory, given the significant maternal contributions of photosynthetic reproductive tissues to the seeds of apiaceous plants (Zangerl et al., 2003). If so, the role of lutein as a photoprotectant could present an environmental constraint on changes in fruit lutein content of *H. lanatum* in montane populations in response to excess light stress that is more likely to occur under the intense UVA light regimes.

In summary, this study demonstrates that both abiotic (UV) and biotic (parasitism) factors can influence patterns of plant chemical defense production. Efforts to characterize mechanisms underlying geographic mosaics of coevolutionary interactions (Thompson, 1999), which focus primarily on biotic factors, must also take into account the abiotic environment in which species interactions are embedded; although abiotic forces themselves do not respond reciprocally, they can have tremendous influence on reciprocal selective responses among interacting species.

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TEMPORAL AND SPATIAL VARIATION IN ALKALOID LEVELS IN *Achnatherum robustum*, A NATIVE GRASS INFECTED WITH THE ENDOPHYTE *Neotyphodium*

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Abstract—The native North American perennial grass *Achnatherum robustum* (Vasey) Barkworth [= *Stipa robusta* (Vasey) Scribn.] or sleepygrass is toxic and narcotic to livestock. The causative agents are alkaloidal mycotoxins produced from infections by a systemic and asexual *Neotyphodium* endophyte. Recent studies suggest that toxicity is limited across the range of sleepygrass in the Southwest USA. We sampled 17 populations of sleepygrass with varying distance from one focal population known for its high toxicity levels near Cloudcroft, NM, USA. For some, we sampled individual plants twice within the same growing season and over successive years (2001–2004). We also determined infection levels in each population. In general, all populations were highly infected, but infection levels were more variable near the focal population. Only infected plants within populations near the Cloudcroft area produced alkaloids. The ergot alkaloid, ergonovine, comprised the bulk of the alkaloids, with lesser amounts of lysergic and isolysergic acid amides and ergonovinine alkaloids. Levels of all alkaloids were positively correlated among individual plants within and between growing seasons. Infected plants that produced no alkaloids in 1 yr did not produce any alkaloids within the same growing season or in other years. Levels of alkaloids in sleepygrass populations declined with distance from the Cloudcroft population, although infection levels increased. Infected plants in populations in northern New Mexico and southern Colorado produced no alkaloids at all despite 100% infectivity. Our results suggest that only specific

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Neotyphodium haplotypes or specific *Neotyphodium*–grass combinations produce ergot alkaloids in sleepygrass. The *Neotyphodium* haplotype or host–endophyte combination that produces toxic levels of alkaloids appears restricted to one locality across the range of sleepygrass. Because of the wide variation in alkaloid levels among populations, interactions between the endophyte and host, and consequences for herbivores, competitors, and pathogens and other components of the community, are likely to vary widely across the geographic range of this native grass.

Key Words—*Achnatherum robustum*, alkaloids, endophyte, ergonovine, geographical variation, lysergic acid amide, native grass, *Neotyphodium*, sleepygrass.

INTRODUCTION

Asexual, vertically transmitted endophytes in the genus *Neotyphodium* are renowned for causing livestock toxicosis and reduced invertebrate herbivory in two introduced and widespread agronomic grasses, tall fescue (*Lolium arundinaceum*) and perennial ryegrass (*Lolium perenne*) (Clay, 1988, 1990; Siegel and Bush, 1996; Schardl and Phillips, 1997; Clay and Schardl, 2002). The toxic and deterrent properties of these *Neotyphodium*-infected agronomic grasses to vertebrate and invertebrate herbivores result from alkaloids produced by the endophytes. Increased resistance to herbivores and seed predators via endophytic alkaloids is generally viewed as the primary route of mutualistic benefits provided by *Neotyphodium* to the host grass (Cheplick and Clay, 1988; Saikkonen et al., 1998; Clay and Schardl, 2002), although *Neotyphodium* infections may also increase resistance to drought (Bacon, 1993) or poor soil nutrient conditions (Malinowski and Belesky, 1999), and generally increase competitive abilities (Marks et al., 1991; Clay et al., 1993). However, because alkaloids are nitrogen-rich compounds, benefits gained via protection against herbivores may be offset by reduced nitrogen availability to other host grass growth and reproductive functions, similar to other defensive allelochemicals produced by the host plant (Faeth, 2002).

The types and levels of alkaloids in agronomic grasses vary with host and especially with endophyte genotype (Siegel et al., 1990; Bush et al., 1993; Christensen et al., 1993; Leuchtman et al., 2000; Bony et al., 2001). Recently, genes for alkaloid production in some *Neotyphodium* endophytes have been identified and manipulated (Spiering et al., 2005). Alkaloid levels in agronomic grasses are also influenced by environmental factors, such as soil moisture and nitrogen availability (Arechavaleta et al., 1992; Agee and Hill, 1994; Roylance et al., 1994; Malinowski et al., 1998; Hunt et al., 2005) and temperature (Huizing et al., 1991). Additionally, alkaloid production in tall fescue can be induced to higher levels by herbivory (Bultman et al., 2004).

Less is known about infection frequencies and alkaloid levels and types in native grasses, and particularly how alkaloids vary geographically or temporally. Some native grasses have long been known anecdotally as toxic to livestock (Hance, 1876; Bailey, 1903; Freeman, 1904; Marsh and Clausen 1929), but only recently has this toxicity been directly linked to infection by *Neotyphodium* endophytes (Kaiser et al., 1996; Miles et al., 1996; Jones et al., 2000; Moon et al., 2002). Early reports implied that infected grasses were toxic throughout their geographic range, and hence, common names such as “sleepygrass” (*A. robustum*) and “drunken horse grass” (*Achnatherum inebrians*) were ascribed to these species. However, recent studies suggest that many, if not all, of “toxic” grass species with *Neotyphodium* infections are toxic in only some parts of their range (Miles et al., 1996; Jones et al., 2000; Faeth, 2002). Limited toxicity in *Neotyphodium*-infected grasses also seems to hold for the widely planted agronomic grasses, perennial ryegrass (Bony et al., 2001), and tall fescue (Saikkonen, 2000; Saikkonen et al., 2004), when examined in their native ranges. This suggests that alkaloid types and levels vary widely within and among populations of *Neotyphodium*-infected native grasses known for high toxicity. Alkaloid concentrations may also vary within and among growing seasons with environmental (e.g., soil moisture and nutrients) and developmental (e.g., as endophytes grow with their hosts) factors (Belesky and Hill, 1997), adding additional variation to alkaloid levels in native grass populations.

To our knowledge, geographic and temporal alkaloid variation in native *Neotyphodium*-infected grasses have yet to be simultaneously documented for any of the known highly toxic grasses (about 9–10 species; Faeth, 2002). Yet, alkaloid variation is the key trait influencing herbivore, seed predator and pathogen resistance, community interactions, and ecosystem functions (Matthews and Clay, 2001; Omacini et al., 2001; Faeth, 2002; Rudgers et al., 2004; Müller and Kraus, 2005). In previous studies, samples from individual plants have been pooled from within or across populations (Jones et al., 2000), individual infected plants have not been sampled over time (Powell and Petroski, 1992), or sampled plants were grown from seeds collected from natural populations (Miles et al., 1996). To assess within and among population variation, as well growing season variation, it is necessary to: (1) sample individual plants from multiple populations and (2) sample the same individual plants repeatedly over time. This populational variation is essential to understanding the coevolution and ecology of species interactions in general (Thompson, 1994, 2005) and, specifically, the outcome of interactions between host grass and systemic endophytes (Faeth and Sullivan, 2003; Sullivan and Faeth, 2004).

To ascertain geographic and temporal variation in alkaloids, we sampled 17 populations of sleepygrass or robust needlegrass, *A. robustum* [Vasey]

Barkworth = *Stipa robusta* [Vasey] Scribn.], radiating outward from the known toxic population in the Sleepygrass Picnic area, Lincoln National Forest, NM, USA, near the town of Cloudcroft (Petroski et al., 1992), from 2001 to 2004. We assessed the frequency of *Neotyphodium* infections in all populations. In some populations, individual plants were marked and resampled both within (seasonal) and among growing seasons (yearly). We correlated within and between growing season alkaloid levels to estimate how much variation is caused by developmental or environmental factors. We also determined how alkaloid levels varied with distance from the Sleepygrass Picnic area site, known for toxicity to livestock.

METHODS AND MATERIALS

Study Grass and Endophyte. *A. robustum* (Vasey) (Pooideae: Tribe Stipeae) is a cool-season, native grass found at high elevations throughout Arizona, New Mexico, Colorado, Wyoming, and Montana on “sky islands” of the Rocky Mountains in the western USA (Jones et al., 2000). Sleepygrass is a perennial bunchgrass found in semiarid pine–grassland habitats and reproduces by seed (USDA, 1988). In some localities in New Mexico, it is referred to as “robust needlegrass” (Jones et al., 2000).

The *Neotyphodium* endophyte is commonly found in sleepygrass populations at high frequencies (Petroski et al., 1992; Jones et al., 2000). *Neotyphodium* in *A. robustum* has yet to be identified to species (Moon et al., 2004; Schardl, personal communication). Morphologically, it appears intermediate in characteristics between *Neotyphodium starii* and *N. chisosum* (Kaiser et al., 1996) and also different from *Neotyphodium* isolated from *A. inebrians*, an Asian grass species known to produce high levels of similar alkaloids (Miles et al., 1996). Moon et al. (2004) found that *Neotyphodium* in sleepygrass was genetically most related to the sexual forms *Epichloë festucae* and *E. elymi*. Here, we refer to the endophyte as simply *Neotyphodium*.

Populations and Sampling. From 2001 to 2004, we sampled 17 populations of sleepygrass in New Mexico and Colorado (Table 1). We centered our sampling on a population that has long been known for high toxicity (Marsh and Clawson, 1929; Smalley and Crookshank, 1976; Jones et al. 2000), Sleepygrass Picnic area in the Lincoln National Forest (SP, Table 1), and then we sampled populations outward from this locality. The SP site is at the southern edge of the Sacramento Mountains, and most populations of sleepygrass occur northward. Three populations (SC, GR, and WD, Table 1), however, were southward, but still occurred within the Sacramento Mountain range. We assumed that these sites were separate populations, given that the closest population (MV) was 2.5 km from SP. *Neotyphodium* in Arizona fescue exhibits low gene flow between

populations (much less than 2.5 km) because seeds in which it is transmitted are not dispersed far (<1 m) and fall near the maternal plant (Sullivan and Faeth, 2004). Seeds of *A. robustum* may be transported further because of relatively large awns that may attach to animal fur, but we expect little movement between these disparate populations.

Number of sampled plants varied among years at the different sites (Table 1) because of time constraints, accessibility, missing plants from year to year, and replacement plants added in successive seasons or years. We sampled 432 plants among the 17 populations. In each population at each sampling period, leaf sheath tissue was removed, and plants were marked with surveyor's whisks and numbered metal tags. Plant tissue was placed on ice and returned to the lab for determination of infection status and for alkaloid analyses. For 4 populations, the same plants were sampled twice within the same growing season, June and September 2003, at the beginning and the end of the growing season, respectively.

Infection and Alkaloid Analyses. Infection status of all plants was determined by a modified tissue immunoblot assay (Gwinn et al., 1991; Schulthess and Faeth, 1998) and was later confirmed by staining and microscopic examination of seeds for characteristic *Neotyphodium* hyphae (Saha et al., 1988). The remainder of all plant samples were frozen until they could be lyophilized. Freeze-dried samples were ground to a fine powder in a Wiley mill. Ergot alkaloids were analyzed by methods adapted from TePaske et al. (1993) and Jones et al. (2000). In brief, 5 ml methanol and 0.050 ml of concentrated ammonium hydroxide were added to 100 mg of ground plant tissue. Samples were rotated overnight and filtered. The filtrate was dried at 60°C under a flow of nitrogen, after which 2 ml of 1% acetic acid and 2 ml of chloroform were added, and the samples were mixed by rotation for 2–3 min. Samples were centrifuged to aid in layer separation, and a 1.0-ml aliquot of the upper acid solution was removed and placed into an high-performance liquid chromatography (HPLC) autosampler vial. Concentrated ammonium hydroxide was added (~0.030 ml) to adjust the pH to 9–10 along with 0.010 ml of a 100-ppm stock solution of monocrotaline (quantitative reference standard). Samples were analyzed by HPLC–mass spectrometry (MS). Separation and detection were achieved with a Betasil C18 reversed-phase HPLC column (100 × 2 mm, Keystone Scientific) and HP 1100 binary HPLC solvent pump coupled to an LCQ (Thermo Finnigan) mass spectrometer. Solvent elution was a gradient of methanol (A) and 20 mM ammonium acetate (B) starting at 25% (A) increasing to 55% (A) from 0 to 10 min. The mass spectrometer was operated under positive electrospray ionization and single ion monitoring of $m/z = 268.3$ and 326.3 . Alkaloids are reported as total alkaloids as a mixture of ergonovine, lysergic acid amide, isolysergic acid amide, and ergonovinine. Quantification of individual alkaloids was made by measurement of peak area vs. calibration

TABLE 1. STUDY SITE DESCRIPTIONS, LOCATION, GPS COORDINATES, ELEVATION, AND SAMPLE SIZES (N) FROM FALL (SEPTEMBER) 2001 TO FALL 2004

Site	Description	City, county, state	GPS	Elevation (m)	Sampling years (N)
SC	Black Bear Campground, Lincoln National Forest	Near Cloudcroft, Otero Co., NM	32° 56.459'N, 105° 44. 474'W	2703	F 2001 (16), F 2002 (17) S 2003 (15), F 2003 (17)
GR	Carrie Green Ranch	Near Cloudcroft, Otero Co., NM	32° 55.170'N, 105° 44.379'W	2642	F 2001 (20), F 2002 (14) F 2003 (12)
MSI	Hwy 244 right of way near Mescalero Apache Reservation	Near Mescalero, Otero Co., NM	33° 01.674'N, 105° 36.408'W	2285	F 2001 (10)
SP	Sleepy Grass Picnic Area, Lincoln National Forest	Near Cloudcroft, Otero Co., NM	32° 57.452'N, 105° 43.092'W	2597	F 2001 (12), F 2002 (22) S 2003 (30), F 2003 (28)
WD	Lincoln National Forest	Near Weed, NM, Otero Co., NM	32° 47.691'N, 105° 35.659'W	2262	F 2001 (12), S 2003 (14), F 2003 (24)

MS2	Hwy 244 right of way near Mescalero Apache Reservation	Near Mescalero, Otero Co., NM	33° 10.740'N, 105° 41.620'W	2312	F 2001 (12), S 2003 (11)
MV	Lincoln National Forest	Near Cloudcroft, Otero Co., NM	32° 56.505'N, 105° 44.275'W	2659	S 2003 (41), F 2002 (39) F 2003 (32)
WR	Walker Ranch, 16 Springs Road	Near Mayhill, Otero Co., NM	33° 25.181'N, 111° 48.147'W	2285	F 2003 (43)
A	Intersection of US 285 and Rd G6	Near Antonito, Conejos Co., CO	37° 05.400'N, 106° 00.584'W	2385	F 2004 (20)
B	Near intersection of US 285/RD 12S	Near Alamosa, Alamosa Co., CO	37° 24.059'N, 105° 54.357'W	2260	F 2004 (20)
C	64/84 Hwy south of I-17	Near Chama, Rio Arriba Co., NM	36° 51.437'N, 106° 34.599'W	2334	F 2004 (20)
D	Hwy 159, South of Hwy 160/159 junction	Near San Luis, Costilla Co., CO	N: 37° 16.713' W: 105° 25.981'W 981'	2414	F 2004 (20)
E	San Isabel National Forest, Hwy 12	Near North Lake, Las Animas Co., CO	37° 14.636'N, 105° 02.302'W	2537	F 2004 (20)
G	Hwy 69, between mm 40-41	Near Westcliffe, Custer Co., CO	37° 54.491'N, 105° 19.770'W	2476	F 2004 (20)
J	US 50 between mm 214-215	Near Salida, Chaffee Co., CO	38° 31.303'N, 106° 05.861'W	2273	F 2004 (20)
K	US 50 between mm 159-160	Near Gunnison, Gunnison Co., CO	38° 32.667'N, 106° 52.743'W	2272	F 2004 (20)
L	Hwy 62, Between mm 5-6	Near Placerville, San Miguel Co., CO	38° 05.042'N, 108° 00.477'W	2323	F 2004 (20)

Samples were collected from some populations in June at the beginning of the growing season in 2003 (S 2003).
GPS = Global Positioning System.

curve generated using ergonovine standards. We assayed only for ergot alkaloids, the main alkaloid type found in *A. robustum* (Petroski et al., 1992; Jones et al., 2000); it is possible that other minor alkaloids were present but not detected by our methods.

Statistical Analyses. We summed all unique infected and uninfected plants ($N = 432$) within each population across seasons and years to determine infection frequencies (that is, no plant was included more than once for determining infection frequency). We determined yearly changes in alkaloids by correlating total levels and individual alkaloids in the same individual plants from 2001 to 2002 and from 2002 to 2003. If plants produced relatively the same levels from year to year, then we expect a strong positive correlation. Likewise, we correlated levels of alkaloids in the same individual plants in June 2003 (beginning of the growing season) to levels in September 2003 (end of the growing season) to ascertain if seasonal levels of alkaloids vary within a growing season.

To determine if alkaloids vary with distance among populations from the SP population, known for its high toxicity, we regressed mean total alkaloids and individual alkaloid fractions from each population with distance (in km) from this focal population.

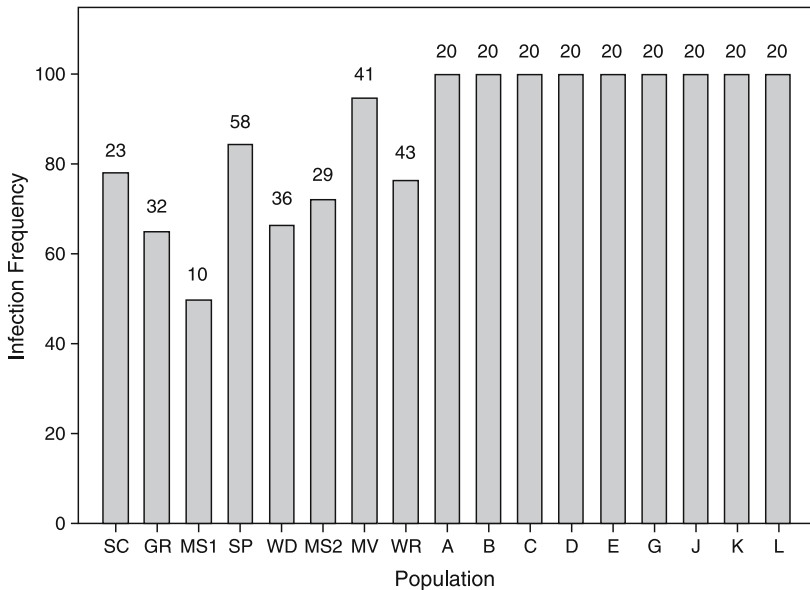


FIG. 1. Infection frequencies and sample sizes (numbers above bars) for the 17 sleepy-grass populations. See Table 1 for site abbreviations and locations.

RESULTS

Infection Frequencies. Infection frequencies among populations were generally high, ranging from 50 to 100% (Figure 1). Notably, the northern New Mexico and southern Colorado populations (A–L, Figure 1) were 100% infected. Populations nearer the focal population SP were much more variable.

Alkaloid Levels. Total alkaloid levels were highly variable across populations (Figure 2). The highest levels were in infected plants in the focal SP population and the nearby MV and MS2 population (Figure 2). Some of the infected plants had >150 µg/g total ergot alkaloids, some of the highest known for *Neotyphodium*-infected plants. However, a few plants in these populations, although infected, never produced alkaloids, notably, the northern New Mexico and southern Colorado populations that were 100% infected (Figure 1, data not shown). Ergonovine was consistently the highest fraction with lesser amounts of lysergic and isolysergic amides (Figure 2). Ergonovine constituted the lowest fraction (data not shown).

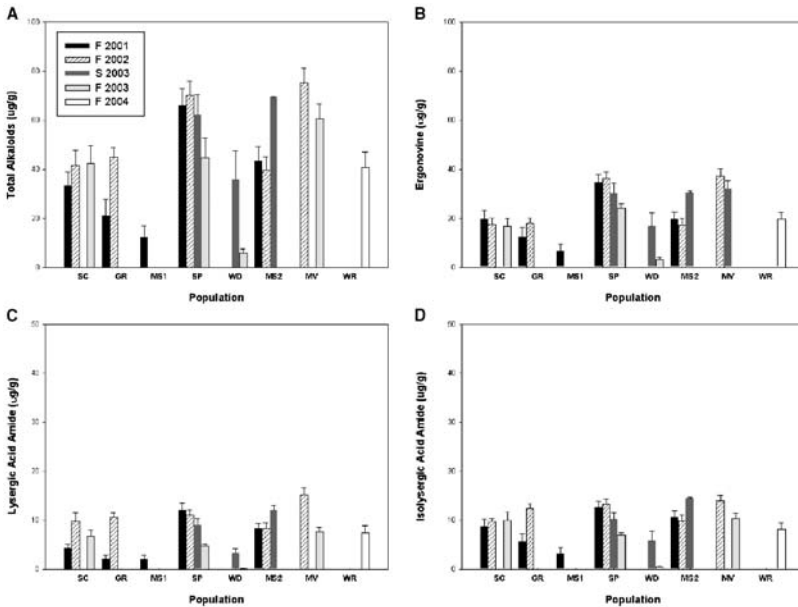


FIG. 2. Mean (±SE) concentrations (µg/g) of total (A), ergonovine (B), lysergic acid amide (C), and isolysergic amide (D) alkaloids from eight populations from 2001 to 2004. Infected plants from nine other populations in northern New Mexico and southern Colorado had zero alkaloids and are not shown. See Table 1 for site abbreviations. Note the difference in scale in boxes A, B and C, D.

Alkaloid levels in infected plants were positively correlated from 1 yr to the next. For the same individual plants sampled in fall of 2001 and 2002 ($N = 16$), concentrations of total (Pearson's correlation coefficient = 0.56, Bonferroni probability = 0.02), ergonovine (0.62, $P = 0.01$), isolysergic acid amide (0.53, $P = 0.04$), and ergonovine (0.54, $P = 0.03$) were positively correlated. Levels of lysergic acid amides were not significantly correlated ($P = 0.26$). For the same individual plants sampled in fall of 2002 and 2003 ($N = 50$), concentrations of total (0.33, $P = 0.02$), ergonovine (0.32, $P = 0.03$), lysergic acid amides (0.42, $P = 0.002$), and isolysergic acid amide (0.38, $P = 0.007$) were positively and significantly correlated. Levels of ergonovine alkaloids in fall of 2002 and 2003 were positively correlated (0.27), but only marginally significantly so ($P = 0.06$).

Within a growing season, alkaloid concentrations of the same individual plants ($N = 20$) were strongly and positively correlated. Levels of total alkaloids (0.71, $P < 0.001$), ergonovine (0.75, $P < 0.001$), lysergic acid amides (0.70, $P = 0.001$), isolysergic acid amides (0.64, $P = 0.002$), and ergonovine (0.69, $P = 0.001$) were positively and significantly correlated in the same plants sampled in the beginning (June 2003) and end (September 2003) of the growing season.

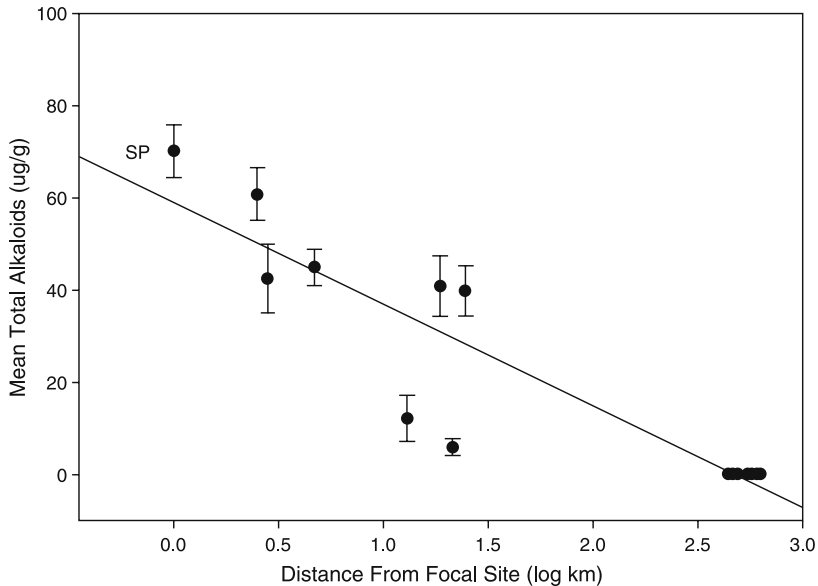


FIG. 3. Regression of mean (\pm SE) total alkaloids and log distance from site SP. Distances (km) were measured from the SP, the population used as the focal site (see text).

Mean Alkaloid Levels and Distance From The Focal Population. Mean levels of alkaloids in infected plants within each sampled population declined with linear distance from the focal population (SP; Figure 3). The best regression fit was alkaloid levels with log distance. Regressions of alkaloids levels with log distance were significant for mean total alkaloids ($N = 17$, adjusted $R^2 = 0.83$, $P < 0.001$, Figure 3), ergonovine (adjusted $R^2 = 0.80$, $P < 0.001$, data not shown), lysergic acid amides (adjusted $R^2 = 0.74$, $P < 0.001$, data not shown), isolysergic acid amides (adjusted $R^2 = 0.74$, $P < 0.001$, data not shown), and ergonovinine (adjusted $R^2 = 0.76$, $P < 0.001$, data not shown). Because all infected plants in the northern New Mexico and southern Colorado populations had no alkaloids, inclusion of all eight data points (Figure 3) could disproportionately skew the regression of alkaloid levels and distance towards a more negative relationship. Therefore, we also performed similar regressions of distance and alkaloid levels, but considered these eight distant populations as a single data point. The results were the same; mean total alkaloids and all mean alkaloid fractions rapidly and significantly declined with distance from the SP population.

DISCUSSION

Systemic fungal endophytes in cool-season, pooid grasses are well known for producing a wide variety of alkaloids that purportedly provide protection for the host against vertebrate and invertebrate herbivores (Cheplick and Clay, 1988; Breen, 1994; Siegel and Bush, 1996; Clay and Schardl, 2002; Faeth, 2002), root nematodes (West et al., 1988; Kimmons et al., 1990), some microbial pathogens (Burpee and Bouton, 1993), and possibly act as allelopathic agents (Peters and Zam, 1981). These effects are well known in two widespread agronomic grasses, tall fescue and perennial ryegrass, where *Neotyphodium* alkaloids are directly linked to livestock toxicoses and neurological disorders and increase resistance to generalized invertebrate pests (Bush et al., 1997; Schardl and Phillips, 1997). Much less is known about alkaloid levels in native grass populations harboring *Neotyphodium*. In general, relatively few *Neotyphodium*-infected native grasses are known for toxicity to livestock (Faeth, 2002). However, one of these, *A. robustum*, is toxic to horses, cattle, and sheep (Bailey, 1903; Marsh and Clawson, 1929; Petroski et al., 1992). Despite the central role of fungal alkaloids in agronomic and livestock production (Schardl and Phillips, 1997), and more generally to *Neotyphodium*-host grass interactions (Clay and Schardl, 2002; Faeth, 2002), there have been no studies of seasonal and yearly variation in alkaloid levels, or the relationship of alkaloid production to infection levels among populations, in infected native grasses.

Here, we show that ergot alkaloid levels in native sleepygrass populations are highly variable both within and among populations. The populations near Cloudcroft, NM, have high levels of ergot alkaloids on average, as previously reported (Petroski et al., 1992; Jones et al., 2000), but levels vary greatly, with some infected plants producing no alkaloids at all, whereas others produce more than 150 $\mu\text{g/g}$ total alkaloids (range = 0–156 $\mu\text{g/g}$). Most of the alkaloid fraction is ergonovine, with lesser amounts of lysergic acid, isolysergic amide, and ergonovine. This is in contrast to reports from Petroski et al. (1992) where ergonovine was lower relative to lysergic and isolysergic amides and other alkaloids. However, those plants were not sampled over time. In a study of the alkaloid content of a congeneric infected native grass from Asia, Miles et al. (1996) found that infected *A. inebrians* had high levels of ergonovine (up to 2500 $\mu\text{g/g}$) and lesser amounts of lysergic acid amide (up to 400 $\mu\text{g/g}$), similar to the relative amounts of alkaloids in sleepygrass but higher in absolute concentrations. Those plants, however, were grown in the greenhouse from seed, so it is not clear if the levels reflect alkaloid levels in natural populations. Nonetheless, in terms of alkaloid profiles, *Neotyphodium* in *A. robustum* and *A. inebrians* appear similar. Molecular studies would be fruitful to determine if *Neotyphodium* in these two geographically disjunct grass species are genetically related.

Lysergic and isolysergic acid amides are likely the alkaloids responsible for the dramatic narcotic effects of infected sleepygrass on livestock (Miles et al., 1996; Jones et al., 2000). Horses that feed on small amounts of infected sleepygrass tissue or seeds from the Cloudcroft region become narcotized and go into a deep sleep (Powell and Petroski, 1992). Consumption of larger amounts causes elevated body temperature, weakness, frequent urination, dizziness, hypersalivation, diarrhea, and possibly death (Smalley and Crookshank, 1976; Miles et al., 1996). Both lysergic acid and ergonovine are vasoconstrictors. Ergonovine is known as a stimulator of smooth muscles and used medically to induce uterine contractions (Powell and Petroski, 1992; Miles et al., 1996). Ergot alkaloids, in general, are deterrent and toxic to some invertebrate herbivores (Siegel et al., 1990), but it is not known if ergonovine generally deters herbivory by vertebrates or invertebrates as do lysergic acid amides, although vasoconstriction is associated with heat stress and tissue gangrene in livestock consuming other *Neotyphodium*-infected grasses. Ergonovine may also increase susceptibility of invertebrate herbivores to entomopathogenic nematodes (Kunkel et al., 2004).

That alkaloid levels are positively correlated both within and among growing seasons suggests that the endophyte species, endophyte haplotype, or the host–endophyte genotypic combination largely determines alkaloid levels. Although alkaloids varied slightly within population within and among growing seasons, in general, levels remained consistent (Figure 2), indicating that

environmental and developmental variation has lesser influences on alkaloid levels in infected sleepygrass. Alkaloid variation in infected agronomic grasses and some other native grasses appears largely dictated by endophyte species or haplotypes (Siegel et al., 1990; Leuchtmann, 1992, 1997; Bush et al., 1993; Christensen et al., 1993) and less so by host genotype and environmental factors (Siegel et al., 1990; Leuchtmann et al., 2000). However, host grass genotype in agronomic grasses (Siegel et al., 1990) and at least one infected native grass may drive alkaloid production (Faeth et al., 2002). Although we cannot distinguish here between endophyte and host genotype effects, or their interactions, on alkaloid production, consistent levels within and between growing seasons suggest that environmental factors play a lesser role. Furthermore, infected plants that produced no alkaloids in June never did so in the fall, and infected plants that produced no alkaloids in 1 yr did not in subsequent years, suggesting that a specific *Neotyphodium* species or haplotypes or specific host and endophyte genotypic combinations are necessary for alkaloid production.

That alkaloid levels in sleepygrass populations decline and disappear with distance from the SP population suggests that the specific strain or haplotype of *Neotyphodium* or endophyte–host genetic combination may be localized near Cloudcroft, NM. This strain or combination rapidly decreases with distance in frequency relative to less toxic and nontoxic strains or combination. The *Neotyphodium* endophyte in sleepygrass has yet to be identified to species or thoroughly examined for geographic genetic variation. Kaiser et al. (1996) found a diversity of spore morphologies in cultures of 10 New Mexico and Colorado populations. Moon et al. (2004) found a hybrid *Neotyphodium* in sleepygrass in one sample from southern Colorado. Based on gene sequences, they postulated that this hybrid was formed from *Epichloë elymi* and *E. festucae*. Hybridization is thought to occur when the sexual and spore-transmitted form *Epichloë* colonizes and hybridizes with *Neotyphodium* in the same host grass or when two *Epichloë* species hybridize. Hybridization is thought to rapidly infuse genetic variation into the population and create novel genetic combinations that may result in new alkaloid types (Schardl and Craven, 2003; Moon et al., 2004). The hybrid *Neotyphodium* in Moon et al. (2004) came from regions in southern Colorado where we found no alkaloids. Furthermore, the hybrid in Moon et al. (2004) does not harbor the gene for ergot alkaloids (Schardl, personal communication). In another native southwest US grass, Arizona fescue (*Festuca arizonica*), genetic variation in *Neotyphodium* is highly spatially structured, with the presence of hybrid and nonhybrid endophytes, and with little or no gene flow between grass populations that are less than 2 km apart (Sullivan and Faeth, 2004). Sullivan and Faeth (2004) also found that hybrids did not differ in levels or types of alkaloids relative to Arizona fescue with nonhybrid *Neotyphodium*. Thus, hybridization at least in native sleepygrass and Arizona fescue apparently is not a prerequisite for novel or high alkaloid

production. Future studies should focus on ascertaining the underlying genetic basis in the *Neotyphodium* endophyte for this steep gradient in alkaloid production in sleepygrass populations.

Interestingly, the sleepygrass populations near Cloudcroft are also more variable in infection frequency than northern New Mexico and southern Colorado populations (Figure 1). Infection frequency in the latter are 100%, but the *Neotyphodium*–grass symbiota produces no alkaloids. The variability in infection frequency and in alkaloid levels among infected plants near the SP population suggests that high alkaloid producing *Neotyphodium* endophytes may also incur substantial costs. Faeth (2002) argued that alkaloid production in infected grasses is costly in terms of nitrogen demand and metabolic costs. High levels may also be costly in terms of toxicity to the host, similar to alkaloidal allelochemicals produced directly by host plants (Karban and Baldwin, 1997). The benefits of high alkaloids may offset these costs when grazing or herbivory is intense and persistent and available soil nutrients are high (Faeth, 2002). The frequency of toxic infected *A. robustum* (Marsh and Clawson, 1929; Jones et al., 2000) and *A. inebrians* (Miles et al., 1996; Nan and Li, 2001) appears to have increased relative to nontoxic individuals because livestock grazing has intensified in the respective geographic range of these host grasses.

Our results indicate that *A. robustum*–endophyte interactions, in terms of alkaloids, are highly spatially structured across a wide geographic range of populations. Thompson's (1994, 2005) geographical mosaic theory predicts that interactions evolve or coevolve in metapopulations, with the interaction between local selection pressures, migration, and genetic drift creating a range of possible outcomes in species interactions geographically. Alkaloid levels in infected sleepygrass vary dramatically, and the notion of sleepygrass as a toxic grass because of endophyte infection only applies in a very restricted part of the geographic range of the host grass. Given that alkaloid production is considered the main trait that dictates the outcome of grasses infected with systemic, seed-borne endophytes with herbivores, pathogens, competing plants, higher trophic levels, community diversity, and even ecosystem functions (Saikkonen et al., 1998; Omacini et al., 2001; Clay and Holah, 1999; Clay and Schardl, 2002; Faeth, 2002; Faeth and Bultman, 2002; Rudgers et al., 2004), we predict that these interactions, and community and ecosystem repercussions, will also be highly variable across the geographic range of the sleepygrass. Understanding populational variation in alkaloids because of endophyte and host genetics, and not just infection status of grasses, will be necessary to unravel these complex host–endophyte interactions and their consequences at the community and ecosystem level.

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IMPACT OF BOTANICAL PESTICIDES DERIVED
FROM *Melia azedarach* AND *Azadirachta indica* PLANTS
ON THE EMISSION OF VOLATILES THAT
ATTRACT PARASITOIDS OF THE DIAMONDBACK
MOTH TO CABBAGE PLANTS

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Abstract—Herbivorous and carnivorous arthropods use chemical information from plants during foraging. Aqueous leaf extracts from the syringa tree *Melia azedarach* and commercial formulations from the neem tree *Azadirachta indica*, Neemix 4.5[®], were investigated for their impact on the flight response of two parasitoids, *Cotesia plutellae* and *Diadromus collaris*. *Cotesia plutellae* was attracted only to *Plutella xylostella*-infested cabbage plants in a wind tunnel after an oviposition experience. Female *C. plutellae* did not distinguish between *P. xylostella*-infested cabbage plants treated with neem and control *P. xylostella*-infested plants. However, females preferred infested cabbage plants that had been treated with syringa extract to control infested plants. Syringa extract on filter paper did not attract *C. plutellae*. This suggests that an interaction between the plant and the syringa extract enhances parasitoid attraction. *Diadromus collaris* was not attracted to cabbage plants in a wind tunnel and did not distinguish between caterpillar-damaged and undamaged cabbage plants. Headspace analysis revealed 49 compounds in both control cabbage plants and cabbage plants that had been treated with the syringa extract. Among these are alcohols, aldehydes, ketones, esters,

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terpenoids, sulfides, and an isothiocyanate. Cabbage plants that had been treated with the syringa extract emitted larger quantities of volatiles, and these increased quantities were not derived from the syringa extract. Therefore, the syringa extract seemed to induce the emission of cabbage volatiles. To our knowledge, this is the first example of a plant extract inducing the emission of plant volatiles in another plant. This interesting phenomenon likely explains the preference of *C. plutellae* parasitoids for cabbage plants that have been treated with syringa extracts.

Key Words—Botanical pesticides, parasitoid behavior, *Plutella xylostella*, induced plant volatiles, elicitor.

INTRODUCTION

The diamondback moth *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is a major pest of crucifer crops and is found throughout the world (Talekar and Shelton, 1993). Natural enemies are recognized as important components in *P. xylostella* management strategies, particularly where control with chemicals has failed. However, they need to be integrated with other strategies for successful control. Botanical pesticides are thought to be compatible with biological control, as they tend to be relatively harmless to parasitoids and predators (Schmutterer, 1995, 1997; Charleston et al., 2005). In South Africa, 21 species of parasitoids are associated with *P. xylostella* (Kfir, 2003), providing a rich and abundant source for biological control. Botanical pesticides have not been registered for use in South Africa. However, the syringa tree *Melia azedarach* L. (Meliaceae) is an invasive plant found throughout South Africa. It has insecticidal properties (Ascher et al., 1995) and may provide the small-scale rural farmer with an alternative control tactic. It is important to investigate the impact that extracts have on the natural enemy fauna. Botanical pesticides act as repellents against a number of pest species, but little information is available concerning their effect on the behavioral responses of natural enemies of pests such as parasitoids and predators (Akol et al., 2003).

Plants influence carnivore behavior (Dicke et al., 1990; Turlings et al., 1990; Steinberg et al., 1993; Dicke and Vet, 1999; Vet, 1999; Dicke, 1999a; Hilker and Meiners, 2002). Plants that are attacked by herbivores emit volatile cues that can be used by natural enemies of the herbivore to find their hosts, and these plant volatiles may contain information on the identity of the herbivore (Turlings et al., 1990, 1993; Vet et al., 1991; Vet and Dicke, 1992; Dicke and Vet, 1999). The main components of the volatile blend released from cabbage plants are terpenoids and green leaf volatiles (Mattiacci et al., 1994; Shiojiri et al., 2001). Terpenoids are a major class among herbivore-induced synomones that attract carnivores (reviewed by Takabayashi et al., 1994; Dicke, 1994;

Turlings et al., 1995; Pichersky and Gershenzon, 2002). Terpenoids are released in analogous amounts in both herbivore-damaged and mechanically-damaged cabbage plants, as well as in undamaged plants (Mattiacci et al., 1994; Shiojiri et al., 2001). Plants emit green leaf volatiles during aging or when injury occurs (Visser and Ave, 1978; Hatanaka, 1993). However, there is a dramatic increase of green leaf volatiles in the headspace of damaged cabbage plants compared to undamaged plants, and this may play a role in parasitoid attraction (Mattiacci et al., 1994; Shiojiri et al., 2001; Smid et al., 2002). For example, *Cotesia rubecula* and *Cotesia glomerata* (Hymenoptera: Braconidae) can distinguish between damaged and undamaged Brussels sprout plants (Steinberg et al., 1992; Geervliet et al., 1996). Artificially damaged cabbage plants also produce volatiles, but these do not show any qualitative differences compared to the volatiles released when the plant is damaged by herbivores (Mattiacci et al., 1994; Geervliet et al., 1997; Shiojiri et al., 2001). However, there are quantitative differences (Mattiacci et al., 1994; Shiojiri et al., 2001). *Cotesia glomerata* did not distinguish between artificially damaged cabbage plants and plants damaged by its host *Pieris rapae* (L.) (Lepidoptera: Pieridae) (Shiojiri et al., 2001). However, *Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae) distinguished between artificially damaged cabbage plants and plants damaged by *Plutella xylostella* (Shiojiri et al., 2001), and the parasitoid spent longer searching infested cabbage plants than artificially damaged plants (Shiojiri et al., 2000a). Chemical analysis of the volatiles released by cabbage plants that had been damaged by two different host species, *P. xylostella* and *Pieris rapae*, indicated that there were slight qualitative differences (Agelopoulos and Keller, 1994) and many quantitative differences in the compounds produced in response to damage from these two herbivores (Agelopoulos and Keller, 1994; Geervliet, 1997; Shiojiri et al., 2001). *Cotesia plutellae* discriminated between cabbage plants infested with *P. xylostella* (host) and plants infested with *Pieris rapae* (nonhost) (Shiojiri et al., 2000b, 2001). The searching time of *C. plutellae* on cabbage leaves infested by host larvae was also longer than on leaves infested with the nonhost (Shiojiri et al., 2000a), which suggests that this parasitoid can discriminate between host- and nonhost-infested plants through antennal contact (Shiojiri et al., 2001, 2000a).

Cotesia plutellae and *Diadromus collaris* (Gravenhorst) (Hymenoptera: Ichneumonidae) are the two parasitoid species most commonly found in cabbage fields around Pretoria, South Africa. We have shown that aqueous leaf extracts from the syringa tree *Mella azedarach* and the neem product, Neemix 4.5[®], taken from the neem tree *Azadirachta indica* Juss. (Meliaceae) do not have a direct impact on the survival of these parasitoids (Charleston et al., 2005), and that they are still able to find their hosts when host-infested plants have been treated with the botanical extracts. In fact, in both greenhouse and field experiments, *C. plutellae* parasitize a greater proportion of *Plutella xylostella*

larvae on cabbage plants treated with syringa extracts than they do on control plants (Charleston et al., 2005). Small differences in plant volatiles can affect the attraction of parasitoids, and treating plants with botanical extracts may change the volatile profiles, which may have an impact on the responses by natural enemies. Here, we investigated the impact that these botanical extracts have on the volatile profile of cabbage plants and how this may influence the attraction of *C. plutellae* and *D. collaris*.

METHODS AND MATERIALS

Experimental Plants and Insects. Cabbage plants *Brassica oleracea* var. *capitata* L. (Cruciferae) were bought as seedlings, planted in black plastic bags, and left in a glasshouse ($30 \pm 5^\circ\text{C}$) to grow. The plants were fertilized with compost when planted and regularly watered. To reduce insect damage, the plants were placed inside a tent-like construction composed of fine nylon netting (mesh size <1 mm).

Plutella xylostella were from a culture originally collected near Pretoria ($28^\circ15'S$; $25^\circ44'E$) and Brits ($25^\circ38'S$; $27^\circ47'E$), South Africa. The laboratory culture was maintained on canola seedlings *Brassica napus* L. (Cruciferae). The two parasitoid species most common in cabbage fields near Pretoria, South Africa, were *Cotesia plutellae* and *Diadromus collaris*. Laboratory cultures of these were established in 1993. Each parasitoid species was kept communally in glass cages ($38 \times 27 \times 28$ cm) and exposed to *P. xylostella* larvae on canola seedlings three times per week. After exposure to *C. plutellae*, the second instar *P. xylostella* larvae were maintained on cabbage leaves in shallow plastic rearing containers until cocoon formation. To obtain fresh pupae for *D. collaris*, they were exposed to late fourth instar *P. xylostella*, which would pupate within 1 d. Cocoons of *C. plutellae* or *P. xylostella* pupae parasitized with *D. collaris* were placed into clean cages, and the emergence of wasps took place in clean cages without any plant or host material. The parasitoids were maintained on a diet of honey and water. Parasitoids used in the bioassays were 2–6 day old mated females.

All insect rearing was carried out in a controlled environment ($24 \pm 2^\circ\text{C}$; $65 \pm 5\%$ RH, 16:8 hr light/dark period).

Botanical Extracts. *Syringa*: *Melia azedarach* (hereafter referred to as syringa) leaves were collected from Rietondale in Pretoria, South Africa ($28^\circ15'S$, $25^\circ44'E$). Leaves were collected from trees at a height of about 1.5–3.5 m at the start of spring flush, in September 2002, placed in a glasshouse ($30 \pm 5^\circ\text{C}$) to dry, after which they were crushed into a fine powder and stored in an airtight container until use. The extract was made with 100 ml of distilled water. The water was heated to 48°C ; 5 g of leaf powder were added to the water, and the mixture was shaken for approximately 1 min. The extract was left in a refrig-

erator ($\pm 4^{\circ}\text{C}$) overnight. The following morning, it was filtered using Advantec[®] filter paper no. 2. Three drops of liquid detergent (Teepol[®]) were added to the final extract to act as a surfactant, without which the extract runs off the surface of the leaf.

Neem: A commercial preparation of *Azadirachta indica*, Neemix 4.5[®] (hereafter referred to as neem), was provided by Thermo Trilogly Corporation, Columbia, MD, USA. A dose of 32 μl per 100 ml of distilled water was used. Three drops of liquid detergent were added to the final solution.

Control: The control treatment used consisted of 100 ml of distilled water mixed with three drops of liquid detergent.

Wind Tunnel Design. A wind tunnel was set up within a tent-like construction (350 \times 300 \times 200 cm). It was placed in a room with a controlled temperature of $24 \pm 2^{\circ}\text{C}$. No daylight could enter the room. Illumination was provided by rows of lights in the roof of the tent, simulating daylight (2300 lx). Two table fans were placed at the end of a table; a sheet of gauze material (mesh size, 3 mm) was placed in front of the fans to reduce the wind speed and to provide a more laminar airflow. The wind speed at the release point was approximately 0.133 m/sec. The cabbage plants were placed on a table (180 \times 900 cm; Figure 1). The table was covered with white plastic sheeting to facilitate cleaning, and thick (4.7 cm) black strips of tape were placed at 30-cm intervals to provide a contrast for the flying insects. To contain the insects within the experimental arena and to create diffuse lighting, white cotton sheeting was placed across two poles 112 cm above the table and left to hang over the sides, enclosing the arena. An equilateral triangle (45 cm) was marked out on the table, and cabbage plants of the same age (± 5 wk after transplant) were placed in groups of four on either side of the base of the triangle. The plants were placed in a square around the mark, with 20 cm between the centers of each plant. One group of plants was treated with the botanical pesticide and the other with control solution. The plants were changed to opposite sides of the triangle after every five observations.

Behavioral Recordings. Parasitoids were released from a platform at a 20-cm height, at the apex of the equilateral triangle at the downwind end of the tunnel (Figure 1). A female parasitoid was faced with a choice between treated and control cabbage plants and was observed to assess which plant she landed on first. "Response" was recorded when the female left the release platform and landed on one of the plants. For each treatment, 60 responding females were observed. The number of females that did not respond was also recorded. "No response" occurred when the female failed to leave the platform after 5 min, or if the female landed on any surface other than a cabbage plant.

Choices between treated and control plants were analyzed by using binomial probability functions to assess a difference from a 50–50 distribution between the treatment and control.

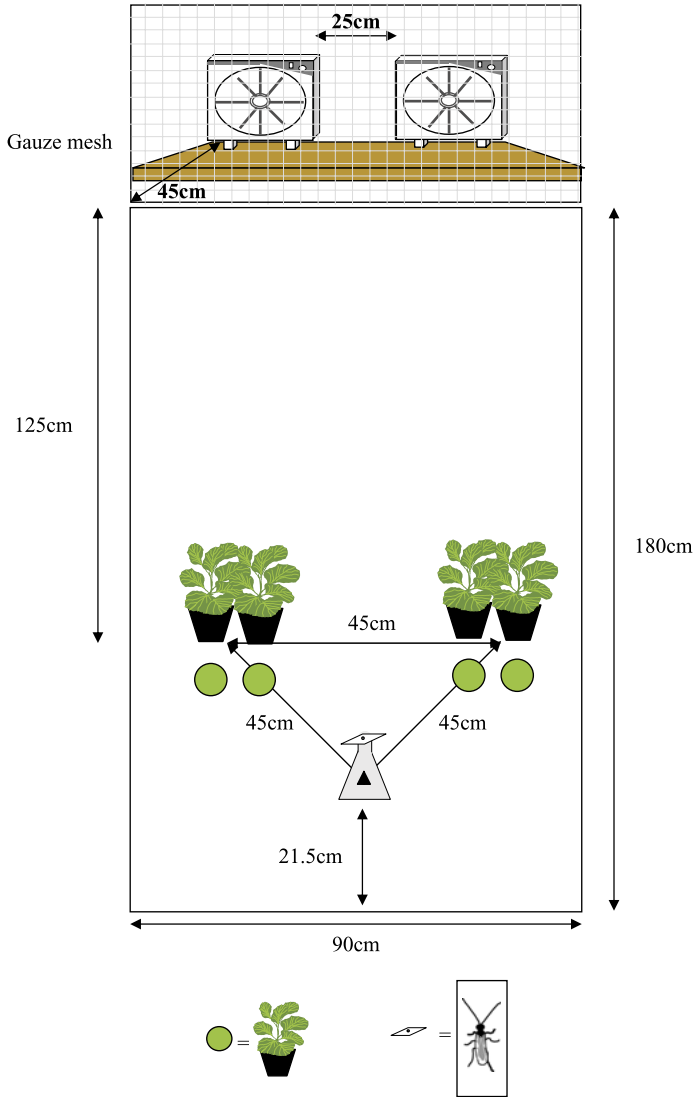


FIG. 1. Diagram showing the design of the wind tunnel.

Previous Experience. We observed that *Cotesia plutellae* did not respond in the wind tunnel without an oviposition experience. Potting et al. (1999) have shown that an oviposition experience significantly increased the response of *C. plutellae* to volatiles. Therefore, each *C. plutellae* female was exposed to a

cabbage leaf with feeding *Plutella xylostella* larvae. Each female was allowed to oviposit two to three times before being removed and placed into a glass vial with some honey for food. Approximately 1–2 hr later, she was released in the wind tunnel and observed.

Diadromus collaris females were also given an experience before they were released in the wind tunnel. In this case, the female was exposed to a cabbage leaf that had been damaged by *Plutella xylostella* larvae, which had subsequently pupated on the leaf just before exposure to *D. collaris*. The female was left to explore the leaf for 3 min, after which she was removed and placed in a glass vial with some honey for food, and released in the wind tunnel 24 hr later.

Experiments

Plant–Host Complex. Cabbage plants were first sprayed with either the botanical pesticide or the control (approximately 100 ml per plant) and then infested with 15 *Plutella xylostella* larvae (second instar for test with *Cotesia plutellae*, or late fourth instar for test with *Diadromus collaris*). Larvae were left to feed on the cabbage plants for 24 hr, after which infested plants were placed in the wind tunnel and the observations began. To compensate for any differences between the cabbage plants themselves, the experiment was carried out over three different days using different plants and different wasps each day.

Effect of Experience. This trial was also used to investigate whether previous experience on cabbage treated with the plant extracts had an influence on the subsequent flight behavior of the parasitoid in the wind tunnel. Each species was given experience with *Plutella xylostella* larvae (for *Cotesia plutellae*) or pupae (for *Diadromus collaris*) on either treated or untreated cabbage leaves, and the subsequent behavior was compared. Data were analyzed with a chi-square test.

Response of Pupal Parasitoid. Because *Diadromus collaris* showed a low responsiveness in the wind tunnel (<50% responded), we carried out an additional experiment to investigate whether this species responded to volatiles released from damaged plants. For this experiment, one group of cabbage plants was undamaged, and one group was damaged by 15 late fourth instar *Plutella xylostella* larvae, which were left to feed and pupate. Approximately 48 hr later, pupae were removed from the cabbage plants, and these plants were exposed to *D. collaris* in the wind tunnel, with undamaged plants for comparison. The groups of plants were replaced three times during the experiment. Again, less than 50% of the *D. collaris* females responded to the plants and, therefore, no further experiments were carried out with this species.

Different Syringa Doses. Further experiments were carried out with *Cotesia plutellae* using two lower doses of the syringa extract. The lower doses

were made with 1 and 3 g of leaf powder and 100 ml of distilled water, and prepared as described above ([Plant–Host Complex](#)).

Damaged Plants without Hosts. To investigate whether the presence of the host itself resulted in an attraction of *Cotesia plutellae*, the experiment was repeated as described above ([Plant–Host Complex](#)). However, *Plutella xylostella* larvae were allowed to feed for 24 hr and then removed from the plant before the plants were exposed to *C. plutellae* in the wind tunnel. Only the host larvae were removed; their by-products (frass, silk, etc.) remained on the plant.

Equally Damaged Plants without Hosts. Schuler et al. (1999, 2003) have shown that the amount of damage is an important factor influencing the response of *Cotesia plutellae*. Because botanical extracts reduce the feeding of *Plutella xylostella* larvae (Charleston, 2004), the experiment was repeated to compensate for any possible differences in the amount of damage. To create cabbage plants that had an approximately equal amount of damage, plants were first infested with 15 second instar *P. xylostella*. The larvae were left to feed on the plants for 24 hr, after which the larvae were removed. The plants were then sprayed with either the treatment or the control. The groups of plants were replaced twice during the experiment.

Undamaged Plants and Filter Paper. To investigate whether botanical pesticides influence parasitoid behavior, irrespective of the presence of damage, undamaged plants were also compared. Three doses of the syringa extract were tested: 1, 3, and 5 g (see description above for preparation of the extracts), and one dose of neem. Finally, a test was performed to investigate whether the syringa extract alone influenced parasitoid behavior. For this test, filter paper was dipped into either the 5-g syringa treatment or into the control, and parasitoid behavior was observed.

Analysis of Plant Volatiles. To investigate differences in volatile emission by cabbage plants (*Brassica oleracea* var. *gemmifera* cv. Cyrus) that had been treated with syringa extracts and control plants, headspace analysis was carried out. The 5-g syringa extract was made as described above, and 100 ml were applied to each clean, undamaged, cabbage plant. Plants that served as controls were sprayed with 100 ml of distilled water mixed with liquid detergent. The plants were left to dry for 60 min before being placed into 30-l collecting jars.

Pressurized air was filtered over silica gel, molecular sieves (8–12 mesh beads, 4-Å pore width, Sigma), activated charcoal, and a disposable Tenax-containing tube (90 mg Tenax TA) before entering the collecting jar. The air-inlet, air-outlet, filters, and sampling jars were connected with 0.8-cm diam Teflon tubing. Prior to the experiments and between each sample, the system was purged with purified air overnight at a flow rate of 500 ml/min.

Cabbage plants were carefully removed from the pots, taking care not to disturb the root system; then the entire soil and root system was covered in

aluminum foil. Plants were placed individually into the collecting jars, which were covered with a loose glass lid. A viton O-ring was placed between the jar and the lid, and the lid was tightly closed with a metal clamp. High-frequency fluorescent lights (30–35 $\mu\text{mol photons/m}^2/\text{sec}$) were placed 15 cm above the collection jars. The system with the plants was purged for 1 hr at 500 ml/min, after which the flow was reduced to 225 ml/min, and a Tenax (90 mg Tenax TA) trap was connected to the air outlet in the lid of the collecting jar. Volatiles were trapped at a rate of 175 ml/min by pulling the air through the trap by using an in-house vacuum. Volatiles were collected for 4.5 hr, after which the traps were refrigerated ($\pm 4^\circ\text{C}$) until they were analyzed. Four independent samples were collected for each treatment (5-g syringa and control). These independent samples were collected on consecutive days, and each was collected in the morning at the same time each day. After the second sample, and again after the final sample, two blank samples were taken from empty collecting jars to ensure that volatiles always present in filtered/clean air were not considered in the final analysis.

Samples of volatiles were also taken from the syringa extract mixed with liquid detergent. For this sample, 25 ml of extract were placed into a glass Petri dish and placed on top of an Erlenmeyer flask to provide sampling at approximately the same height as the cabbage plant. The Petri dishes were placed into the collecting jars, and the system was purged for 1 hr. After 1 hr, the airflow was reduced to 225 ml/min. A Tenax TA trap was used to collect the volatiles, and air was pulled through the trap at 175 ml/min. Volatiles were collected for 1 hr, after which the Tenax traps were refrigerated ($\pm 4^\circ\text{C}$) until they were analyzed. Four samples were collected from the syringa extract.

Volatiles were released from the Tenax traps with a thermodesorption cold trap setup (Markes, UK) by heating at 200°C for 10 min, with a He-flow of 30 ml/min. Desorbed volatiles were collected in the cold trap at -100°C . Volatiles were injected in splitless mode into the RTX-5Silms column (Restec, 30 m \times 0.32 mm ID, 0.33- μm film thickness) by heating the cold trap to 270°C . After an initial column temperature of 40°C for 2 min, the temperature was raised to 95°C at $3^\circ\text{C}/\text{min}$, then to 165°C at $2^\circ\text{C}/\text{min}$, and subsequently to 250°C at $15^\circ\text{C}/\text{min}$. The column was directly coupled to the ion source of a Finnigan quadrupole mass spectrometer, which was operating in the 70-eV EI ionization mode and scanning from mass 33 to 300 at 3 scans/sec. Compounds were identified by comparison of mass spectra with those in the NIST 98 and Wiley 7th edition spectral libraries and by checking the retention indices with those of authentic reference compounds.

For each compound, the mean peak area was calculated, and each compound was classified as being emitted in larger amounts by control or syringa-treated cabbage plants, based on mean peak area. A sign test was used to determine whether the number of compounds that were emitted in larger amounts by control or syringa-treated plants differed from a 50–50 distribution over the two treatments

(Sokal and Rohlf, 1995). To compare the total headspace composition of plants with and without syringa extract treatment, a principal component analysis (PCA) was carried out according to the description by Mumm et al. (2004).

RESULTS

Plant–Host Complex. Cabbage plants were sprayed with the botanical extracts or control solution and then infested with *Plutella xylostella*. Parasitoids were exposed to the entire plant–host complex in the wind tunnel. *Cotesia plutellae* did not show a preference for the treated or control plants when the plants were treated with the neem extract ($P = 0.093$; Figure 2). However, they did show a preference for plants that had been treated with the 5-g syringa extract ($P < 0.001$; Figure 2). When lower doses of the syringa extract were tested, *C. plutellae* still showed a clear preference for the 3-g syringa dose ($P = 0.029$). Differences were not significant at the lowest 1-g dose ($P = 0.052$; Figure 2). For further experiments with *C. plutellae*, the highest syringa dose (5 g) was used.

Diadromus collaris did not show a preference for the control or the treated cabbage plant for either of the botanical pesticides [neem, $P = 0.37$; syringa (5-g dose), $P = 0.36$].

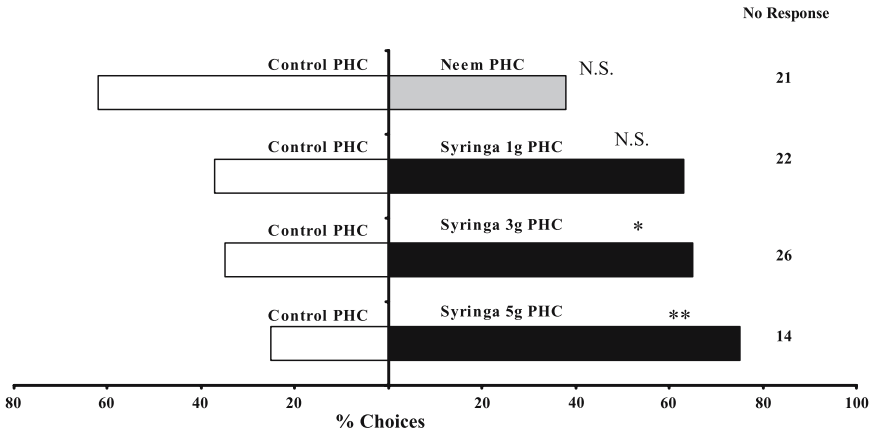


FIG. 2. Effect of syringa extract and neem on the response of female *Cotesia plutellae* to cabbage plants infested with *Plutella xylostella*. Each plant was sprayed with the control or the botanical pesticide and then infested with *P. xylostella*. Parasitoids were exposed to the entire plant–host complex (PHC). Percentage indicates total number of landings on target per treatment group (no. of responding wasps = 60). Significant differences are indicated in the graph (binomial test, N.S., $P > 0.05$; * $P < 0.05$; *** $P < 0.001$). Number of females not responding is indicated on the right side of the figure.

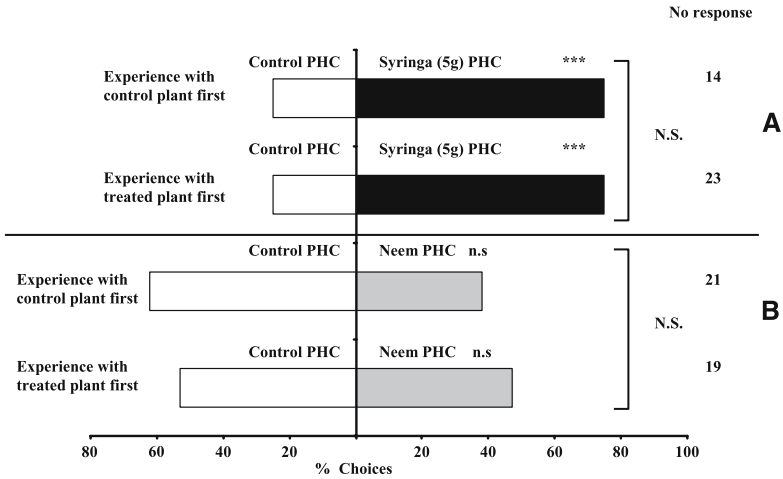


FIG. 3. Effect of previous experience on the response of female *Cotesia plutellae* to cabbage plants infested with *Plutella xylostella*. (A) Response to plants treated with syringa extract (5-g dose); (B) response to plants treated with neem. Each plant was sprayed with the control or the botanical pesticide and then infested with *P. xylostella*. Parasitoids were exposed to the entire PHC. Percentage indicates total number of landings on target per treatment group (no. of responding wasps = 60). Significant differences are indicated in the graph (χ^2 , N.S., $P > 0.05$; binomial test: ns $P > 0.05$; *** $P < 0.001$). Number of females not responding is indicated on the right side of the figure.

Effect of Experience. The previous experience of female *Cotesia plutellae* with treated or control cabbage plants did not affect the response (neem, $\chi^2 = 0.93$, $df = 1$, $P = 0.64$; syringa, $\chi^2 = 1.679$, $df = 1$, $P = 0.44$; Figure 3A and B). Similar results were found for *Diadromus collaris*; the previous experience of the female with treated or control cabbage plants did not influence the subsequent behavior (neem, $\chi^2 = 0.031$, $df = 1$, $P = 0.97$; syringa, $\chi^2 = 0.304$, $df = 1$, $P = 0.86$; Figure 4A and B).

Response of Pupal Parasitoid. *Diadromus collaris* did not respond well in the wind tunnel, which is clear from the large number of “no responses” ($\pm 50\%$; Figure 4A and B). When damaged and undamaged cabbage plants were compared, females did not distinguish between them ($P = 1.0$), and less than 50% responded (Figure 4C). We, therefore, abandoned this parasitoid species for the remaining trials.

Damaged Plants without Hosts. Plants were sprayed with the botanical extract or control solution and damaged by *Plutella xylostella*, but before exposure to *Cotesia plutellae* in the wind tunnel, the *P. xylostella* larvae were removed. The removal of the hosts did not alter the response of the parasitoid.

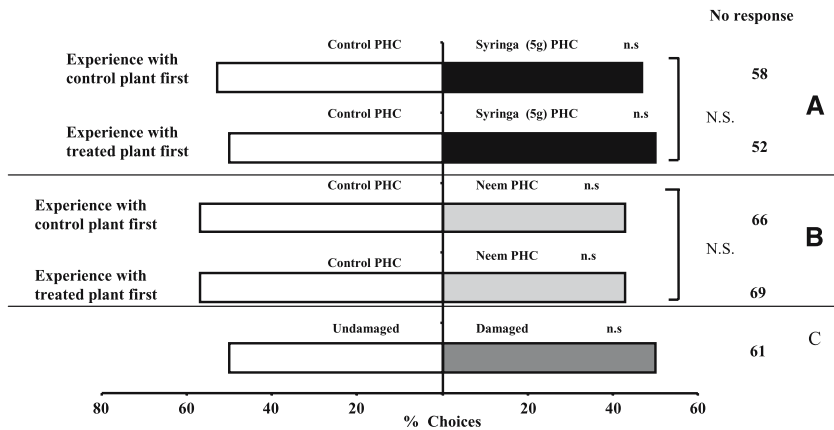


FIG. 4. Effect of previous experience on the response of female *Diadromus collaris* to cabbage plants infested with *Plutella xylostella*. (A) Response to plants treated with syringa extract (5-g dose); (B) response to plants treated with neem. Each plant was sprayed with the control or the botanical pesticide and then infested with *P. xylostella*. Parasitoids were exposed to the entire PHC. (C) Caterpillar damaged vs. undamaged cabbage plants; these plants were not sprayed with any treatment. Percentage indicates total number of landings on target per treatment group (no. of responding wasps = 60). Significant differences are indicated in the graph (χ^2 , N.S., $P > 0.05$; binomial test: ns, $P > 0.05$). Number of females not responding is indicated on the right side of the figure.

Again, *C. plutellae* showed a preference for the plants that had been treated with the syringa extract ($P < 0.001$), whereas they did not for the treated or control plants if the plants had been sprayed with the neem extract ($P = 0.52$; Figure 5A).

Equally Damaged Plants without Hosts. To create equally damaged plants, the plants were infested with *Plutella xylostella* larvae for 24 hr, larvae were removed, and then plants were sprayed with botanical extract or control solution. *Cotesia plutellae* did not show a preference for the treated or the control plant when the plants had been treated with the neem extract ($P = 0.7$), but they again showed a highly significant preference for plants that had been treated with syringa extract ($P < 0.001$; Figure 5B).

Undamaged Plants and Filter Paper. *Cotesia plutellae* was exposed to undamaged cabbage plants that had been sprayed with the botanical extracts or the control, or to filter paper that had been dipped in the treatments. Despite the lack of damage, *C. plutellae* still showed a preference for plants that had been treated with the syringa extract, except at the lowest dose (1-g dose, $P = 0.092$; 3-g dose, $P = 0.027$; 5-g dose, $P < 0.001$). Again, there was no effect of treatment with the neem solution ($P = 0.52$; Figure 6). The number of females

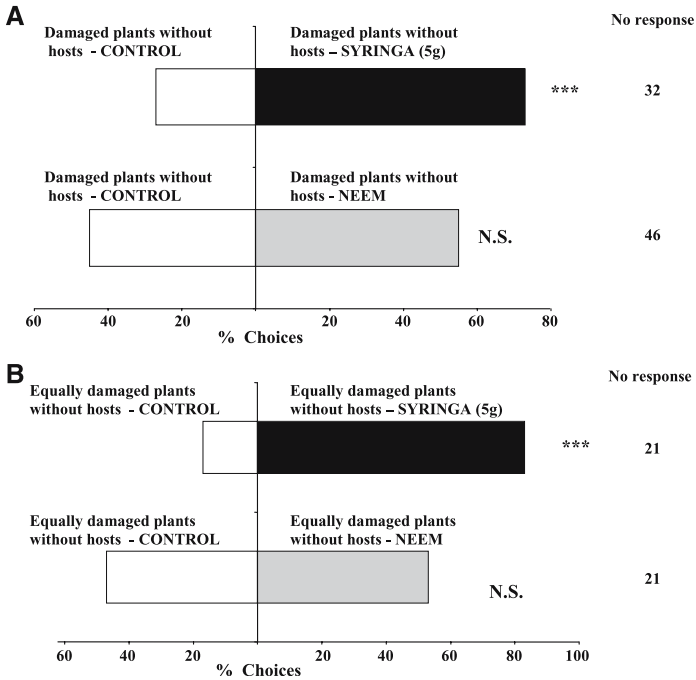


FIG. 5. Effect of syringa (5-g dose) and neem on the response of female *Cotesia plutellae* to treated cabbage plants. Percentage indicates total number of landings on target per treatment group (no. of responding wasps = 60). Significant differences are indicated in the graph (binomial test, N.S., $P > 0.05$; *** $P < 0.001$). Number of females not responding is indicated on the right side of the figure. (A) Cabbage plants had previously been infested with *Plutella xylostella* hosts. Each plant was sprayed with the control or the botanical pesticide and then infested with *P. xylostella*. The *P. xylostella* hosts were removed just before the cabbage plants were exposed to the parasitoids. (B) Cabbage plants were equally damaged. Each plant was infested with *P. xylostella* larvae for 24 hr, the larvae were removed, and then the plants were sprayed with the botanical pesticide or the control.

responding to undamaged plants was less than the number responding to caterpillar-damaged plants.

The attraction of the parasitoid to the syringa extract was lost when it was offered on filter paper instead of cabbage plants ($P = 0.70$; Figure 6).

Analysis of Plant Volatiles. Only compounds that were detected in two or more samples per treatment were included in the analysis. Headspace compositions of the control cabbage and syringa-treated cabbage were clearly different. The PCA resulted in a model with three significant principal factors,

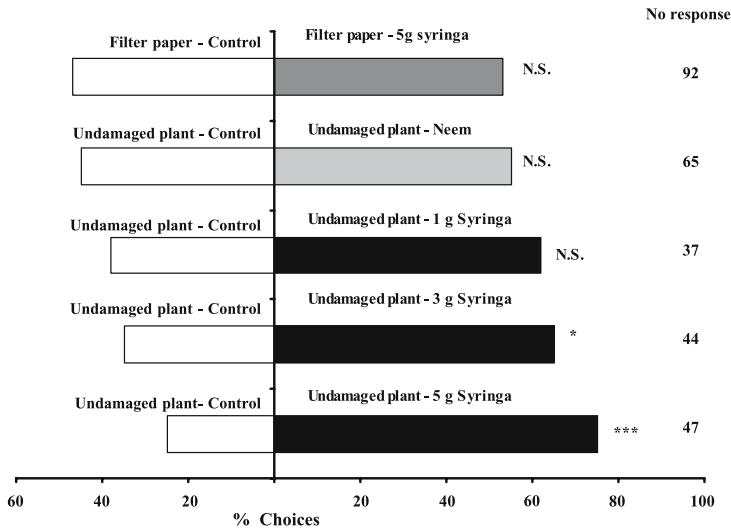


FIG. 6. Effect of syringa and neem on the response of female *Cotesia plutellae* to undamaged cabbage plants or to filter paper dipped in the highest dose of the syringa treatment. Each plant was sprayed with the control or the botanical pesticide, but plants were not infested with *Plutella xylostella*. The filter paper was dipped into the control or the syringa extract (5-g dose). Percentage indicates total number of landings on target per treatment group (no. of responding wasps = 60). Significant differences are indicated in the graph (binomial test, N.S. $P > 0.05$; * $P < 0.05$; *** $P < 0.001$). Number of females not responding is indicated on the right side of the figure.

explaining 72.5% of the variance in the data (Figure 7). A total of 49 compounds, alcohols, aldehydes, ketones, esters, terpenoids, sulfides, and an isothiocyanate were found in both control and treated plants (Table 1). Fifteen of the volatiles in the headspace from cabbage plants that had been treated with the syringa extract were also present in the syringa extract itself: four alcohols, two aldehydes, two esters, two ketones, three terpenoids, and two unidentified compounds (Table 1). Six additional compounds were found in plants that had been treated with syringa extract but not in control cabbage plants (Table 1). The syringa extract itself produced 25 compounds that were not found in the control plants or in the cabbage plants that had been treated with the syringa extract (Table 2). The total quantities of volatiles produced by plants that had been treated with the syringa extract were significantly higher than the quantities produced by control plants: of the 49 compounds, 38 compounds were emitted in larger quantities (based on mean peak areas) by syringa treated plants, and 11 were emitted in larger quantities by control plants (sign test, $P < 0.001$).

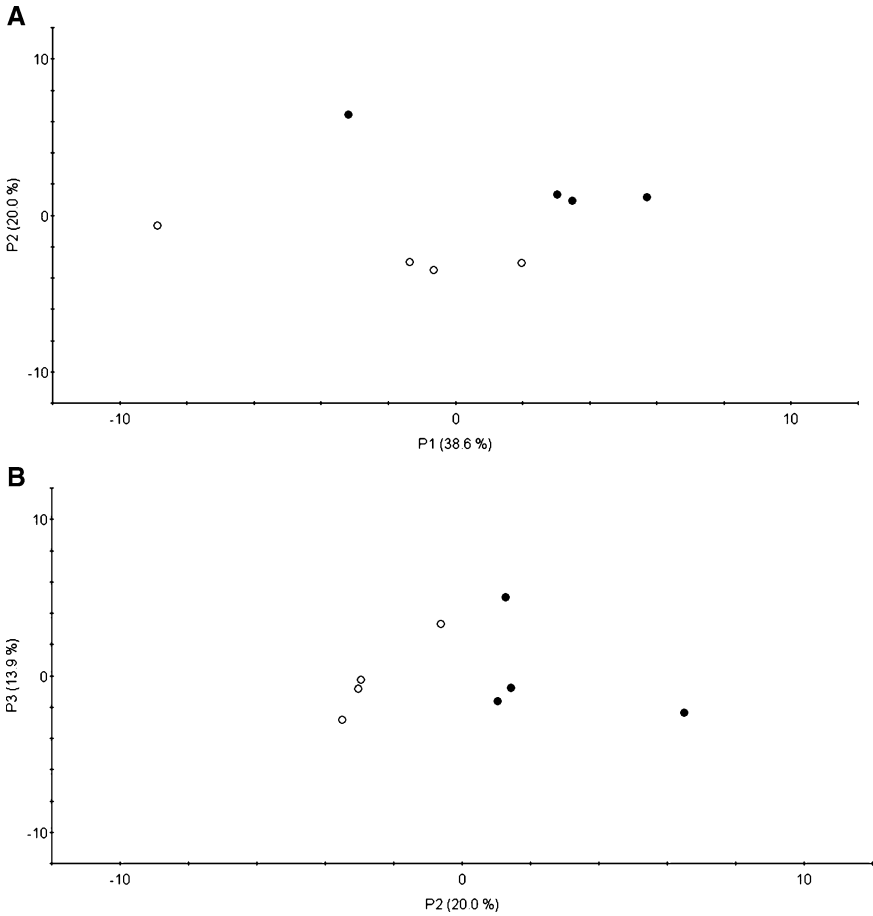


FIG. 7. Principal component analysis of the volatile patterns of differently treated cabbage plants. Score plots for the first (P1) vs. second (P2) significant principle factor (A) and for the second (P2) vs. the third (P3) significant principle factor (B) from partial-least-squares discriminant analysis based on absolute amounts of volatile constituents. Open circles represent samples of control cabbage plants, and filled circles represent samples for syringa-treated cabbage plants.

DISCUSSION

After an oviposition experience, *Cotesia plutellae* responded well in the wind tunnel. Females did not distinguish between cabbage plants that had been treated with the neem solution and plants that had been sprayed with the control.

TABLE 1. MEAN (\pm SE) OF GC PEAK AREA (ARBITRARY UNITS) FOR COMPOUNDS DETECTED IN HEADSPACE OF CONTROL CABBAGE PLANTS AND CABBAGE PLANTS TREATED WITH AQUEOUS LEAF EXTRACTS FROM *Melia Azedarach*

Number	Cabbage volatiles	Control cabbage	N	Treated cabbage	N	Syringa extract	N
<i>Alcohols</i>							
1	1-Hexanol ^a	8.30 \pm 3.33	4	13.00 \pm 1.15	3	69.00 \pm 11.63	4
2	2-Ethyl hexanol ^a	624.00 \pm 89.54	4	807.50 \pm 3.50	2		
3	1-Pentanol ^a	4.25 \pm 1.11	4	7.53 \pm 0.32	4	100.75 \pm 22.35	4
4	1-Penten-3-ol ^a	193.75 \pm 69.28	4	339.25 \pm 7.23	4	459.50 \pm 47.06	4
5	3-Hexen-1-ol ^a	69.25 \pm 29.68	4	113.50 \pm 12.58	4	3.08 \pm 0.46	4
<i>Aldehydes</i>							
6	2-Ethyl hexanal ^a	52.75 \pm 8.67	4	177.50 \pm 66.19	4	364 \pm 43.58	4
7	Hexanal ^a	9.25 \pm 1.38	4	14.50 \pm 1.55	4	343.50 \pm 69.50	4
<i>Esters</i>							
8	1-Butanol-3methyl acetate	2.65 \pm 1.05	2	2.38 \pm 0.60	4		
9	2-Penten-1-ol acetate ^a	47.10 \pm 24.15	4	92.00 \pm 16.05	4		
10	3-Hexen-1-ol acetate ^a	420.25 \pm 116.57	4	608.75 \pm 39.70	4	10.57 \pm 2.69	3
11	3-Hexen-1-ol-propanoate ^a	1.13 \pm 0.38	3	2.45 \pm 0.75	2		
12	2-Ethyl acetate ^a	580.25 \pm 86.18	4	733.20 \pm 410.67	3		
13	Acetic acid pentyl ester ^a	2.95 \pm 0.91	4	6.15 \pm 0.85	2	11.4 \pm 3.29	4
14	Heptyl acetate ^a	2.25 \pm 0.67	4	7.03 \pm 0.54	3		
15	Methyl salicylate ^a	2.40 \pm 1.00	3	5.25 \pm 2.21	4		
<i>Isothiocyanate</i>							
16	Methyl isothiocyanate	8.65 \pm 2.25	4	8.33 \pm 1.09	4		
<i>Ketones</i>							
17	2-Heptanone ^a	3.75 \pm 0.75	2	10.03 \pm 1.23	4	36.25 \pm 8.06	4
18	3-Heptanone ^a	15.75 \pm 3.17	4	52.50 \pm 10.84	4	311.75 \pm 49.43	4
19	3-Pentanone ^a	101.75 \pm 38.10	4	244.50 \pm 32.85	4		
<i>Sulfides</i>							
20	Dimethyl disulfide ^a	28.50 \pm 12.51	4	42.75 \pm 6.61	4		
21	Dimethyl trisulfide ^a	4.05 \pm 1.41	4	10.68 \pm 2.90	4		
<i>Terpenoids</i>							
22	1,8-Cineole	167.00 \pm 52.25	4	150.00 \pm 30.82	4	1.03 \pm 0.23	4
23	2- β -Pinene	77.75 \pm 22.40	4	57.75 \pm 8.81	4		
24	α -Phellandrene ^a	1.73 \pm 0.32	3	18.35 \pm 17.22	4		

TABLE 1. CONTINUED

Number	Cabbage volatiles	Control cabbage	<i>N</i>	Treated cabbage	<i>N</i>	Syringa extract	<i>N</i>
25	α -Terpinolene ^a	0.70 ± 0.15	3	15.63 ± 15.18	3		
26	α -Pinene	201.00 ± 57.79	4	186.50 ± 37.38	4		
27	α -Thujene	206.25 ± 63.60	4	185.75 ± 30.03	4		
28	β -Myrcene ^a	5.73 ± 1.07	3	33.28 ± 29.58	4		
29	γ -Terpinene ^a	295.50 ± 53.45	4	303.75 ± 41.35	4	5.25 ± 0.55	2
30	l-Limonene	4.15 ± 0.55	2	3.23 ± 0.35	3		
31	Mix α -terpineol	507.00 ± 76.95	4	392.50 ± 111.45	4		
32	(<i>E</i>)- β -Ocimene ^a	4.00 ± 1.56	4	7.98 ± 4.39	4		
33	α -Terpinene ^a	2.15 ± 0.15	2	2.58 ± 0.32	4		
34	Sabinene ^a	2.60 ± 0.45	4	7.48 ± 3.58	4		
35	<i>trans</i> -4-thujanol	17.25 ± 5.57	4	13.57 ± 2.43	3		
36	Unknown terpenoid 1 <i>Others</i>	106.00 ± 28.54	4	81.00 ± 11.60	4	4.93 ± 0.77	4
37	Unknown 1 ^a	28.00 ± 1.00	3	44.67 ± 13.68	3		
38	Unknown 2 ^a	1.38 ± 0.25	4	2.25 ± 0.38	4		
39	Unknown 3 ^a	56.75 ± 23.78	4	105.75 ± 24.87	4		
40	Unknown 4 ^a	8.30 ± 0.85	3	27.25 ± 3.30	4		
41	Unknown 5 ^a	23.00 ± 8.74	3	40.67 ± 12.99	3	12.50 ± 0.50	2
42	Unknown 6 ^a	4.93 ± 1.07	3	6.70 ± 2.10	2		
43	Unknown 7	1.20 ± 0.20	2	1.03 ± 0.17	4		
44	Unknown 8 ^a	1.47 ± 0.26	3	5.77 ± 0.23	3		
45	Unknown 9 ^a	25.67 ± 15.27	2	32.70 ± 18.18	3		
46	Unknown 10 ^a	27.33 ± 11.26	3	29.63 ± 15.65	3		
47	Unknown 11 ^a	51.67 ± 9.60	3	481.33 ± 150.93	3	631.67 ± 65.17	3
48	Unknown 12 ^a	13.00 ± 4.56	4	28.00 ± 3.46	3		
49	Unknown 13 ^a	17.25 ± 8.02	4	31.67 ± 4.48	3		
Compounds only found in cabbage plants treated with syringa					<i>N</i>		
1	Methyl pyrrole			18.40 ± 11.60	2		
2	3-Methyl butanal			9.00 ± 1.57	3		
3	Quinhydrone			6.83 ± 2.04	4		
4	1-Octen-3-ol			4.23 ± 0.69	3		
5	Benzoxazole			2.65 ± 0.75	2		
6	<i>trans</i> -Caryophyllene			1.85 ± 0.55	2		

N = no. of samples out of 4, in which the compound had been identified.

^aCompounds emitted in larger amounts by syringa-treated plants (sign test, $P < 0.001$).

TABLE 2. MEAN (\pm SE) OF GC PEAK AREAS (ARBITRARY UNITS) FOR COMPOUNDS DETECTED IN HEADSPACE OF SYRINGA EXTRACT ONLY

Number	Compounds only in Syringa	Syringa	N
	<i>Alcohols</i>		
1	3-Methyl-1-butanol	34.00 \pm 8.33	3
2	1-Nonanol	11.13 \pm 2.58	4
	<i>Aldehydes</i>		
3	(<i>E</i>)-2-Hexenal	8.58 \pm 2.00	4
4	2-Pentenal	124.75 \pm 31.68	4
	<i>Ketone</i>		
5	6-Methyl-5-hepten-2-one	184.50 \pm 41.05	4
	<i>Others</i>		
6	Unknown 14	18.15 \pm 4.66	4
7	Unknown 15	24.00 \pm 5.03	3
8	Unknown 16	116.25 \pm 27.16	4
9	Unknown 17	52.50 \pm 10.25	4
10	Unknown 18	132.75 \pm 10.35	4
11	Unknown 19	3.43 \pm 1.15	3
12	Unknown 20	11.00 \pm 1.00	3
13	Unknown 21	16.53 \pm 8.24	3
14	Unknown 22	8.47 \pm 3.50	3
15	Unknown 23	12.93 \pm 2.15	3
16	Unknown 24	11.60 \pm 3.24	3
17	Unknown 25	13.25 \pm 2.21	4
18	Unknown 26	19.33 \pm 0.67	3
19	Unknown 27	1.33 \pm 0.35	4
20	Unknown 28	311.50 \pm 57.12	4
21	Unknown 29	3.43 \pm 1.04	4
22	Unknown 30	4.98 \pm 1.27	4
23	Unknown 31	483.00 \pm 84.00	2
24	Unknown terpenoid 2	23.50 \pm 5.24	4
25	Unknown terpenoid 3	4.60 \pm 3.70	3

N = no. of samples out of 4, in which the compound had been identified.

However, *C. plutellae* always preferred cabbage plants that had been sprayed with the syringa extract to the control plants, except at the lowest dose. *C. plutellae* appears to detect and respond differently to volatiles from plants treated with these two botanical extracts. Akol et al. (2003) have shown that *Diadegma molipla* is able to detect and distinguish between volatiles emitted by cabbage plants sprayed with two different neem formulations. In their experiment, they found that a neem seed oil formulation had a negative effect on the foraging of *D. molipla*, as females significantly preferred volatiles from control plants over those from plants sprayed with neem formulation. However, when *D. molipla* had a choice between plants sprayed with a solution from a neem kernel cake powder and control plants, they did not distinguish between

sprayed and control plants (Akol et al., 2003). *Uscana lariophaga*, an egg parasitoid of the bruchid *Callosobruchus maculatus*, was repelled by neem seed oils on cowpea beans, but the larval parasitoid *Dinarmus basalis* did not discriminate between control and neem-treated beans (Boeke, 2002; Boeke et al., 2003). In our study, the neem formulation Neemix 4.5[®] did not appear to have an adverse effect on foraging by *C. plutellae*.

Pre-flight experience can have a distinct effect on parasitoid behavior (reviewed by Turlings et al., 1993 and Vet et al., 1995). However, learning is thought to have a less important role for specialist parasitoids (Geervliet et al., 1998). Both *Cotesia plutellae* and *Diadromus collaris* are relatively specialized parasitoids attacking mainly *Plutella xylostella*, and results from this study show no indication that these parasitoids acquired a response to syringa through associative learning.

The response of *Cotesia plutellae* to volatiles from herbivore-damaged plants that had been treated with syringa extract was independent of the presence of host larvae. Previous studies have also shown that herbivore-damaged plants are attractive to *C. plutellae* (Shiojiri et al., 2001; Vuorinen et al., 2004) even after removal of the hosts (Potting et al., 1999).

Schuler et al. (1999, 2003) found that the amount of damage was an important factor influencing the flight responses of *Cotesia plutellae*. In previous studies, we have shown that *Plutella xylostella* damage is lower on cabbage plants treated with botanical extracts (Charleston, 2004). However, our data show that the response of *C. plutellae* was similar whether the cabbage plants had been sprayed with the botanical extracts first or whether plants were first damaged by *P. xylostella* larvae and then sprayed with the botanical extract. The difference in feeding damage between the treated and control cabbages may have been too small to affect the flight response of *C. plutellae*.

The pupal parasitoid *Diadromus collaris* was not attracted to volatiles emitted by cabbage plants in the wind tunnel and did not distinguish between caterpillar-damaged and undamaged plants. *D. collaris* is a pupal parasitoid, and while there is a wealth of knowledge available on the role of volatile cues used by parasitoids attacking larval stages of herbivores (Dicke and Vet, 1999), there is not much information about the chemical cues used by pupal parasitoids (Vet et al., 1995). Some pupal parasitoids have been shown to respond to plant volatiles. The stemborer pupal parasitoid *Dentichasmias busseolae*, for example, makes use of plant volatiles from maize and sorghum and is particularly attracted to herbivore-damaged plants (Gohole et al., 2003). For pupal parasitoids, as for all parasitoids, the possibilities of using direct host-derived cues are limited, and in addition, larvae often pupate away from the site of damage, restricting the use of predictable indirect cues by pupal parasitoids (Vet et al., 1995). The opportunity of using volatiles from larval feeding damage is limited to situations where the pupae stay in or on the plant, and larval and pupal stages co-occur. *Plutella xylostella* tend to pupate on the plant (although not directly

near feeding damage), and, because of fast development and overlapping generations, larval and pupal stages are often found together in the field. Hence, *D. collaris* could make use of plant volatiles to find its host. Electroantennogram (EAG) studies indicate that *D. collaris* does respond to cabbage volatiles (Lecomte and Pouzat, 1985), which further suggests that this parasitoid may make use of plant volatiles. However, in our studies, *D. collaris* did not appear to use herbivore-induced plant volatiles. Therefore, it is possible that *D. collaris* makes use of other strategies to find its host. *Cotesia plutellae* was attracted to cabbage plants treated with syringa extract. Even undamaged cabbage plants treated with syringa extract attracted the parasitoids. In contrast, *C. plutellae* did not show a preference for filter paper dipped in the syringa extract. Headspace analysis yielded a total of 49 compounds from both undamaged control cabbage plants and undamaged cabbage plants that had been treated with syringa extract. The volatile bouquet emitted after treatment with syringa extract is composed of the same components as emitted by untreated cabbage plants. This is comparable to what happens in response to herbivory: the composition of the bouquet is qualitatively similar to that emitted by intact or mechanically damaged plants, and consists of fatty acid derivatives, terpenoids, and a few sulfur-containing compounds such as methyl isothiocyanate and sulfides. However, the quantity of volatiles was significantly higher in the plants treated with the syringa extract. Some of the volatiles found in cabbage plants treated with the syringa extract were also present in the syringa extract itself. Yet, many of the volatiles emitted by the syringa extract were not emitted, or were only emitted at low rates, from cabbage plants treated with the syringa extract. This indicates that the enhanced emission of the plant volatiles from cabbage plants treated with the extract is caused by an induction of these volatiles in cabbage rather than an evaporation of the syringa extract from the treated plants. To our knowledge this is the first example of a plant extract causing an increased emission of plant volatiles, which is likely to explain the significant attraction of *C. plutellae* to plants that have been treated with the syringa extract. Which of the emitted volatiles attract the parasitoids remains to be elucidated. For crucifer–parasitoid interactions, a gas chromatography (GC)–EAG approach has limited the total number of compounds to be tested in behavioral tests to about 20 for the parasitoids *Cotesia glomerata* and *Cotesia rubecula* (Smid et al., 2002). A recent field study in an open field and a hop yard showed that 11 carnivorous arthropods were attracted to 13 herbivore-induced plant volatiles (James, 2005).

It is well-known that elicitors can induce parasitoid-attracting plant volatiles. Among these elicitors are, for example, compounds from herbivore regurgitant (Alborn et al., 1997; Mattiacci et al., 1995) or phytohormones (Dicke et al., 1999; Gols et al., 1999; Ozawa et al., 2000; Horiuchi et al., 2001). Furthermore, there is evidence that plants can affect the emission of volatiles in downwind neighboring plants (Dicke and Bruin, 2001; Pichersky and

Gershenzon, 2002; Choh et al., 2004; Engelberth et al., 2004). Some green leaf volatiles, i.e., (*Z*)-3-hexenol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate, induce gene expression in plants that are exposed to these compounds in an airtight container (Arimura et al., 2001). We have recorded (*E*)-2-hexenal in the headspace of the syringa extract. However, whether this compound is responsible for inducing the emission of parasitoid attractants in cabbage remains to be investigated.

Our data show that treatment of cabbage with syringa extract or Neemix 4.5[®] would not impair the process of host habitat location by *Cotesia plutellae* or *Diadromus collaris*. In fact, *C. plutellae* was always attracted to cabbage plants treated with the syringa extract, even when the plants were undamaged, which may indicate an interaction between a plant and a botanical extract that enhances natural enemy activity. This may have negative implications for biological control. If the parasitoids do not discriminate between infested and uninfested plants, then they may waste time searching uninfested plants, which may result in a reduction of the parasitization rate (Dicke et al., 1990). Treatment of plants with the plant hormone jasmonic acid results in an increased emission of plant volatiles, and this results in an attraction of parasitoids and predators (Dicke et al., 1999; Gols et al., 1999, 2003; Thaler, 1999; Ozawa et al., 2004). Yet, in field-grown tomatoes, a blanket treatment with jasmonic acid resulted in higher parasitization levels of *Spodoptera exigua* compared to control plots (Thaler, 1999). In our study, the parasitoids were only given a short preoviposition experience with cabbage leaves that were infested with host insects and were not given the opportunity to discriminate between damaged and undamaged plants. This is not representative for most foraging decisions in nature, where previous foraging experiences, both positive and negative, can be integrated (Vet et al., 1998; Dicke, 1999b). Under field conditions, infested and uninfested plants are likely to be in close proximity, and the parasitoid may soon learn to discriminate. It would be interesting to expand these observations to investigate the impact that these botanical pesticides have on parasitoid foraging behavior in field situations.

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A NEW POTENTIAL ATTRACTANT FOR *Anastrepha obliqua* FROM *Spondias mombin* FRUITS

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Abstract—Nonirradiated males and females of *Anastrepha obliqua* (Macquart) were attracted to and landed more frequently on ripe fruits of *Spondias mombin* L. than on artificial fruit in wind tunnel bioassays. Porapak Q volatile extracts of *S. mombin* were also attractive and elicited landing on artificial fruit for both sexes. Combined gas chromatographic–electroantennographic detection (GC-EAD) analysis of volatile extracts showed that nine volatile compounds elicited repeatable antennal responses from females and males. The EAD-active compounds were identified by GC–mass spectrometry (MS) as follows: ethyl butyrate, isopropyl butyrate, hexan-1-ol, propyl butyrate, isobutyl butyrate, ethyl hexanoate, isopentyl butyrate, ethyl benzoate, and ethyl octanoate. In wind tunnel bioassays, males and females were attracted and landed more frequently on lures containing the nine-component blend of synthetic compounds than on unscented controls. Field cage bioassays showed that multilure traps baited with the nine-synthetic blend captured significantly more *A. obliqua* than traps baited with hydrolyzed protein or water.

Key Words—*Anastrepha obliqua*, mombin fruit, attractants, host volatiles, gas chromatographic–electroantennographic detection, wind tunnel.

INTRODUCTION

The West Indian fruit fly *Anastrepha obliqua* (Macquart) (Diptera: Tephritidae) has a wide distribution in the Americas. It has been recorded from the USA

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(Florida and Texas) to South America, including the Caribbean Islands (Hernández-Ortiz and Aluja, 1993). *A. obliqua* is a highly polyphagous insect attacking fruits of 13 families (Norrbom et al., 2000), but females usually prefer to oviposit in fruit belonging to the Anacardiaceae family (Jirón et al., 1988; Hernández-Ortiz and Aluja 1993).

In Mexico, *A. obliqua* is considered a key pest of mango (*Mangifera indica* L.) and mombin (hogplum) fruits (*Spondias* spp.; Hernández-Ortiz and Aluja, 1993), making the production of some varieties unprofitable. Moreover, its presence in mango orchards is motive for strict quarantine from the USA, the main importer of the Mexican mango (APHIS, 1994). The most widely used system for monitoring *Anastrepha* fruit flies during the last 35 yr consists of glass and plastic versions of the McPhail trap containing a mixture of protein and water (Aluja, 1994). However, difficulties in handling, low capture efficiency, and capture of nontarget organisms (Epsky et al., 1993; Thomas et al., 2001) lead to investigations of potential attractants from different sources such as bacteria, bird feces, and fruits (e.g., Robacker et al., 1990, 1992; Nigg et al., 1994; Epsky et al., 1997). Most of the effort in the search for attractants of *Anastrepha* flies has concentrated on *Anastrepha ludens* (Loew) and *A. suspensa* (Loew), and few studies have been carried out with *A. obliqua* (Hedström, 1988; Ortega-Zaleta and Cabrera-Mireles, 1996; DeMilo et al., 1997). Ortega-Zaleta and Cabrera-Mireles (1996) evaluated the attractiveness of pineapple (*Ananas comosus* Merr.) juice, fermented pineapple juice, cow milk serum, and ether and ethanol extracts of *Spondias purpurea* L., *Spondias mombin* L., and *M. indica* as potential attractants for *A. obliqua*. They found that only the ethanolic extract of *S. purpurea* was more attractive than hydrolyzed protein when evaluated in a mango orchard.

We conducted this study to search for potential attractants in the fruit of *S. mombin*, which is a native and highly preferred host of *A. obliqua* (Hernández-Ortiz and Aluja, 1993). The objectives were to (1) evaluate the attraction of *A. obliqua* to ripe fruits of *S. mombin* and its volatile extracts in a wind tunnel, (2) locate the electrophysiological active compounds from fruit volatile extracts using gas chromatographic–electroantennographic detection (GC-EAD), (3) identify chemically the EAD-active compounds by GC-MS, and (4) evaluate the response of the flies to synthetic compounds in wind tunnel and field cage tests.

METHODS AND MATERIALS

Insects. Flies used for experiments came from a strain obtained from the Subtropical Agricultural Research Laboratory (ARS-USDA, Weslaco, TX). Insects were originally collected from infested *S. mombin* fruits in the State of

Veracruz, Mexico (Moreno et al., 1997). Prior to our experiments, this strain had been reared on an artificial diet for at least 110 generations at the Moscafrut mass-rearing facilities (SAGARPA-IICA) located in Metapa de Dominguez, Chiapas, without subsequent introductions of wild material (Dominguez et al., 2000). After emergence, fertile adults were placed in wooden cages with mesh sides ($65 \times 65 \times 45$ cm) and kept at $25 \pm 1^\circ\text{C}$, 60–70% relative humidity, and 12-hr light/12-hr dark photoperiod. Adults were fed *ad libitum* with a mixture of enzymatic yeast hydrolysate (ICN Biomedical, Costa Mesa, CA, USA), sucrose (1:3), and water provided in test tubes covered with cotton wicks. Males and females of 10–19 d old and 7–13 d old were used for laboratory assays (wind tunnel and GC-EAD analysis) and field cage tests, respectively.

Fruits. We obtained fruit specimens from mombin trees located near Tapachula, Chiapas, Mexico. Fruits were collected when they were orange in color (ripe), placed in plastic bags, and immediately carried to the laboratory for bioassays and volatile collection. To quantify the degree of ripeness more accurately, we measured the Brix value to determine sugar content. This value is used to measure liquid density, especially sugar concentration in fruit and vegetable juices (Hogness and Jones, 1984). The Brix values of the mombin fruits used ranged from 14.0 to 16.5 as measured by a manual refractometer (Iroscope, Mexico City).

Volatile Collection. We collected fruit volatiles using the dynamic head-space technique. Ripe healthy mombin fruits (400 g) were placed into a cylindrical glass aeration chamber (58 cm long \times 18.5 cm i.d.). A charcoal-filtered air stream (1 l/min) was maintained through the glass aeration chamber for 16 hr. Fruit volatiles were collected on 350 mg of Porapak Q (50–80 mesh, Water Associates, Inc., Milford, MA, USA) packed between plugs of silanized glass wool in a Pasteur pipette. The Porapak Q was previously cleaned using the procedure of Blight (1990). Volatiles were eluted from the Porapak Q with 2 ml of diethyl ether (HPLC grade, Aldrich, Toluca, Mexico). Final concentration to 600 μl was made by slow evaporation under a gentle stream of nitrogen. The extract was stored at a -20°C until bioassays, and chemical analysis could be performed. Samples were collected from five different lots of mombin fruits.

Wind Tunnel Bioassays. Behavioral observations were carried out in a flight wind tunnel, 120 cm long and 30 cm high and wide. The wind tunnel was constructed from 1 cm thick clear Plexiglas. A fan was used to pull air, filtered by activated charcoal, through the tunnel at a velocity of 0.4 m/sec. Illumination was provided by four fluorescent bulbs mounted 60 cm above the wind tunnel giving a light intensity of 2380 lx. We evaluated the insects in groups of 25 individuals (males or females) 18 hr after placing them in a plastic container (6 cm high \times 8-cm diam release cylinder) with screen top. Water, but no food, was provided in the containers. Flies were allowed to acclimatize in the wind tunnel under room condition ($25 \pm 1^\circ\text{C}$ and $60 \pm 5\%$ relative humidity) for at least 1 hr before

being assayed. In three different trials, the response of flies to host fruits, Porapak Q volatile extracts, and the synthetic blend was evaluated in no-choice tests. Polystyrene spheres (5-cm diam.), painted with vinyl acrylic water-based paints matching (as detected by the human eye) the orange color of the mombin fruit, were used for dispensing either the mombin extracts (second trial) or the synthetic blend (third trial), and as controls (in all trials). One hundred microliters of mombin extract (equivalent to 0.5 mg of fruit) or 1 mg of the synthetic blend prepared according to the relative proportions of each compound in the natural extracts (Table 1) were loaded on a rubber septum (Agrisense, England). The rubber septum was inserted into an orange-painted sphere. A rubber septum loaded with 100 μ l of diethyl ether and placed in an orange sphere was used as control. The target stimulus (e.g., fruit, unscented sphere, or scented sphere) was hung in the center of the wind tunnel, 16 cm from the upwind end.

Each observation was started by placing the release cylinder on a 12 cm high platform at the downwind end of the tunnel, and insects were released and observed for 10 min. The behavior of the insects was recorded during the test, specifically for upwind flight and for landing. Upwind movement was noted if insects passed a point two thirds of the distance from the release cylinder toward the odor source or control (Robacker and Fraser, 2002). Landing behavior was scored for either landing or walking onto the target stimuli. Generally, flies that responded remained in the upwind section of the tunnel or on the odor source for the rest of the bioassay. Ten replicates for each sex and treatment were carried out. The flies were only used once during the bioassays. All bioassays were conducted between 0800 and 1300 hr.

TABLE 1. EAD-ACTIVE COMPOUNDS FROM MOMBIN FRUIT DETERMINED BY INTEGRATION OF GC-MS TOTAL ION CURRENT USING ADSORBENT FIVE SAMPLES FROM RIPE FRUITS

Peak no. ^a	Compound	GC-MS mean (%)	WT and FC ^b
1	Ethyl butyrate	36.8	40
2	Isopropyl butyrate	0.8	1
3	Hexan-1-ol	0.8	1
4	Propyl butyrate	1.3	1
5	Isobutyl butyrate	3.6	5
6	Ethyl hexanoate	36.7	40
7	Isopentyl butyrate	3.8	5
8	Ethyl benzoate	12.6	15
9	Ethyl octanoate	3.6	5

^aNumbers correspond to labeled peaks in Figure 1.

^bWT and FC indicate the proportion of each compound in the synthetic blend used in wind tunnel and field cage experiments.

Chemicals. Synthetic chemicals were purchased from Sigma-Aldrich (Toluca, Mexico) and Bedoukian Research (Danbury, CT, USA), and the purities were >95% based on the results with the capillary gas chromatography.

Combined Gas Chromatographic-Electroantennographic Detection (GC-EAD). GC-EAD analysis (Arn et al., 1975) was carried out to locate the antennal active components from Porapak Q extracts. The system consisted of a gas chromatograph (Varian 3600, Palo Alto, CA, USA) coupled to an electroantennogram apparatus (Syntech, Hilversum, The Netherlands). The GC was equipped with a nonpolar DB-5 capillary column (30 m × 0.25 mm i.d., film thickness 0.5 µm; J&W Scientific, Folsom, CA, USA), a flame ionization detector (FID) and a split/splitless injector. The temperature oven was programmed at 50°C for 2 min, then 3°C/min to 280°C, and held for 10 min. Injector and detector temperatures were at 250 and 300°C, respectively. The injector was operated in splitless mode. Helium was used as carrier gas at 2 ml/min. At the end of the capillary column, a fixed outlet splitter (VSOS, Scientific Instruments Services, Ringoes, NJ, USA) distributed the effluent from the column to FID and to a transfer line towards the EAD preparation. Both connections were made of deactivated fused silica with the same length and diameter such that the column effluent was split in a ratio of 1 (EAG) to 1 (FID). Before injection of a sample, the antenna was stimulated with linalool to check sensitivity. If the antennae elicited a clear response different from background, then the mombin fruit extracts were injected. A minimum of 16 antennae per sex were used.

Chemical Analysis. Gas chromatography–mass spectrometry was conducted with a Varian Star 3400 CX gas chromatograph linked to a Varian Saturn 4D mass spectrometer (Palo Alto, CA, USA). The samples were analyzed using a nonpolar DB-5 capillary column (identical to the one used in the GC-EAD system described above) or a polar HP-FFAP capillary column (25 m × 0.25 mm i.d., film thickness 0.5 µm; J&W Scientific, Folsom, CA, USA). The temperature program was the same as that described for the GC-EAD analyses. The carrier gas was helium at constant flow rate (1.0 ml/min). The injector port temperature was held at 200°C. Compounds were identified by comparison of retention times and mass spectral matches of available standards. Quantification of the relative proportions of the EAD-active compounds was made from mombin extracts. Compounds quantification was made from the ion abundance from GC-MS analyses, or FID responses, according to the standard curves made from external standards of known concentrations.

Field Cage Tests. The attractiveness of the mombin blend was compared against hydrolyzed protein and water in no-choice and three-choice tests. Tests were performed in a seminatural condition using cylindrical clear nylon screen field cages (2.85-m diam. × 2 m high). In no-choice tests, one coffee (*Coffea arabica* L.) and mango-potted trees (1.20 m tall) were placed in the center of each cage. In three-choice tests, five coffee and six mango-potted trees were

placed inside of each cage, one tree of each species in each cardinal point, and two trees (one in the case of coffee) in the center of the cage. In no-choice tests, one Multilure trap (Better World Manufacturing Inc., Fresno, CA, USA) was placed 50 cm above the trees. In three-choice tests, three Multilure traps were placed 30 cm from the periphery of the cage with 185 cm between each trap. The traps were hung at 10 cm from the top of cage. The synthetic mombin blend was prepared according to the relative proportions in the Porapak extracts (Table 1), and 100 mg of this blend were loaded in rubber septa. The hydrolyzed protein Captor 300 (Promotora Agropecuaria Universal S.A. de C.V., Mexico City) was prepared using 5 g of borax mixed with 10 ml of hydrolyzed protein dissolved in 235 ml of water per trap. Water plus Tween 80 (ICI, Wilmington, DE, USA) was used to retain the flies in the traps baited with the synthetic mombin blend and water. About 25 (12 or 13 of each sex, in no-choice tests) and 150 flies (75 of each sex, in three-choice tests) with 15 hr of starvation were released in the center of the cage in the morning. The lures were placed in traps 15 min before the flies were released into the cage. Traps were hung from 0800 hr, and the number of insects caught by each trap was counted 24 hr later. Trap positions were rerandomized daily to reduce positional effects. During the period of the tests, the temperature ranged from 23 to 31°C and relative humidity from 60 to 95%. The experiments were replicated 12 times.

Data Analysis. All statistical analyses were carried out using the computer program Statistica (StatSoft, 2003). The wind tunnel data were analyzed using a *t* test to compare the proportions of fruit flies that were attracted and landed on odor source (fruit, extract, or synthetic blend) or sphere control. The proportions were transformed by arcsine of the square root before statistical analysis. Proportions of zero were replaced with $1/4n$ before transformation (Robacker and Fraser, 2002). Because an analysis by *t* test showed that the number of flies caught by traps did not significantly differ between sex in the field cage experiments, data for male and female were pooled and transformed with $\sqrt{x + 0.5}$

TABLE 2. MEAN RESPONSE (\pm SE) OF *Anastrepha obliqua* TO MOMBIN FRUITS AND SPHERE CONTROL, AND EXTRACT VOLATILES AND SPHERE CONTROL (WITH SOLVENT) WHEN OFFERING IN NO-CHOICE TESTS IN A WIND TUNNEL

Treatment	Moved upwind		Landed on source	
	Female	Male	Female	Male
Fruit	34.4 \pm 3.56b	24.0 \pm 3.44b	25.5 \pm 3.48b	9.2 \pm 2.3b
Control	11.2 \pm 1.64a	6.8 \pm 1.04a	2.0 \pm 1.04a	0.44 \pm 0.04a
Porapak Q extract	30.4 \pm 3.73b	20.8 \pm 2.94b	19.6 \pm 3.97b	9.4 \pm 1.67b
Control	12.0 \pm 1.68a	6.4 \pm 1.06a	6.8 \pm 1.68a	1.6 \pm 0.64a

Means followed by different letters within each comparison are significantly different at $P < 0.05$ level (*t* test). Total number of flies released was 250 per sex for each test.

before analyzing by one-way analysis of variance (ANOVA). When significant treatment effects were observed, means were separated by Tukey test ($P < 0.05$).

RESULTS

Response to Fruits and Porapak Q volatile Extracts. Flights consisted of either a general movement upwind towards the odor source or a direct upwind flight. In general movement upwind, females would often take flight from the platform, alight on the sides of the wind tunnel, and display cleaning of antennae and legs before resuming upwind flight. Oriented upwind flights were either straight line or a side-to-side zigzag movement. Sometimes, insects flew directly from the platform to the odor source. Flies that were not attracted normally flew to the floor, side, or top of the tunnel or remained in the release cylinder. Both

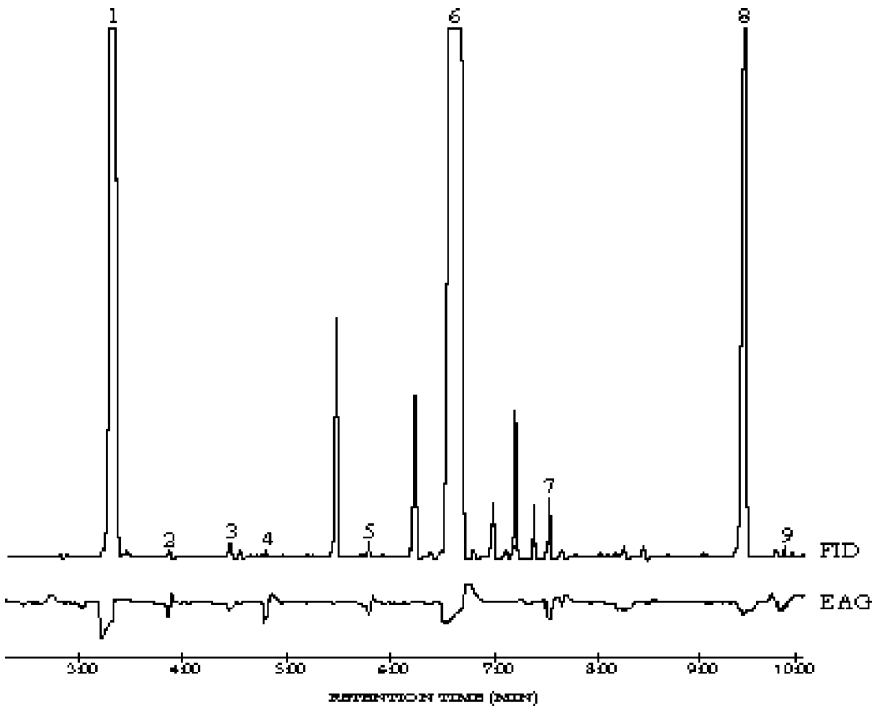


FIG. 1. Simultaneous responses of flame ionization detector (FID) and electroantennographic detection (EAD) using the antennae of female *Anastrepha obliqua* to headspace collected volatile from *Spondias mombin* fruits on a nonpolar capillary column. Numbered peaks indicate EAD-active compounds (see also Table 1).

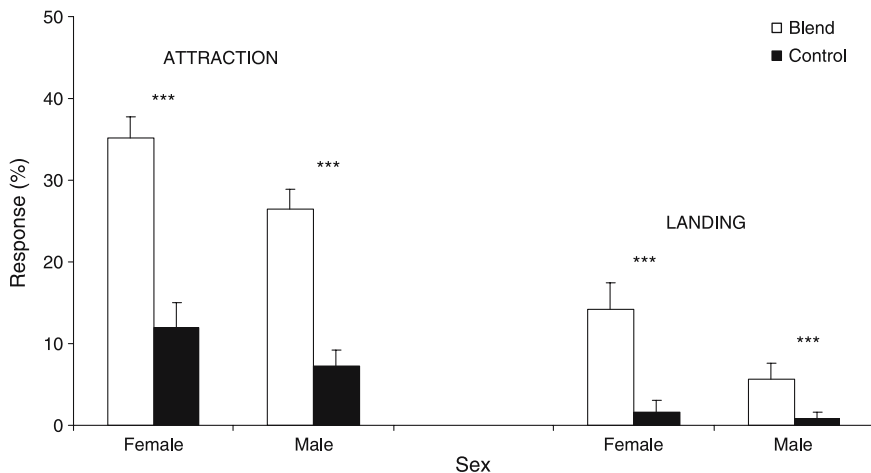


FIG. 2. Mean percent response (\pm SE) of *A. obliqua* to sphere dispensing the mombin synthetic blend and sphere control (with solvent) when offering in no-choice tests in a wind tunnel. Differences between paired bars (*t* test) indicated by ***, $P < 0.001$. Total number of flies released was 250 per sex for each test.

females ($t = 6.0$, $df = 18$, $P < 0.001$) and males ($t = 4.8$, $df = 18$, $P = 0.001$) were more attracted to mombin fruits than to artificial fruits. Similarly, both sexes (females: $t = 4.6$, $df = 18$, $P < 0.001$; males: $t = 4.6$, $df = 18$, $P < 0.001$) were more attracted to the Porapak Q volatile extracts than to solvent control. Landing was also observed more frequently on fruits (females: $t = 7.2$, $df = 18$, $P < 0.001$; males: $t = 3.7$, $df = 18$, $P = 0.001$) and extracts (females: $t = 2.7$, $df = 18$, $P = 0.015$; males: $t = 3.8$, $df = 18$, $P = 0.001$) than on controls (Table 2).

GC-EAD analysis. The analyses by GC-EAD of the headspace sample of *S. mombin* fruits showed that *A. obliqua* male and female antennae responded strongly (>1 mV) to three compounds (peaks 1, 4, and 6; Figure 1). Weaker (<1 mV) but repeatable responses were also found to six other components (peaks 2, 3, 5, 7, 8, and 9). The corresponding EAD-active compounds were identified as ethyl butyrate (peak 1), isopropyl butyrate (peak 2), hexan-1-ol (peak 3), propyl butyrate (peak 4), isobutyl butyrate (peak 5), ethyl hexanoate (peak 6), isopentyl butyrate (peak 7), ethyl benzoate (peak 8), and ethyl octanoate (peak 9). The relative ratios of the EAD-active compounds in five adsorbent extracts of volatiles from *S. mombin* fruits are listed in Table 1. The EAD activity of compounds was verified by GC-EAD analysis with authentic standards.

Response to Mombin Blend in a Wind Tunnel. Attraction of *A. obliqua* to the nine-synthetic blend was different from control (females: $t = -8.5$, $df = 18$, $P < 0.001$; males: $t = 8.6$, $df = 18$, $P < 0.001$). Landing of both sexes on scented

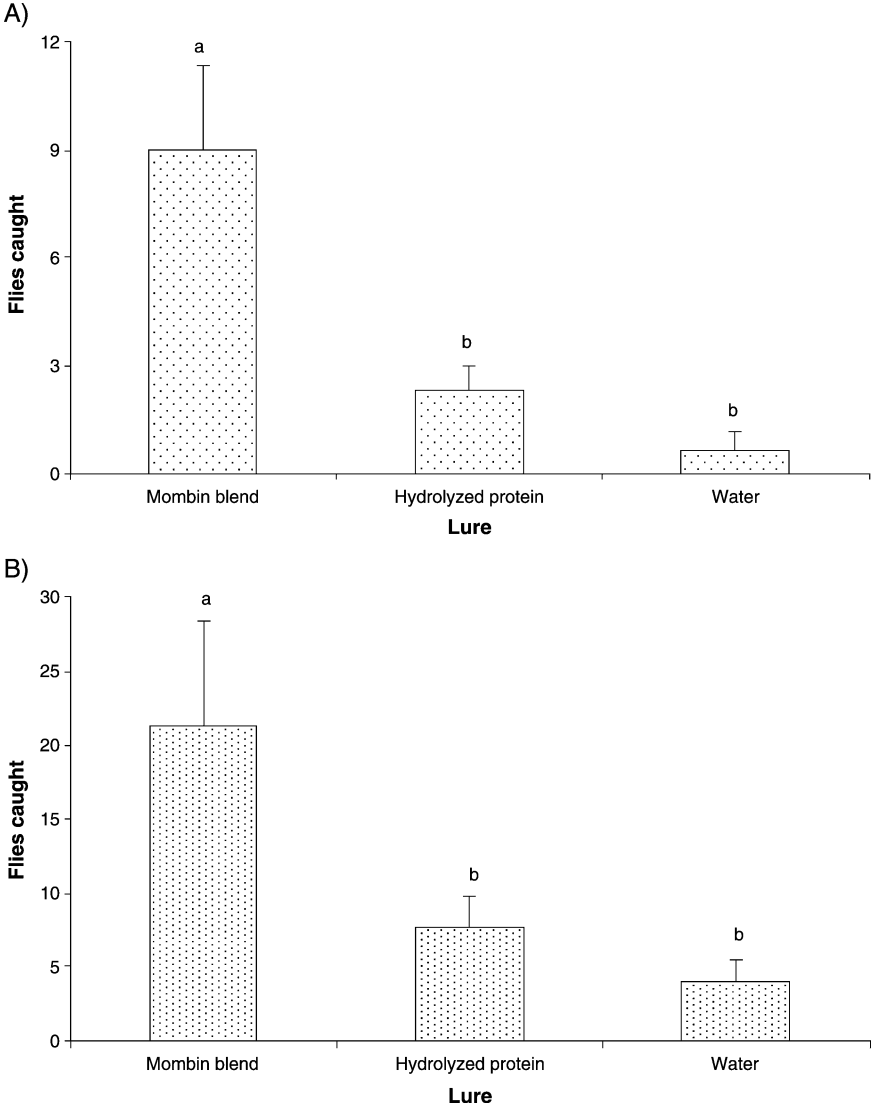


FIG. 3. Mean number (\pm SE) of males and females *A. obliqua* caught in Multilure traps baited with mombin volatiles blend, hydrolyzed protein and water in no-choice (A) and choice (B) tests. Columns with the same letters are not significantly different by Tukey test, $P > 0.05$. Total number of flies released was 300 and 1800 in no-choice and three-choice tests, respectively.

lures was different from the controls (females: $t = -6.13$, $df = 18$, $P < 0.001$; males: $t = 5.9$, $df = 18$, $P < 0.001$; Figure 2).

Field Cage Test. Field cage experiments showed that traps baited with the mombin blend caught more flies than traps baited with hydrolyzed protein and water in both no-choice ($F = 17.1$, $df = 2,35$, $P < 0.001$) and three-choice ($F = 7.5$, $df = 2,35$, $P = 0.001$) tests (Figure 3).

DISCUSSION

The results demonstrated that both sexes of *A. obliqua* were attracted to mombin fruits and Porapak Q volatile extracts in a wind tunnel. Furthermore, our approach of coupled GC-EAD analysis revealed that nine volatile constituents of the mombin extracts were EAD-active on the antennae of males and females. The compounds were identified as ethyl butyrate, isopropyl butyrate, hexan-1-ol, propyl butyrate, isobutyl butyrate, ethyl hexanoate, isopentyl butyrate, ethyl benzoate, and ethyl octanoate. Additionally, the nine-component blend of the synthetic compounds was attractive to both sexes of *A. obliqua* when evaluated in a wind tunnel, and field cage tests traps baited with the mombin blend caught significantly more flies than traps baited with hydrolyzed protein or water.

One potential criticism of our results is the fact that the behavioral and electrophysiological tests were made with flies reared for several generations in the laboratory. The biology of insects maintained in the laboratory may differ from the biology of wild insect of the same species. For instance, Briceño and Eberhard (2002) found that the mating behavior of mass-reared *Ceratitis capitata* (Wiedemann) males was different from that of wild males. We do not know if the host-finding behavior of *A. obliqua* is affected by the mass rearing. However, preliminary trials showed that wild males are caught by traps baited with nine-component blend of synthetic compounds in mango orchards (Toledo et al., unpublished data).

For most species of tephritid fruit flies, host plants and trees are important foraging sites (Jang and Light, 1996). We do not know whether the mombin volatiles identified here are used by *A. obliqua* as cues during the searching for food, mates, and/or oviposition sites. We observed that both sexes of *A. obliqua* fed on fruits and females attempted to oviposit on mombin fruits during the wind tunnel experiments. Therefore, it is possible that attraction of *A. obliqua* to fruit odors may represent food foraging for both sexes and oviposition foraging for females. Fruit volatiles also may affect the sexual behavior of *A. obliqua* as documented for other tephritid fruit flies (Robacker and Garcia, 1990; Landolt et al., 1992; Papadopoulos et al., 2001). For example, chapote odor almost

completely inhibited attraction of sexually mature *A. ludens* females to male pheromone (Robacker and Garcia, 1990). Future studies using fruits with different degree of ripeness (e.g., green, green-orange, and orange) and flies of different physiological state (age, mating status, and hunger level) are needed to investigate whether *A. obliqua* used the host volatiles as cues during the search for food, mates, and/or oviposition sites.

The predominant volatile compounds in the extracts of mombin fruits were esters and alcohols. In fact, of the compounds that elicited electrophysiological and behavioral responses from *A. obliqua*, eight were esters and only one was an alcohol. The volatiles of mango, another highly preferred host of *A. obliqua*, are characterized by monoterpenes and esters (MacLeod et al., 1988; Andrade et al., 2000; Jirovets, 2003). Three of the EAD-active compounds identified in the mombin volatiles have been found in mango volatiles: hexan-1-ol, ethyl hexanoate, and ethyl octanoate. Preliminary GC-EAD analysis of mango volatiles (CV Ataulfo) showed that six compounds elicited repeatable antennal responses from sexes of *A. obliqua* (Galindo-López, 2004). The EAD-active compounds were identified as ethyl hexanoate, *cis*-ocimeno, isoterpinolene, terpinolene, methyl octanoate, and ethyl octanoate. The six-component blend was more attractive for *A. obliqua* males and females than hexane control. The results with mango and mombin volatiles suggest that this generalist fruit fly species may use a wide range of fruit volatiles during its host location behavior. Díaz-Fleischer et al. (2000) proposed that fruit fly species with a narrow host range tend to be specialists in terms of host cues to which they respond during the host-finding process, whereas polyphagous species tend to be generalists. With regard of the chemical cues, the limited information available suggests that generalist fruit fly species respond to the odor of a broad range of unrelated volatiles released by ripening host fruits, whereas specialist species respond to narrow range of host fruit volatiles (Díaz-Fleischer et al., 2000).

Inexpensive attractants for fruit flies have been the objective of much research and would benefit fruit production and support fruit exports in many countries (e.g., McPhail, 1943; Steiner, 1952; Chambers, 1977). The mombin volatiles identified here may be used for developing a dry synthetic attractant that avoids the difficulties associated with the use of aqueous protein baits (Heath et al., 1995). For example, bait solutions may be spilled readily while managing the trap, and spilled bait offers an attractant source outside the trap. Also, it is expected that traps baited with fruit volatiles will capture mostly *A. obliqua*, whereas traps baited with hydrolyzed protein capture many other nontarget insects (Aluja, 1999). However, we recognize that the cost of synthesizing a bait from the mombin volatiles identified here may prove too expensive for practical use in the short term; particularly to poor fruit growers in Latin America.

Effective attractants for tephritid fruit flies have been developed from host fruits previously (Robacker et al., 1990, 1992; Nigg et al., 1994), and the most

remarkable, effective, and useful attractant developed from a host plant is that for the apple maggot *Rhagoletis pomonella* (Walsh) (Fein et al., 1982; Zhang et al., 1999). Initially, Fein et al. (1982) found that a seven-component blend of esters was highly attractive to both sexes of *R. pomonella* in laboratory bioassays and field trials (Reissig et al., 1982). The multicomponent lure was commercialized as an attractant for use in sticky red-sphere monitoring traps and for control programs (Jang and Light, 1996). Recently, a new five-component mix of volatiles from apples has been found by using solid-phase microextraction and GC-EAD techniques (Zhang et al., 1999). This five-component blend contains three compounds (propyl hexanoate, butyl hexanoate, and hexyl butanoate) in common with the previous seven-component blend, as well as two new components (butyl butanoate and pentyl butanoate). Wind tunnel experiments showed that the new blend was more attractive than the seven-component mix and butyl hexanoate, and field trials revealed that the five-component blend was superior to butyl hexanoate.

The results obtained here are encouraging in the search of a new attractant for males and females of *A. obliqua*. However, further research is needed before the mombin blend can be evaluated in mango and mombin orchards. Future trials will determine the minimal blend that effectively mimics the total blend in eliciting a behavioral response for *A. obliqua*. It is well known that, although a complex mixture of volatiles rather than one single compound is fundamental for the attraction of the phytophagous insects (Visser, 1986), not all the components contribute to the attractiveness of the blend in the same way (Phelan et al., 1991; Hartlieb and Rembold, 1996; Nojima et al., 2003). For example, Nojima et al. (2003) found that *R. pomonella* was attracted to a blend of six components identified from hawthorn fruit volatiles. Selected subtraction bioassays demonstrated further that the four-component blend of 3-methylbutan-1-ol, 4,8-dimethyl-1,3(*E*),7-nonatriene, butyl hexanoate, and dihydro- β -ionone elicited levels of attraction equivalent to the six-component mixture. Subtraction of the first component from the four-component mix resulted in complete loss of activity. Removal of the last three compounds from the four-component blend resulted in significant decreases in attraction compared to the four- or six-component mixtures. The optimal concentration and the ratios of the mombin components will be investigated in future experiments.

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FORAGING BEHAVIOR OF *Anastrepha ludens*,
A. obliqua, AND *A. serpentina* IN RESPONSE TO FECES
EXTRACTS CONTAINING HOST MARKING PHEROMONE

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Abstract—Following oviposition, females of many Tephritid flies deposit host marking pheromones (HMPs) to indicate that the host fruit has been occupied. We describe the foraging behavior of these three economically important species (*Anastrepha ludens* and *A. obliqua* from the *fraterculus* species group and *A. serpentina* from the *serpentina* species group) when they encounter an artificial fruit (green agar spheres wrapped in Parafilm) marked with intra- and interspecific feces extracts that contain, among other substances, host marking pheromone. When flies encountered fruit treated with either 1 or 100 mg/ml feces extract, there were drastic and statistically significant reductions in tree residence time, mean time spent on fruit, and in the number of oviposition attempts or actual ovipositions when compared to the control treatment (clean fruit). These responses were almost identical irrespective of extract origin (i.e., fly species), indicating complete interspecific HMP cross-recognition by all three *Anastrepha* species tested. We discuss the ecological and practical implications of our findings.

Key Words—*Anastrepha*, Tephritidae, foraging behavior, host marking pheromones, infochemicals, evolution.

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INTRODUCTION

When resources for foraging insects are fixed in space (e.g., leaf, fruit, larvae), the efficiency of females searching for an adequate oviposition substrate increases if they are able to quickly recognize and avoid already occupied hosts. A successful strategy to minimize overcrowding and to elicit movement away from areas with already occupied resources is the use of epideictic pheromones (among them host marking pheromones or HMPs) (Prokopy, 1981). According to Nufio and Papaj (2001), a marking pheromone is a “chemical signal associated with the host resource that signals occupation by conspecifics. Typically perceived by contact chemoreception, these pheromones are generally produced by females and placed onto or within larval resources following egg-laying.” Interestingly, these infochemicals can either deter or enhance oviposition depending, among other factors, on concentration (Papaj and Aluja, 1993). The existence of HMPs has been reported in the insect orders Coleoptera, Diptera (particularly in the family Tephritidae), Hymenoptera, Lepidoptera, and Neuroptera (Prokopy, 1981; Nufio and Papaj, 2001).

In the case of true fruit flies (Diptera: Tephritidae), host marking behavior has been described in the frugivorous genera *Anastrepha*, *Bactrocera*, *Ceratitis*, *Paraceratitella*, and *Rhagoletis* (work reviewed by Aluja et al., 2000a; Díaz-Fleischer and Aluja, 2000; Díaz-Fleischer et al., 2000; Nufio and Papaj, 2001). In nonfrugivorous tephritids, the phenomenon is less well studied but has been reported for *Tephritis bardanae* (Schrank) (Straw, 1989), *Chaetorellia australis* Hering (Pittara and Katsoyannos, 1990), and *Terellia ruficauda* (Fabricius) (Lalonde and Roitberg, 1992). Flies in the genera *Anastrepha*, *Ceratitis*, and *Rhagoletis* mark hosts by dragging of the aculeus tip on the fruit surface (Roitberg and Prokopy, 1987; Aluja et al., 2000a), while in the case of the olive fruit fly, *Bactrocera oleae* Gmelin, marking is achieved through labellar spreading of fruit juices oozing from an oviposition puncture (Cirio, 1971; Girolami et al., 1981).

The most striking behavioral responses exhibited by foraging female fruit flies upon encountering fruit covered with HMP are as follows: an increase in overall movement (e.g., number of leaf landings per minute, distance of between-tree displacements) and fruit visitation rates, reduction in tree residency time (Roitberg and Prokopy, 1984; Aluja and Boller, 1992a), reduction or increase in clutch size depending on HMP concentration and fruit size (Papaj et al., 1990, 1996; Papaj and Aluja, 1993), and a reduction in the propensity of a female to initiate oviposition (Nufio and Papaj, 2001). HMP recognition is contingent upon previous experience (Roitberg and Prokopy, 1981) but sensitivity to the pheromone is reduced if exposure is continual, apparently due to central habituation or peripheral adaptation of certain sensilla in the tarsi (Aluja and Boller, 1992a). In the case of males of species exhibiting

a resource–defense mating system (e.g., *R. pomonella*, *R. cerasi*), an encounter with an HMP-marked fruit causes arrestment (Prokopy and Bush, 1972; Katsoyannos, 1975).

Host marking behavior has been described for *A. suspensa* (Prokopy et al., 1977), *A. fraterculus* (Prokopy et al., 1982a), and *A. ludens* (Papaj and Aluja, 1993). In *Anastrepha*, as with *Ceratitis capitata* and some *Rhagoletis* species (Prokopy, 1972; Papaj et al., 1989), the presence of the HMP does not automatically deter other conspecifics from ovipositing and re-marking a fruit. *A. ludens* females laid eggs in grapefruit marked up to three previous times by conspecifics (Papaj and Aluja, 1993). Low concentrations of the HMP seem to stimulate continued host marking and an upper concentration threshold must be reached to deter further oviposition. These findings lead Papaj and Aluja (1993) to postulate that host marking in *A. ludens* is regulated by sensory adaptation or habituation to HMP, in conjunction with dosage-dependent restoration of inhibition of locomotory behavior.

The HMPs of *R. cerasi* (*N*[15(β -glucopyranosil)oxy-8-hydroxypalmitoyl]-taurine) and *A. ludens* (2-[2,14-dimethyl-pentadecanoylamino]-pentanedioic acid) were extracted from the feces of sexually mature females (Boller and Hurter, 1985; Hurter et al., 1987; Aluja et al., 2003; Díaz-Fleischer et al., 2004). They show some degree of similarity as both contain a long fatty acid and an amino acid. The *Anastrepha* HMPs are polar compounds (easily dissolved in water and methanol), which are remarkably stable under varying pH values and environmental light and temperature conditions (Díaz-Fleischer et al., 2004).

A topic of particular interest in the study of host marking behavior is the phenomenon of interspecific pheromone recognition. In some insects, this was attributed to phylogenetic relatedness among species (i.e., lineage effect). For example, in *Rhagoletis*, females within a species group (e.g., the *cingulata* group) recognize HMPs of heterospecifics, while females from unrelated groups (e.g., *cingulata* vs. *pomonella* groups) are usually not deterred by each other's HMP (Prokopy et al., 1976). In other insects, interspecific pheromone recognition may reflect an adaptive response to competition for common oviposition resources (see Nufio and Papaj, 2001 for details).

Here, we describe the foraging behavior of *A. ludens*, *A. obliqua*, and *A. serpentina* in response to their own and heterospecific fecal extracts, as these are known to contain the HMPs (Aluja et al., 2003; Díaz-Fleischer et al., 2004). Our hypothesis, based on the work on *Rhagoletis* and recent *Anastrepha* phylogenies (Norrbon et al., 2000; Norrbom, 2002; McPheron et al., 2000), was that interspecific recognition of the pheromone would exist for two species from the same intrageneric group (*A. ludens* and *A. obliqua*) but not for a species from a different group (*A. serpentina*) (Norrbon et al., 2000) given that in nature these three species rarely compete for resources.

METHODS AND MATERIALS

Study System. Fruit flies of the genus *Anastrepha* (Diptera: Tephritidae) are distributed from southern United States to northern Argentina (Hernández-Ortíz and Aluja, 1993). Of the ca. 200 species described so far (Norrbom et al., 2000), seven are considered important agricultural pests: *A. grandis* (Macquart), *A. fraterculus* (Wiedemann), *A. ludens* (Loew), *A. obliqua* (Macquart), *A. striata* Schiner, *A. suspensa* (Loew), and *A. serpentina* (Wiedemann) (Aluja, 1994). The three *Anastrepha* species we selected for this study are polyphagous species with different oviposition strategies. While *A. obliqua* deposits a single egg per oviposition bout, *A. ludens* and *A. serpentina* females can lay from 1 to over 20 eggs per oviposition (Aluja et al., 2000a). *A. ludens* attacks mainly fruit in the family Rutaceae (e.g., *Sargentia greggii* Coult., *Casimiroa edulis* Llave & Lex., *Citrus* spp.). It also attacks mangoes (*Mangifera indica* L.), but mainly at higher elevations (>800 m) (Aluja et al., 1987, 1996). *A. obliqua* preferentially attacks fruit in the family Anacardiaceae, especially in the genus *Spondias* (e.g., *S. mombin* L., *S. purpurea* L., *S. radlkoferi* J.D. Smith) (Díaz-Fleischer and Aluja, 2003b). It also attacks mangoes in lowland areas (therefore, simultaneous use of a mango fruit by *A. obliqua* and *A. ludens*, although possible, is not common) (Aluja et al., 1996). We note that mangoes were introduced by the Spaniards ca. 500 yr ago and therefore, from an evolutionary perspective, the interaction between the latter two *Anastrepha* species in such an exotic host is quite recent. Finally, *A. serpentina* attacks fruit in the family Sapotaceae (e.g., *Chrysophyllum caimito* L., *Manilkara zapota* (L.) P. Royen, *Mammea americana* L.) (Aluja et al., 1987, 2000b).

Study Site. All experiments were performed on the grounds of the Medfly Program at Metapa de Domínguez, Chiapas, México.

Research Arena. General bioassay protocols are described by Aluja and Boller (1992a). Eighteen potted mango trees (*M. indica* cultivar “criollo”) were placed in the center of a cylindrical 3 m (diam) \times 2 m (height) field cage (Chambers et al., 1983), on top of wooden stools at different heights so as to mimic the canopy of a mango tree (ca. 2 m \times 1.90 m). The cage was protected by a 25-m² metal frame with a corrugated fiberglass roof, and shading was provided by black greenhouse screen (2 mm fabric) placed on both the sides and the top. Approximately 35% of all leaves were removed to facilitate detection of a foraging fly within the canopy (details in Aluja et al., 1989 and Aluja and Prokopy, 1993), and trees were arranged so that a 50-cm walkway was left free to allow an observer to move around without encountering obstacles. Throughout this study (i.e., during fly maintenance phase and experiments), 3-cm-diam fruit mimics made of agar (Bacteriological Agar; Sigma, St. Louis, MO, USA) colored with green food dye (Colorante Alimenticio, Verde, McCormick, Herdez, Mexico) and wrapped in Parafilm (American National Can, Neenah,

WI, USA) (Boller 1968) were used instead of real fruit to control for intrinsic variations in both fruit quality and chemistry, as these influence fruit fly oviposition behavior (Papaj et al., 1992). There is ample evidence indicating that female flies accept such artificial oviposition substrates without perceptible behavioral differences with respect to natural hosts (Freeman and Carey, 1989; Jácome et al. 1999; Díaz-Fleischer and Aluja, 2003a).

Insects. All flies used were collected from infested fruit in the Soconusco Region, Chiapas and the vicinity of Xalapa, Veracruz, Mexico: *A. ludens* from *C. aurantium* L. and *M. indica*, *A. obliqua* from *Spondias* spp., and *M. indica*, whereas *A. serpentina* came from *M. americana* L. Details on handling of larvae and pupae are described elsewhere (Aluja et al., 1987).

Newly emerged females and males (25 of each sex per cage) were placed in 30 × 30 × 30 cm wooden screened cages with unlimited access to water and a sucrose/hydrolyzed protein mixture (3:1) (yeast hydrolysate enzymatic; ICN Biochemicals, Aurora, OH, USA) and held at 26°C, 70–85% RH, 12L:12D until 1 d prior to testing. Each cage had one *M. indica* branch with ca. 8 leaves to provide resting places for flies and maintain high RH levels. The latter was important because flies in bare cages break their wings by constantly hitting the walls, and relative air humidity influences the function of the tarsal chemoreceptors (Städler et al., 1987). In addition, 40 agar spheres were placed in each cage (ca. 10 hung from the roof and the rest on the floor) and replaced at least twice daily to avoid HMP accumulation on them, as flies maintained in an HMP-saturated environment become habituated/desensitized and lose their ability to discriminate between marked and clean fruit (Aluja and Boller, 1992a). The oviposition substrates were also important to familiarize females with bioassay conditions, and allow them to oviposit and mark fruit. Both aspects are critical in foraging behavior studies related to HMPs, because host acceptance in flies with a full complement of eggs increases after a period of host deprivation (Roitberg and Prokopy, 1983), which in turn “can make an individual prone to accept a host that would otherwise be rejected” (Jaenike and Papaj, 1992). Furthermore, females require prior experience with the HMP before they discriminate between marked and unmarked fruit (Roitberg and Prokopy, 1981).


































Only sexually mature, mated females were used and their ages varied between 16 and 30, 18 and 32, and 22 and 30 d for *A. obliqua*, *A. ludens*, and *A. serpentina*, respectively. On the day prior to testing, two cages with flies and agar spheres were manually transferred from the lab to a field cage to allow flies to acclimate to test conditions. During this period, we constantly provided them with fresh oviposition substrates to avoid high oviposition drive and habituation/desensitization to high pheromone concentrations.

Test Compounds. Crude pheromonal extracts were obtained from *A. ludens*, *A. obliqua*, and *A. serpentina* feces, which, as documented by Aluja et al. (2003)

and Díaz-Fleischer et al. (2004), contain HMPs. For each species, ca. 3500 adult flies (extremely high density) were placed in a $30 \times 30 \times 30$ cm glass cage with two 13×25 cm sheets of glass to increase the exposed surface to flies. Food and water were offered continuously and after 30 d, the feces on the glass surfaces (without insect parts) were meticulously collected by scraping with a razor blade or a metal spatula. The samples were transferred to a ca. 500-ml glass bottle and frozen at -15°C until needed (further details in Aluja et al., 2003). The pheromone extracts (100 mg/ml) were prepared by mixing 1 g feces with 10 ml distilled water or methanol and were shaken manually for 15 min. The liquid was then centrifuged at 12,000 rpm for 20 min, and the resulting supernatant was used to treat test fruit for the bioassays. The same technique was used to prepare the 1 mg/ml concentration, but in this case only methanol was used as it proved to be a more efficient solvent than water (Díaz-Fleischer et al., 2004).

Bioassay Procedure. Following the protocol of Aluja and Boller (1992a), a single female was released into the cage and her behavior observed for a maximum of 1 hr or until it left the tree and flew to the cage walls. Every fly was released at exactly the same location (i.e., release leaf) and only used once (i.e., no repeated measures on same individual throughout study). In each assay, there were eight spheres, hung equidistant and at ca. the same height from branches in the external part of the canopy. The spheres were either pheromone treated (raw HMP extracts of the three fly species used during this study) or clean (treated with either water or methanol depending on which had been used as the solvent). On any given day, we tested all four treatments. An individual fly was either exposed to eight agar spheres treated with the HMP of one of the three *Anastrepha* species, or eight untreated control spheres (i.e., no choice conditions). Once observations with a particular fly were concluded (i.e., 1-hr time limit reached or fly flying to cage walls before time limit was over), another individual was released and exposed to spheres treated with the HMP of a different *Anastrepha* species or to untreated spheres. At the end of the daily ca. 4-hr observation period, the raw HMP extracts of all three *Anastrepha* species as well as the control treatment were tested (i.e., one replicate per treatment per day). Order of testing was randomized every day. Parameters recorded were total time on tree, time spent on fruit, time spent moving, cleaning and resting, and number of fruit visited. These variables are good descriptors of foraging activity (Roitberg et al., 1982) and, indirectly, of fly oviposition “drive” as flies will visit more fruits and generally spend more time on trees with unmarked fruits, than in those with HMP-marked ones (Prokopy et al., 1987; Aluja and Boller, 1992a). Once a fly landed on a sphere, time spent exploring [actively moving while head butting (repeatedly touching surface of fruit with frons)] or resting, attempting to oviposit or actually ovipositing and time spent dragging (walking on fruit surface with extruded aculeus through which HMP is released) were recorded. We also computed a “fruit irritation

TABLE 1. ENVIRONMENTAL CONDITIONS AT METAPA DE DOMÍNGUEZ, CHIAPAS, MEXICO, WHEN STUDYING THE RESPONSES OF *A. ludens*, *A. obliqua*, AND *A. serpentina* FEMALES TO THEIR OWN AND HETEROSPECIFIC FECAL HOST MARKING PHEROMONE (HMP)

Month and year	Aug. 1993	Sep. 1993	Oct. 1993	Nov. 1993	Jul. 1994	Aug. 1994	Sep. 1994	Oct. 1994	Nov. 1994	Dec. 1994	Jan. 1995
Temperature (°C)	24.4	26.31	25.7	25.26	26.33	25.7	24.98	24.7	23.76	22.42	20.97
RH (%)	77.34	76.25	71.48	68.56	71.57	77.34	82	81.48	80.48	79.37	77.79
<i>A. obliqua</i>											
<i>A. serpentina</i>											
<i>A. ludens</i>											

Time at which tests were conducted indicated for every *Anastrepha* species. Values represent mean.

index'' (number of behavioral transitions while on fruit [walking–cleaning–resting–walking–cleaning] (Boller et al., 1987), which is a good indicator of host acceptance (Aluja and Boller, 1992a).

Assays were conducted approximately between 08:15 and 12:15 hr every day, but the actual time varied according to the different species-specific biorhythms (i.e., *A. obliqua* starts ovipositing earlier than *A. ludens* and *A. serpentina*; Aluja and Birke, 1993; Aluja et al., 2000a). Experiments were conducted between July and January, depending on host availability, with the 100 mg/ml HMP concentration assays being carried out in 1993 and the 1 mg/ml concentration during 1994–1995. In all cases, there was sufficient overlap in time of day and time of year to warrant formal comparisons between species (Table 1).

Data Analysis. As the two HMP concentrations were not simultaneously tested, we only compared one concentration at a time. Data were first analyzed by means of a two-way MANOVA (fly species \times HMP origin), followed by a two-way ANOVA given the significance detected (O'Brien and Kaiser, 1985). Multiple comparisons were carried out using a least square means *t*-test (SAS, 2003).

RESULTS

All HMP extracts tested, at concentrations of 1 and 100 mg/ml, elicited strong behavioral responses in the three *Anastrepha* species studied. MANOVA revealed differences among species (Pillai trace = 0.295, $F_{12,568} = 8.2$; $P < 0.001$; Pillai trace = 0.129; $F_{12,562} = 3.2$; $P < 0.001$; for 1 and 100 mg/ml, respectively) and HMP origin (Pillai trace = 0.921, $F_{18,855} = 21.0$; $P < 0.001$; Pillai trace = 0.975; $F_{18,846} = 22.6$; $P < 0.001$; for 1 and 100 mg/ml, respectively). There was also a significant interaction between fly species and HMP origin (Pillai trace = 0.546, $F_{36,1728} = 4.8$; $P < 0.001$; Pillai trace = 0.432; $F_{36,1710} = 3.7$; $P < 0.001$; for 1 and 100 mg/ml, respectively). Two-way ANOVA (summarized in Tables 2 and 3) revealed that clean fruit mimics (untreated agar spheres), treated with both HMP concentrations, elicited a drastic and statistically significant reduction in female tree residence time. Importantly, differences between species were not significant, independent of pheromone concentration. With respect to the interaction, it was nonsignificant at a concentration of 1 mg/ml and significant at 100 mg/ml (Figure 1, Tables 2 and 3). In the case of mean time spent on fruit, we found a statistically significant reduction in HMP-treated hosts at both pheromone concentrations. As was the case with total time spent on the tree, we found no differences among species when comparing total time spent on fruit. Nevertheless, the interaction was nonsignificant at a concentration of 1 mg/ml and significant at 100 mg/ml

TABLE 2. TWO-WAY ANOVA OF THE BEHAVIORAL RESPONSES EXHIBITED BY *A. ludens*, *A. obliqua*, AND *A. serpentina* FEMALES WHEN FORAGING IN FIELD-CAGED MANGO TREES AND ENCOUNTERING ARTIFICIAL FRUIT (I.E., GREEN-COLORED AGAR SPHERES WRAPPED IN PARAFILM) COVERED WITH THEIR OWN AND HETEROSPECIFIC FECAL HOST MARKING PHEROMONE (HMP) EXTRACTS (VALUES ROUNDED TO SECOND DECIMAL)

Dependent variable	Source of variation	df	Sum of squares	Mean square	F value	P value
Tree residence time	HMP	3	263,456,037.13	87,818,679.04	220.3	<0.0001
	Species	2	1,441,810.33	720,905.16	1.8	0.16
	HMP * Species	6	1,853,392.74	5308,898.79	0.8	0.59
	Residual	288	114,814,184.64	398,660.36		
Fruit residence time	HMP	3	197,119,923.48	65,706,641.16	204.6	<0.0001
	Species	2	340,946.13	170,473.06	0.5	0.59
	HMP * Species	6	2,373,760.70	395,626.78	1.2	0.29
	Residual	288	92,467,245.04	321,066.82		
Fruit visited	HMP	3	73.29	24.43	8.7	<0.0001
	Species	2	5.46	2.73	1.0	0.38
	HMP * Species	6	56.01	9.33	3.3	0.003
	Residual	288	803.52	2.79		
Oviposition attempts	HMP	3	14,513.77	4837.92	61.4	<0.0001
	Species	2	895.02	447.51	5.7	0.004
	HMP * Species	6	2766.02	461.00	5.8	<0.0001
	Residual	288	22,683.92	78.76		
Ovipositions	HMP	3	1100.84	366.95	142.2	<0.0001
	Species	2	198.29	99.14	38.4	<0.0001
	HMP * Species	6	483.07	80.51	31.2	<0.0001
	Residual	288	743.04	2.58		
Irritation index	HMP	3	0.18	0.06	9.9	<0.0001
	Species	2	0.01	0.05	7.8	0.0005
	HMP * Species	6	0.04	0.01	1.0	0.42
	Residual	288	1.75	0.016		

HMP extracts tested at a concentration of 1 mg/ml.

(details on degrees of freedom, F and P values in Tables 2 and 3). *A. obliqua* females behaved differently after landing on unmarked control hosts when compared to the other two fly species (Figure 2).

Presence of HMP significantly altered the total number of fruit visited (Figure 3). Females visited more control fruit than treated ones at both HMP concentrations (details on statistical analyses in Tables 2 and 3). In the case of the number of oviposition attempts, we found differences between treatments and fly species at both HMP concentrations (also the case with respect to the interaction between factors). Consistent with the fact that flies tested here exhibit different oviposition strategies in nature (i.e., *A. obliqua* invariably lays

TABLE 3. TWO-WAY ANOVA OF THE BEHAVIORAL RESPONSES EXHIBITED BY *A. ludens*, *A. obliqua*, AND *A. serpentina* FEMALES WHEN FORAGING IN FIELD-CAGED MANGO TREES AND ENCOUNTERING ARTIFICIAL FRUIT (I.E., GREEN-COLORED AGAR SPHERES WRAPPED IN PARAFILM) COVERED WITH THEIR OWN AND HETEROSPECIFIC FECAL HOST MARKING PHEROMONE (HMP) EXTRACTS (VALUES ROUNDED TO SECOND DECIMAL)

Dependent variable	Source of variation	df	Sum of squares	Mean square	F value	P value
Tree residence time	HMP	3	253,603,018.41	84,534,339.47	174.8	<0.0001
	Species	2	363,233.01	181,616.50	0.4	0.69
	HMP *	6	24,686,197.26	4,114,366.21	8.5	<0.0001
	Species					
	Residual	285	139,282,133.52	483,618.52		
Fruit residence time	HMP	3	300,068,550.08	100,022,850.02	64.5	<0.0001
	Species	2	7,318,537.71	3,659,268.85	2.4	0.10
	HMP *	6	23,081,114.72	3,846,852.45	2.5	0.02
	Species					
	Residual	285	446,587,792.08	1,550,652.06		
Fruit visited	HMP	3	213.10	71.03	10.3	<0.0001
	Species	2	13.45	6.72	1.0	0.38
	HMP *	6	109.01	18.17	2.6	0.02
	Species					
	Residual	285	1991.68	6.92		
Oviposition attempts	HMP	3	32,850.55	10,950.19	68.0	<0.0001
	Species	2	2105.08	1052.54	6.5	0.002
	HMP *	6	6143.73	1023.96	6.4	<0.0001
	Species					
	Residual	285	45,912.23	161.1		
Ovipositions	HMP	3	1765.64	588.546	48.0	<0.0001
	Species	2	296.60	148.302	12.1	<0.0001
	HMP *	6	712.67	118.779	9.7	<0.0001
	Species					
	Residual	285	3495.11	12.264		
Irritation index	HMP	3	0.35	0.115	31.3	<0.0001
	Species	2	0.01	0.002	0.6	0.52
	HMP *	6	0.01	0.002	0.6	0.69
	Species					
	Residual	285	1.06	0.004		

HMP extracts tested at a concentration of 100 mg/ml.

eggs singly, whereas *A. ludens* and *A. serpentina* tend to do so in clutches), responses to the control treatment (unmarked fruit) varied among species (Figure 4). With respect to actual ovipositions (aculeus insertion followed by dragging), we found significant differences when comparing all four treatments at both HMP concentrations. We also detected differences between species, as

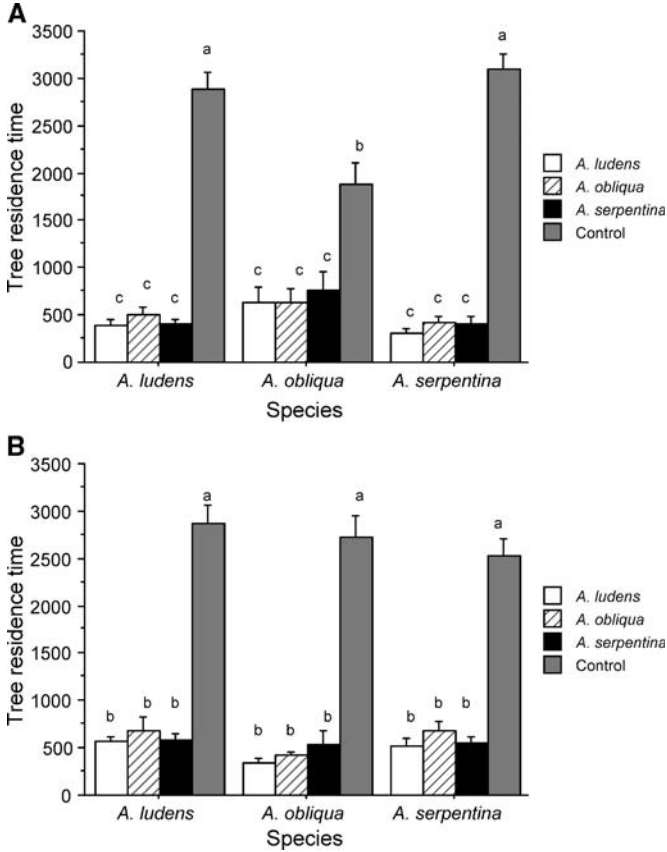


FIG. 1. Tree residence time (sec) (mean \pm SE) exhibited by three *Anastrepha* species exposed to untreated and HMP-treated oviposition substrates. (A) HMP crude extract dissolved in H₂O at 100 mg/ml; (B) HMP crude extract dissolved in MeOH at 1 mg/ml. $N = 25$ in every bar. Means followed by the same letter are not significantly different (least square means t -test).

A. obliqua females oviposited more often than *A. ludens* and *A. serpentina* females (Figure 5). The interaction was significant at both concentrations (Tables 2 and 3). Remarkably, these responses were almost identical irrespective of HMP origin (least square means t -test; SAS, 2003). That is, there was complete interspecific HMP cross-recognition by all three *Anastrepha* species tested.

Finally, the irritability index was significantly higher in HMP-treated than in control (clean) fruit at both concentrations (Figure 6). *A. serpentina* females were significantly less irritated than females of the other two species when

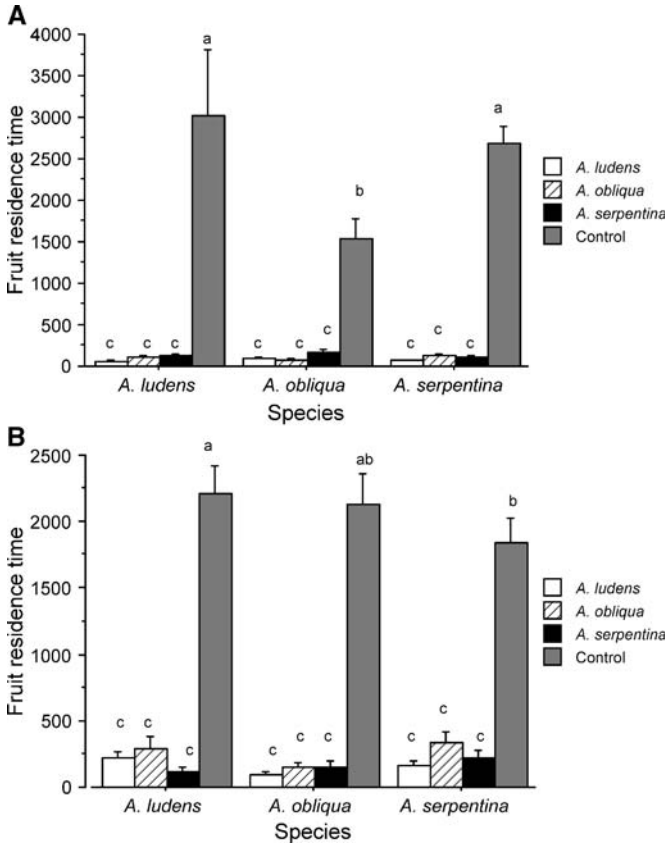


FIG. 2. Fruit residence time (sec) (mean \pm SE) exhibited by three *Anastrepha* species exposed to untreated and HMP treated oviposition substrates. (A) HMP crude extract at 100 mg/ml; (B) HMP crude extract at 1 mg/ml. $N = 25$ in every bar. Means followed by the same letter are not significantly different (least square means t -test).

exposed to fruit covered with HMP at a concentration of 1 mg/ml, but not at a higher concentration. The interaction was not significant at either concentration (Tables 2 and 3).

DISCUSSION

We were able to document a clear pattern of HMP cross-species recognition between *A. ludens*, *A. obliqua*, and *A. serpentina*. Interestingly, although

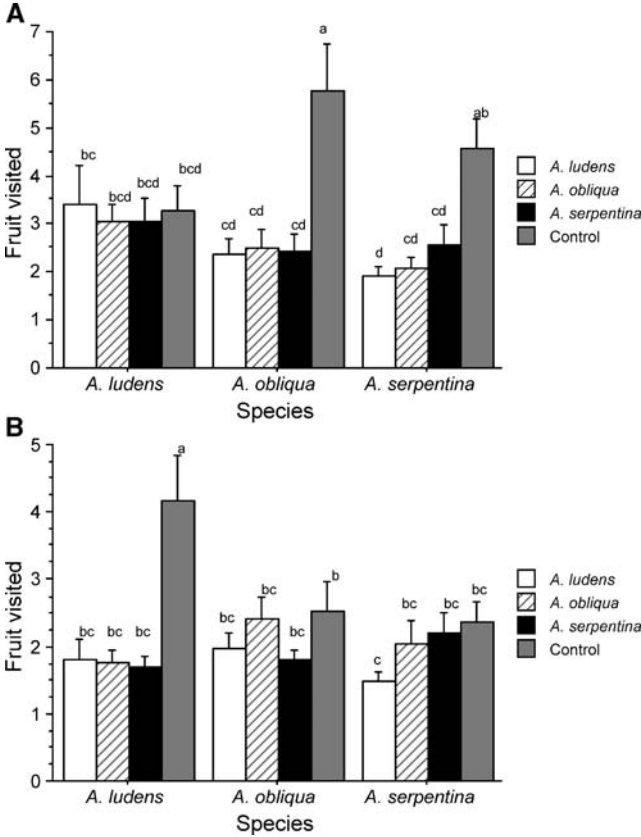


FIG. 3. Mean number (\pm SE) of host visited by three *Anastrepha* species exposed to untreated and HMP treated oviposition substrates. (A) HMP crude extract at 100 mg/ml; (B) HMP crude extract at 1 mg/ml. $N = 25$ in every bar. Means followed by the same letter are not significantly different (least square means t -test).

responses to fruit treated with the two HMP concentrations were practically identical among the three *Anastrepha* species (complete cross-recognition), when comparing the data on untreated, control fruit, clear behavioral differences were detected between the single egg/clutch layer *A. obliqua* and the multiple egg/clutch layers *A. ludens* and *A. serpentina* (discussion at the end of this section). Overall, the response pattern of females to HMP-marked and clean fruit closely resembled the one previously reported for *R. cerasi* (Aluja and Boller, 1992a and references therein). Results from the present study suggest that *Anastrepha* females increase patch persistence time when encountering unmarked hosts, but decrease search allocation following encounters with

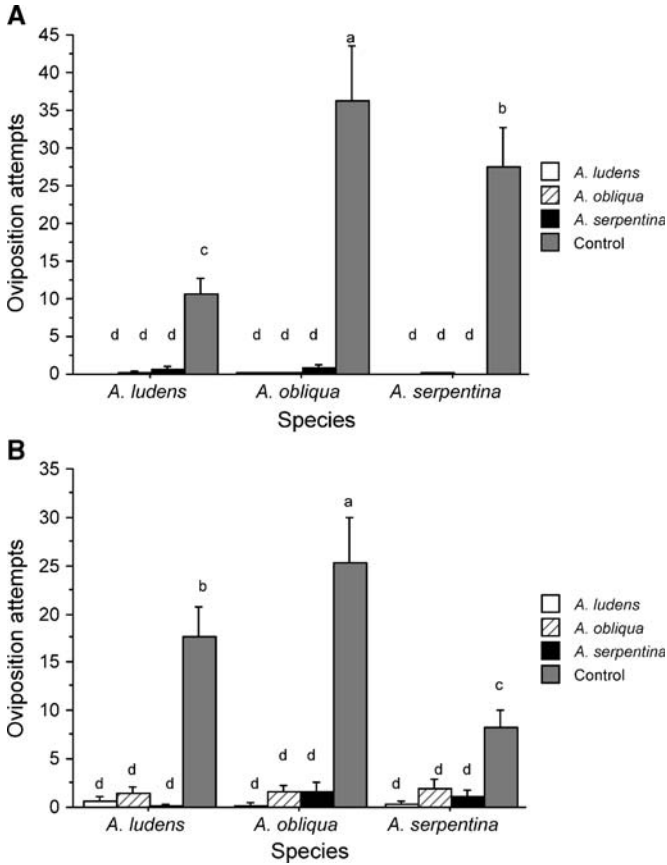


FIG. 4. Mean number (\pm SE) of oviposition attempts by three *Anastrepha* species exposed to untreated and HMP treated oviposition substrates. (A) HMP crude extract at 100 mg/ml; (B) HMP crude extract at 1 mg/ml. $N = 25$ in every bar. Means followed by the same letter are not significantly different (least square means t -test).

marked hosts. Furthermore, females not only increase host-searching time, but they also visited more hosts when they were unmarked. These responses are parallel to those reported by Prokopy et al. (1987) for *C. capitata* flies. Importantly, both HMP concentrations tested, although different by a hundred-fold factor, deterred oviposition in treated fruit. Flies respond strongly to the infochemical, even when present at low concentrations (1 mg/ml HMP in our case). Such a pattern of response to HMPs is similar to the one reported in other insects such as parasitoids (Bernstein and Driessen, 1996).

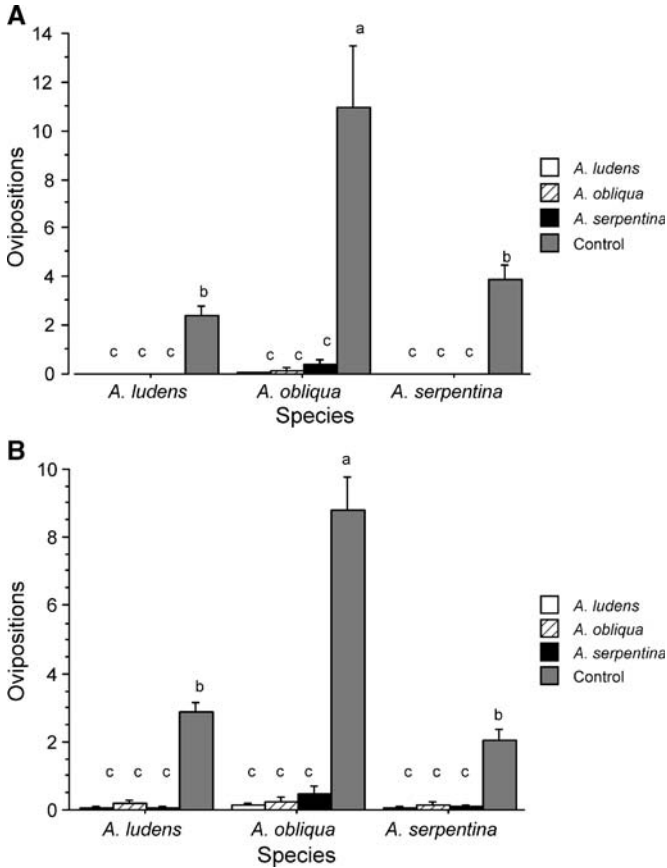


FIG. 5. Mean number (\pm SE) of ovipositions by females of three *Anastrepha* species exposed to untreated and HMP treated oviposition substrates. (A) HMP crude extract at 100 mg/ml; (B) HMP crude extract at 1 mg/ml. $N = 25$ in every bar. Means followed by the same letter are not significantly different (least square means t -test).

The fact that interspecific HMP cross-recognition exists in *Anastrepha* is particularly interesting given that individuals of the species studied rarely exploit the same resources in nature (Norrbon, 2003) and, at least in two cases, are not closely related according to the most recent phylogenies for the group (McPherson et al., 2000; Norrbom et al., 2000). Our prediction that due to a lineage effect there would be HMP cross-recognition between *A. ludens* and *A. obliqua* (*fraterculus* species group), but not *A. serpentina* (*serpentina* species group), was not supported. This finding, together with additional data (M. Aluja and F. D  az-Fleischer, unpublished data) suggests the possibility of shared

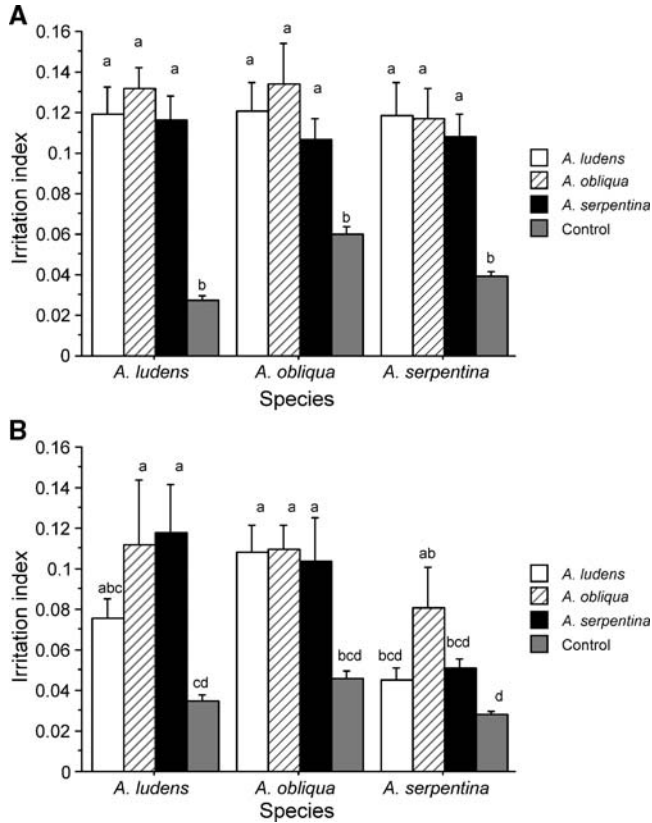


FIG. 6. Mean irritation index (\pm SE) exhibit by females of three *Anastrepha* species exposed to untreated and HMP treated oviposition substrates. (A) HMP crude extract at 100 mg/ml; (B) HMP crude extract at 1 mg/ml. $N = 25$ in every bar. Means followed by the same letter are not significantly different (least square means t -test).

active molecules in the HMP of all *Anastrepha* species). As only the HMP of *A. ludens* has been described (Aluja et al., 2003), it is too early to postulate meaningfully whether this pattern can be explained by convergent evolution or represents a plesiomorphy or synapomorphy. Our results differ from those for other tephritid flies (various species of the genus *Rhagoletis*), as Prokopy et al. (1976) found that “individuals from different species belonging to different species groups did not recognize each other’s marking pheromones” and that “different species within the same species group varied in reaction from complete to no cross-recognition.” However, it is important to note that these differences could be possibly related to bioassay conditions. Prokopy et al.

(1976) worked under artificial laboratory conditions using real fruit marked by live females (resulting in a variable HMP concentration and a possible effect of fruit volatiles on fly response), while we exposed live females to known concentrations of HMP extracts applied on artificial hosts in a field cage containing abundant foliage and foraging space. Thus, these different approaches could affect results, as deterrent activity of HMPs is dose-dependent (Bowdan, 1984; Schoonhoven, 1990; Papaj and Aluja, 1993), and varying numbers of natural marks are needed to obtain similar discrimination coefficients, at least when comparing different *Anastrepha* species (M. Aluja and F. Díaz-Fleischer, unpublished data).

The HMP cross-recognition pattern we report for *A. ludens*, *A. obliqua*, and *A. serpentina* is similar to several cases seen in Lepidoptera. In one case, two species within the same genus were involved (*Pieris rapae* L. and *P. brassicae* L.), with females of *P. rapae* recognizing the crude pheromone extracts of *P. brassicae* (Schoonhoven, 1990; Schoonhoven et al., 1990). In another case, a number of tortricid species from different genera, *Lobesia botrana* (Denis & Schiffermüller), *Eupoecilia ambiguella* (Hübner), *Cydia molesta* (Busck), and *C. pomonella* L., also showed cross-recognition (Thiery and Gabel, 1993; Gabel and Thiery, 1994). In the latter case, Thiery et al. (1992, 1995) and Gabel and Thiery (1996) suggested that a mixture of straight chain fatty acids and esters of fatty acids were most likely responsible for the observed deterrent effect, and the same mechanism may be working in *R. cerasi* and *A. ludens*, as the HMP of these two fruit fly species also contain long chain fatty acids (Hurter et al., 1987; Aluja et al., 2003).

Thiery and Gabel (1993) proposed the term “oviposition regulating synomone” for molecules or blends that influence the interspecific spacing of eggs when females of different species compete for the same oviposition resources in nature. However, in the genus *Anastrepha*, this condition only occurs occasionally. For example, *A. striata* and *A. fraterculus* may compete for various species of fruit in the genus *Psidium* (*P. guajava*, *P. sartorianum*, *P. guineense*) (Aluja et al., 2000b; Sivinski et al., 2004). Consequently, one must ask why is there such strong interspecific HMP recognition between *A. ludens*, *A. obliqua*, and *A. serpentina* when they rarely share the same oviposition resources in nature. As mentioned earlier, one explanation is the presence of shared active molecules in the HMP of all *Anastrepha* species. All HMP may contain substances intimately related to oogenesis (as they are only released by sexually mature females and not by males; Boller and Hurter, 1985; Städler et al., 1992), and could explain, in part, the presence of HMP in feces of species that do not exhibit marking behavior (Aluja et al., 2003; M. Aluja and F. Díaz-Fleischer, unpublished data). HMP is secreted into and accumulated in the gut lumen, to be released with other gut contents in both the marking trail and feces (Prokopy et al., 1982b). The fact that host marking is present in several unrelated groups of insects (Diptera, Lepidoptera, Coleoptera) and that

HMPs may have similar components (fatty acids in Diptera and Lepidoptera) suggest that this could reflect an ancestral physiological process that has remained unchanged over evolutionary time (a plesiomorphy). Such speculation is supported by the fact that, at least in the case of *R. cerasi*, HMP is related to cerebrosides and gangliosides, compounds found in nerve cell membranes (Hurter et al., 1987). Differences in the life histories of species in the genera *Rhagoletis* and *Anastrepha* may also help explain why the chemical structures of their HMPs differ, as most *Rhagoletis* species are monophagous and univoltine (Zwölfer, 1983; Prokopy and Papaj, 2000), whereas the *Anastrepha* species under study here are polyphagous and multivoltine (Aluja et al., 2000a). Thus, digestive processes used to deal with fruit defenses may be quite different as specialist insects develop specific adaptations to confront plant defenses (Lambdon, 2001). If this is the case then, the specialization in *Rhagoletis* flies could have resulted in the HMP molecule, purportedly a by-product of the digestive process, becoming more complex than that observed in *Anastrepha*.

Anastrepha obliqua, an egg-limited species (Aluja et al., 2001; Díaz-Fleischer and Aluja, 2003b), exhibited more oviposition attempts and had more oviposition bouts than either *A. ludens* or *A. serpentina*, two time-limited species (Figures 4 and 5). These behaviors are probably adaptations to cope with highly ephemeral resources [fruit that appear and disappear (fall to the ground) within 4 wks] and could partly explain why *A. obliqua* exhibited a high and uniform irritation index when exposed to low concentrations of all three pheromone sources (it is highly sensitive to fruit quality) (Figure 6).

HMPs have potential as a means of control of insect pests, as shown for *R. cerasi* (Aluja and Boller, 1992b; Aluja et al., 2003). Our present results show that, there is a possibility that one synthetic HMP could offer a viable alternative to the widespread use of insecticides for the management of all three *Anastrepha* species examined in this study. However, when designing deployment strategies, one must remember that HMPs evolved as messages not deterrents (Roitberg and Prokopy, 1987; Schoonhoven, 1990). Consequently, females deprived of suitable oviposition sites may end up exploiting available fruit even in the presence of HMPs (i.e., there is no absolute oviposition deterrence). Furthermore, and as documented by Quiring and McNeil (1984) and Averill and Prokopy (1987), heavy rains can potentially reduce efficacy due to wash off given the water-soluble nature of the substance (but see Katsoyannos and Boller, 1976, 1980), and plants along crop edges should remain unsprayed to maintain high response thresholds to the HMP. Additional research is required to exploit the full potential of this management tool.

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FEEDING BEHAVIOR OF LAMBS IN RELATION TO KINETICS OF 1,8-CINEOLE DOSED INTRAVENOUSLY OR INTO THE RUMEN

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Abstract—The monoterpene 1,8-cineole is a major constituent of the essential oils that adversely influence intake of sagebrush by herbivores, but little is known about the mechanisms of its action. We investigated the influence of 1,8-cineole on the feeding behavior of two groups of sheep, one group dosed intravenously and the other intraruminally. In the first study, we infused 40 mg/kg BW of 1,8-cineole intravenously into four lambs on wk 1, 2, and 4. In the second, we administered 125 mg/kg BW of 1,8-cineole into the rumen of four lambs as a single-bolus dose in wk 1 and 2. Lambs dosed intravenously spent less time feeding than controls (28 vs. 60 min; $P < 0.05$), as did lambs dosed intraruminally (35 vs. 60 min; $P < 0.05$). Dosed lambs ate less than controls during rumen dosing studies ($P < 0.05$). For the intravenous infusion studies, rates of elimination did not differ among weeks ($P < 0.05$). For the rumen infusion studies, however, the absorption rate constant increased from 0.035/min to 0.076/min from wk 1 to 2, while the absorption half-life declined from 24 to 10 min ($P < 0.05$). Maximum plasma concentrations and time to reach maximum plasma concentrations were no faster in wk 2 than wk 1, but the primary elimination rate constant was 2.3 times higher in wk 2 (0.058/min) than in wk 1 (0.025/min) ($P < 0.05$). Dosed lambs exhibited clinical effects—licking of lips, drowsiness, staggering, and 1,8-cineole-

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smelling breath—that were much more pronounced with intravenous than rumen infusions. Dosing did not affect the acid–base balance. Collectively, these data suggest 1) rapid absorption and distribution of 1,8-cineole was responsible for initiating satiety, while more prolonged excretion was responsible for the duration of the satiety effect, and 2) lambs more readily adapted to 1,8-cineole in the rumen-dose study than in the intravenous-dose study.

Key Words — 1,8-Cineole, monoterpenes, feeding, kinetics, elimination, rumen microbes, sheep.

INTRODUCTION

The monoterpene cyclic ether 1,8-cineole is a major component of essential oils from sagebrush (*Artemisia tridentata* Nutt) (Welch and Pederson, 1981; Kelsey et al., 1982; Welch et al., 1983; Personius et al., 1987). Along with several other monoterpenes, it adversely affects the preferences of herbivores for sagebrush (Welch and Pederson, 1981; Personius et al., 1987), but its mechanisms of action are not well known. In general, terpenes are toxic when consumed or dosed at too high concentrations (Johnson et al., 1976). However, animals never consume sagebrush in quantities large enough to result in lethal effects. Rather, they limit intake, at least in part, due to aversive postingestive feedback from compounds such as terpenes (Provenza, 1995). Possible postingestive mechanisms of action of terpenes include toxicity (Johnson et al., 1976), suppressed rumen microbial activity (Ngugi et al., 1995), and disturbed acid–base balance (Foley et al., 1987, 1995; Illius and Jessop, 1997). These mechanisms are not mutually exclusive and may act in concert to cause postingestive effects that lead to cessation of feeding.

Our objectives were to investigate the effects on feeding behavior, systemic kinetics, clinical effects, and systemic pH balance of repeated exposure of lambs to 1,8-cineole administered intravenously or into the rumen. These studies are essential for understanding how monoterpenes limit intake of sagebrush and for developing ways to increase intake of sagebrush as a means to enhance and maintain biodiversity in landscapes dominated by the sagebrush steppe (Provenza et al., 2003; Dziba et al., 2006).

We hypothesized that 1,8-cineole: (1) causes toxin satiation (Freeland and Janzen, 1974; Provenza, 1995, 1996), (2) inhibits rumen microbial activity (Nagy et al., 1964; Oh et al., 1968; Ngugi et al., 1995), (3) inhibits elimination pathways (Pass et al., 1999), and (4) challenges acid–base balance (Foley et al., 1995, 1999). Based on these hypotheses, we predicted that: (1) we would find evidence of centrally mediated satiation due to an increase in systemic concentrations of 1,8-cineole, (2) exposure to 1,8-cineole would suppress rumen

microbes due to the bacteriocidal effect of essential oils, (3) kinetic elimination rates would decline with repeated exposure to 1,8-cineole due to inhibition of elimination pathways, and (4) blood pH would decline as lambs metabolize 1,8-cineole, producing hydrogen ions that disturb the acid–base balance. We predicted these mechanisms would lead to a decline in food intake following dosing with 1,8-cineole.

METHODS AND MATERIALS

Animals and Diet. Twelve lambs weighing 45.6 kg (SE = 0.97) were used for intravenous infusion and eight lambs weighing 34.0 kg (SE = 0.83) were used for rumen dosing. Lambs were exposed as a group to experimental conditions until they were familiar with alfalfa pellets and with the experimental diet, which was similar in nutrient content to sagebrush in the fall (Welch and Pederson, 1981; Kelsey et al., 1982; NRC, 1985). The diet was formulated from a mixture of beet pulp, grape pomace, and soybean meal at a ratio of 49:43:8 to provide 2.44 Mcal/kg digestible energy (DE), 8.77% crude protein (CP), and 52% total digestible nutrients (TDN) (NRC, 1985). During the first week of conditioning, lambs were also fed alfalfa pellets to meet their daily maintenance requirements, until intake of the experimental diet was adequate. During the second week of conditioning and throughout the trials, lambs were fed only the experimental diet from 0900 until 1200 hr. They were habituated to individual metabolism cages (1.5 × 1.0 m) and housed in a lighting-controlled room (12-hr light–dark cycle) for 1 wk prior to dosing. Feeding behavior was monitored for 1 hr during the intravenous infusions and for 1 hr immediately following rumen dosing. After 1 hr, lambs were allowed access to the experimental diet for another 2 hr. They were deprived of food overnight to ensure that food refusals during testing were due to treatment effects. We observed the nature and extent of any clinical signs exhibited by animals in response to 1,8-cineole during and following dosing. Throughout conditioning and during infusions and dosing, lambs had free access to water and trace mineralized salt blocks (American Stockman[®], Overland Park, KS, USA). This study was approved by the Institutional Animal Care and Use Committee at Utah State University (IACUC # 1078).

Dose and Dosing Materials. Intralipid (20% emulsion; CAS# 68890-65-3) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and 1,8-cineole (99% purity; CAS# 470-82-6) from Sigma-Aldrich (Milwaukee, WI, USA). High-performance liquid chromatographic (HPLC)-grade ethyl acetate and HPLC-grade water were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

For the intravenous infusion experiment, 2 g of 1,8-cineole were placed in 50 ml intralipid (1,8-cineole to intralipid concentration of ~2 g/52 ml), and the

solution was thoroughly mixed using a vortex mixer. For rumen administration, 5 g 1,8-cineole were mixed thoroughly in 20 ml vegetable oil (1,8-cineole to oil concentration of ~5 g/25 ml). Intravenous and rumen doses were established via preliminary dosing trials. The starting point was based on known dietary intake from previous feeding experiments with sagebrush monoterpenes (Dziba and Provenza, 2006), and the knowledge that marsupials exhibit a significant first-pass effect of approximately 80% for 1,8-cineole (Stuart McLean, personal communication). The rate and dose used were based on trials with lambs fed diets containing different concentrations of terpenes that resulted in cessation of feeding in less than 1 hr in previous trials (Dziba and Provenza, 2006).

Intravenous Infusion. The objective of the intravenous infusion experiment was to determine the clinical effects, kinetic rates, effects on systemic pH, and influence on feeding behavior of 1,8-cineole in lambs with repeated administration. Intravenous infusion avoids effects due to taste, rumen degradation, absorption, and first-pass metabolism. Twelve lambs were randomly assigned to three treatments: (1) 1,8-cineole in intralipid, (2) intralipid only, and (3) control, no infusion. Intravenous infusions were administered $\times 3$ on the same day of each week on wk 1, 2, and 4 to test for potential systemic inhibition of elimination pathways. This was assessed by comparing alpha and beta elimination rates, as well as feeding behavior, over the three periods. The control treatment was repeated $\times 3$ to compare feeding behavior of dosed lambs to that of controls over the three trials. The intralipid-only treatment was not repeated because lambs in this treatment behaved the same way as non-treated control lambs.

Infusion was used instead of bolus i.v. administration to slowly increase plasma concentrations without the potential of acutely toxic effects. The solution of 1,8-cineole and intralipid was delivered at a constant rate infusion of 1 ml/min for 1 min/kg BW using a VWR[®] variable flow peristaltic pump (VWR Scientific Products, Buffalo Grove, IL, USA). This provided a total dose of 40 mg/kg over a period of 40–50 min. To ensure that the intravenous formulation was sterile, the dosing materials were mixed in the lab each morning of the experiment. A sterile needle was used to transfer 1,8-cineole directly into the bottle of intralipid used for the infusion. All surgical materials were sterile.

The infusion line was attached to an Abbocath[®]-T, 18 G \times 2 in. indwelling intravenous jugular catheter (Abbott Ireland, Sligo, Ireland) and secured to the back and the ear of the lamb to ensure the line stayed in place. The catheter was fitted with a lock-surflo injection plug (Terumo[®] Medical Group, Elkton, MD, USA) for repeated blood collection. A control blood sample was collected before the infusion. Postinfusion sampling was initiated at the time infusion was stopped and denoted as time = 0. Blood samples (10 ml) were collected into EDTA-coated blood collection tubes (Vacutainer[®]; Tyco Healthcare Group LP, Mansfield, MA,

USA) at 0, 1, 3, 6, 12, 18, 30, 45, 60, 90, 120, 180, 240, 360, and 480 min after the infusion. Heparized 0.9% NaCl injectable solution (3 ml) was used to flush the catheter after the infusion and after each blood sample collection to prevent clotting. A total of 16 blood samples were collected from each lamb for systemic pH and pharmacokinetic analyses.

During the infusion, lambs were offered the experimental diet for 1 hr. Feeding behavior was monitored throughout dosing and for 1 hr thereafter. The variable we monitored was feeding time, which we defined as the time it took lambs to stop feeding. The criterion for stopping was 60 sec of continuous nonfeeding activity. We also recorded the time it took lambs to return to feeding after the end of the infusion.

Rumen Dosing. The objective of the rumen dosing experiment was to determine the absorption and elimination rates of 1,8-cineole administered directly into the rumen, as well as effects on feeding behavior. To do so, eight lambs were randomly assigned to two treatments: (1) four lambs dosed with 1,8-cineole in vegetable oil and (2) four control lambs that were not infused. Based on previous studies, we expected no effect of vegetable oil on feeding behavior, and so a vegetable oil control was deemed unnecessary (Dziba and Provenza, 2006). Rumen dosing was repeated 7 d after the first exposure in wk 1 to determine whether repeated exposure to 1,8-cineole altered the kinetics or feeding behavior. By comparing rumen dosing and intravenous infusions, we were able to indirectly assess the effects of degradation of 1,8-cineole in the rumen.

The solution of 1,8-cineole was delivered as a single bolus dose of 0.625 ml/kg BW directly into the rumen with the needle of an Abbocath[®]-T 16 G × 2 in. catheter (Abbot Ireland). This dose provided 1,8-cineole at 125 mg/kg BW. An Abbocath[®]-T, 18 G × 2 in. indwelling intravenous jugular catheter (Abbott Ireland) was inserted for sample collection. Blood samples (10 ml) were collected into EDTA-coated Vacutainer[®] blood collection tubes at 2, 4, 6, 12, 18, 30, 45, 60, 90, 120, 180, 300, and 420 min following the single bolus dose. Heparized 0.9% NaCl injectable solution (3 ml) was used to flush the catheter after each blood sample collection to prevent clotting.

Lambs were offered food at the beginning of the trial, and the remaining feed was weighed every 30 min until all food was consumed. Intake (g) of the experimental diet was measured by difference. Feeding behavior, as previously defined, was monitored for 1 hr following dosing. Lambs had *ad libitum* access to food for the duration of the trials.

Sample Processing and Analyses. Blood samples were analyzed for pH immediately after collection during intravenous infusions. A Beckman Instruments (Irvine, CA, USA) Φ 44 pH Meter with a Thermo Orion Combination pH Probe (Beverly, MA, USA) was used for pH analyses. The probe was rinsed and dried prior to each analysis. The instrument was calibrated daily by using three

buffer solutions of pH 4, 7, and 10. Blood samples were centrifuged for 10 min at 3000 rpm with an IEC HN-SII series centrifuge (Damon/IEC Division, Needham, MA, USA).

Plasma was separated, frozen immediately, and stored at -18°C until gas chromatographic (GC) analysis. The analytical method for 1,8-cineole in plasma is described in detail by Kimball et al. (2004). Briefly, Isolute Solid Phase Extraction columns (C18, 500 mg sorbent, 10-ml reservoir) were used for the extractions (Argonaut Technologies, Foster City, CA, USA). The SPE columns were conditioned with 5 ml HPLC-grade methanol followed by 10 ml HPLC-grade water. Three ml of plasma were passed through the column followed by 10 ml HPLC-grade water (column wash). The columns were allowed to dry under vacuum for 10 min and then immediately eluted with 1.00 ml HPLC-grade ethyl acetate or stored frozen at -18°C for later elution. The column eluate was placed in a GC autosampler vial.

The chromatographic system consisted of a Shimadzu GC 17A Series (Shimadzu Corporation, Columbia, MD, USA) equipped with an FID and an autosampler. The analytical column was a 30 m \times 0.25 mm DB-5.625, 0.25 μm (J&W Scientific, Folsom, CA, USA). One- μl splitless injections (split time of 1.0 min) were made under the following conditions: injection temperature of 200°C and detector temperature of 325°C . The initial oven temperature of 40°C was maintained for 0.5 min. The first oven ramp was at $5^{\circ}\text{C}/\text{min}$ to 110°C . The final ramp was at $20^{\circ}\text{C}/\text{min}$ to a final temperature of 300°C . The total run time was 24 min. The carrier gas was helium delivered at a constant 39 cm/sec by using electronic pressure control. The detector gases were hydrogen (30.0 ml/min), nitrogen (makeup; 30.0 ml/min), and air (400 ml/min).

To provide an analytical matrix match for the eluted samples, five 1,8-cineole standards in ethyl acetate in the range of interest (1.0, 5.0, 10.0, 30.0, and 60.0 $\mu\text{g}/\text{ml}$) were injected at the beginning of each run and after half of the samples had been run. Ethyl acetate blanks preceded and followed each of the standard runs to ensure the column was free of contamination. Linear regression analyses for the standards were used to verify linearity and consistency, and were then used to calculate concentrations from the sample area obtained during the GC-FID analyses.

The plasma concentration–time data for 1,8-cineole were fitted to the following equation using PK Solutions 2.0 for Noncompartmental Pharmacokinetics Data Analysis (Summit Research Services, Montrose, CO, USA):

$$C_t = D \cdot e^{-\alpha t} + E \cdot e^{-\beta t}$$

where C_t is 1,8-cineole plasma concentration at time = t ; α and β are the exponential equation constants representing alpha elimination–distribution and beta elimination, respectively. D and E represent the y -intercept for the alpha

elimination–distribution and beta elimination, respectively. The intravenous study was based on an infusion method of dosing, so we determined only αk_e (alpha elimination–distribution rate constant) and βk_e (terminal or beta elimination rate constant). We used an equation for intravenous bolus dosing, based on the instantaneous cessation of infusion, because no data were collected during the infusion. Data were only collected immediately after the intravenous infusion. Time = 0 is the time of the cessation of infusion.

For the rumen dosing studies, the following kinetic parameters were determined: k_a (absorption rate constant), αk_e (primary or alpha elimination rate constant), and βk_e (terminal or beta elimination rate constant). The plasma concentration–time data for 1,8-cineole were fitted to the following equation:

$$C_t = A \cdot e^{-at} + D \cdot e^{-\alpha t} + E \cdot e^{-\beta t}$$

where C_t is 1,8-cineole plasma concentration at time = t ; a , α , and β are the triexponential equation constants of absorption, alpha elimination–distribution, and beta elimination, respectively. **A**, **D**, and **E** represent the y -intercepts for absorption, alpha elimination–distribution, and beta elimination, respectively. The absorption intercept, **A**, is mathematically derived as a negative value by the PK Solutions software. Thus, the mathematical term, $A \cdot e^{-at}$ represents the amount remaining to be absorbed at time = t . Area under the curve ($AUC_{0-\infty}$) was determined by the trapezoidal rule.

Statistical Analyses. Kinetic data were analyzed with one-way ANOVA. The response variables for both the intravenous infusion and rumen dosing experiments were α and β elimination rates and half-lives. Absorption rates and half-lives were additional response variables for the rumen dosing experiments. Feeding behavior data for the intravenous infusion experiment were analyzed using repeated measures mixed ANOVA with two treatments (dosed lambs and controls). Feeding time was the response variable. Feeding behavior of controls and carrier controls did not differ, so carrier controls were excluded from the analyses. The experiment was repeated on wk 1, 2, and 4, and week was the repeated measure. The error term for treatments was lambs nested within treatments, whereas the error term for week and the Week \times Treatment interactions was lambs nested within Treatments \times Weeks. The fixed effects were treatment and week and lambs (4/treatment) were the random effect. For the rumen dosing experiment, the data were analyzed similarly, except that there were only 2 wk (1 and 2); response variables were feeding time and food intake. Blood pH was analyzed by a paired Student's t -test to determine if the pH of postinfusion blood samples differed from that of the predosing control sample. Mean comparisons were conducted using the Tukey procedure, where significant differences existed among treatments.

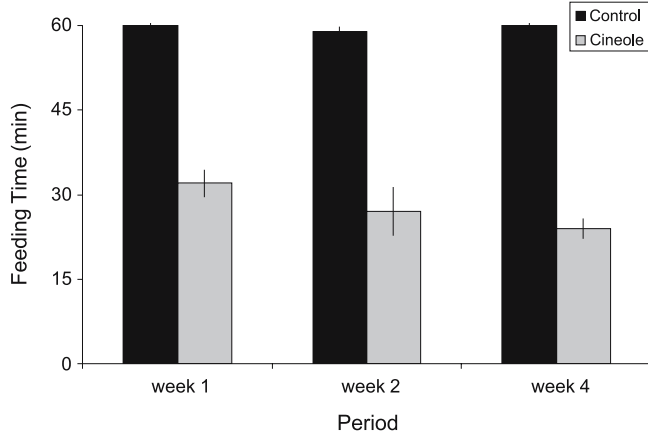


FIG. 1. Mean feeding time of four lambs dosed intravenously with 40 mg/kg BW 1,8-cineole compared to controls over the three periods. Bars are standard errors.

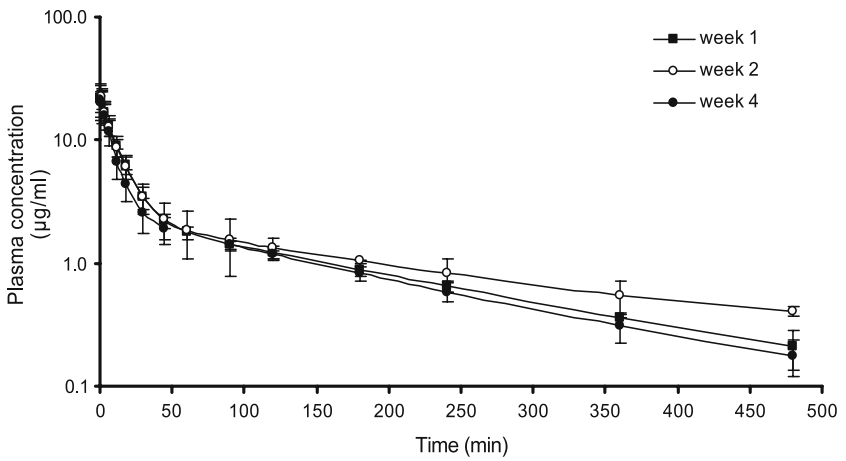


FIG. 2. Mean intravenous plasma concentration time curves of four lambs dosed with 40 mg/kg BW 1,8-cineole during wk 1, 2, and 4.

RESULTS

Intravenous Infusion

Feeding Behavior. On average, lambs dosed intravenously stopped feeding at 28 min (SE = 1.8), whereas controls spent 60 min (SE = 0.7) feeding ($P < 0.05$). Time spent feeding tended to decline over the three periods, although the differences were not significant ($P = 0.29$; Figure 1). The time lambs took to resume feeding following dosing decreased over the three periods from 18 min (SE = 3.5) in wk 1 to 8 min (SE = 1.5) and 7 min (SE = 2.0) in wk 2 and 4, respectively ($P < 0.05$).

Clinical Signs and Blood pH. All treatment lambs licked their lips during and shortly after the infusion. They were all rather sleepy and slightly ataxic, and two of them were also tachypnic. All treatment lambs had the distinct smell of 1,8-cineole on their breath following the infusion. Staggering and licking of lips were also apparent, and to the same extent, in wk 2 and 4. Blood pH remained relatively stable and was not different from the baseline following infusion ($P > 0.05$). Blood pH remained within expected physiological extracellular fluid pH of 7.42 (SE = 0.059).

Pharmacokinetics. The elimination rate curves were similar for each week the lambs were dosed. The plasma time curve for 1,8-cineole was best fit by a biexponential model with a rapid primary (α) elimination phase, and a relatively slow terminal (β) elimination phase (Figure 2). Primary elimination rates did not differ among weeks ($P > 0.05$); the rate constants were 0.074/min in wk 1, 0.099/min in wk 2, and 0.087/min in wk 4 (Table 1). The average primary elimination half-life of 9 min did not change among periods ($P > 0.05$; Table 1). Concentrations of 1,8-cineole declined more slowly during the terminal elimination phase, and the terminal elimination rate constants were 0.003/min in wk 1, 0.005/min in wk 2, and 0.003/min in wk 4 (Table 1). Terminal elimination half-lives differed among weeks, with a lower half-life in wk 2 (153 min) than in wk 1 (216 min) and 4 (236 min) ($P < 0.05$; Table 1).

TABLE 1. COMPARATIVE PHARMACOKINETIC PARAMETERS FOR LAMBS DOSED INTRAVENOUSLY WITH 40 MG/KG BW OF 1,8-CINEOLE

Week	$\alpha_{ke}(1/\text{min})$	$\alpha t_{1/2}(\text{min})$	$\beta_{ke}(1/\text{min})$	$\beta t_{1/2}(\text{min})$
1	0.074 (0.0081)	10 (1.1)	0.003 (0.0004)	216 (29.5)
2	0.099 (0.0110)	7 (0.9)	0.005 (0.0008)	153 (20.3)
4	0.087 (0.0163)	9 (1.9)	0.003 (0.0001)	236 (9.2)
Mean	0.087 (0.0071)	9 (1.3)	0.004 (0.0004)	202 (26.8)

Standard errors in parentheses.

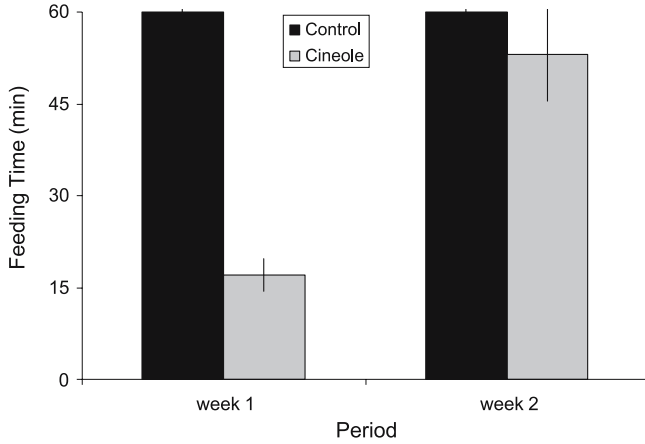


FIG. 3. Mean feeding time of four lambs dosed with 125 mg/kg BW 1,8-cineole compared to control lambs during rumen dosing over the three periods. Bars are standard errors.

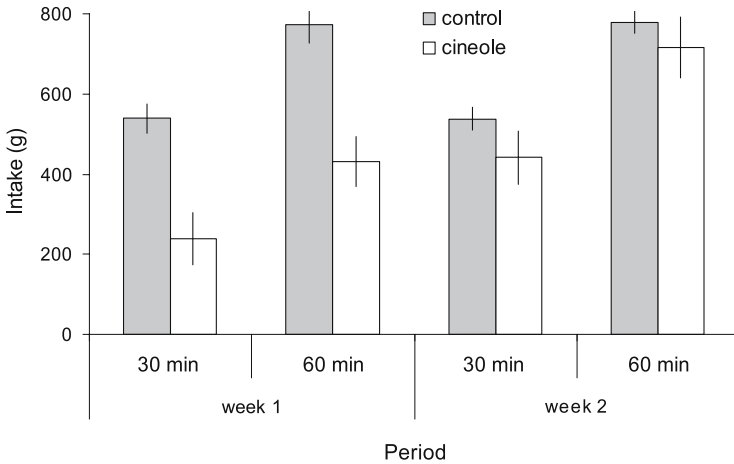


FIG. 4. Mean cumulative feed intake of four lambs dosed intraruminally with 125 mg/kg BW 1,8-cineole compared to controls during wk 1 and 2. Bars are standard errors.

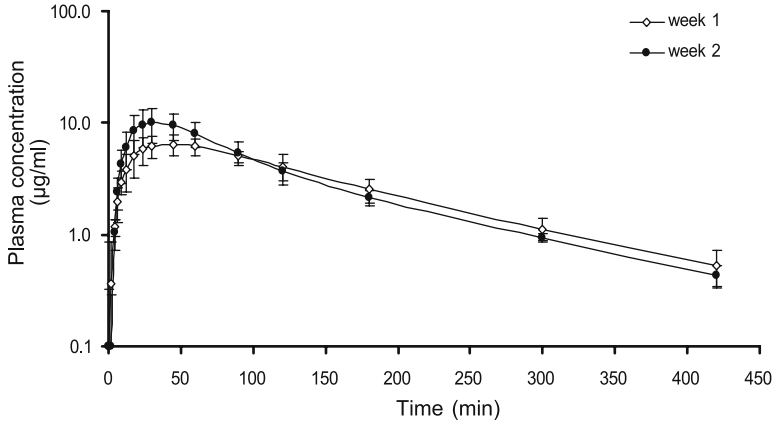


FIG. 5. Mean plasma concentration time curves of four lambs dosed intraruminally with 125 mg/kg BW 1,8-cineole during wk 1 and 2.

Rumen Dosing

Feeding Behavior. Cineole influenced the feeding behavior of lambs when administered as a single bolus dose into the rumen ($P < 0.05$). Treatment lambs stopped feeding sooner than controls (Figure 3). Feeding time increased from wk 1 (17 min; SE = 2.7) to wk 2 (53 min; SE = 7.5) for treatment lambs ($P < 0.05$; Figure 3). During wk 1, controls consumed more food than treatments at both 30 and 60 min ($P < 0.05$), while during wk 2 lambs did not differ in the amount of food they consumed, although the trend was still decreased intake by lambs dosed with 1,8-cineole (Figure 4).

Clinical Effects. All lambs licked their lips in the first week of the rumen dosing, but during the second week, none did. We saw none of the other clinical signs apparent during the intravenous infusion experiment.

TABLE 2. MEAN KINETIC PARAMETERS FOR FOUR LAMBS ADMINISTERED A SINGLE RUMEN DOSE OF 125 MG/KG BW 1,8-CINEOLE

Week	k_a (1/min)	$A_{t1/2}$ (min)	A_{ke} (1/min)	$\alpha_{t1/2}$ (min)	β_{ke} (1/min)	$\beta_{t1/2}$ (min)	C_{max} (µg/ml)	T_{max} (min)
1	0.035 (0.0087)	24 (5.5)	0.025 (0.0075)	39 (11.5)	0.009 (0.0011)	85 (10.1)	7.7 (0.56)	68 (22.8)
2	0.076 (0.0113)	10 (1.1)	0.058 (0.0204)	19 (7.6)	0.007 (0.0007)	111 (12.4)	12.6 (3.29)	42 (16.2)
Mean	0.056 (0.0084)	17 (5.3)	0.041 (0.0097)	29 (10.4)	0.008 (0.0006)	98 (12.5)	10.2 (1.80)	55 (13.9)

Standard errors in parentheses.

Pharmacokinetics. The plasma level–time curve fitted a triexponential mathematic model, with an exponential absorption component and biexponential distribution–elimination components. Absorption was rapid followed by a rapid primary (α) distribution–elimination phase and a relatively slow terminal (β) elimination phase (Figure 5). The absorption rate constant increased from 0.035 to 0.076/min from wk 1 to wk 2, while the absorption half-life declined from 24 to 10 min ($P < 0.05$; Table 2). C_{\max} , calculated maximum plasma concentration for 1,8-cineole, did not differ between weeks (Table 2). Time to reach maximum plasma concentration (T_{\max}) for 1,8-cineole was no faster in wk 2 (42 min) than wk 1 (68 min). The primary elimination rate constant was 0.025/min in wk 1 and 0.058/min in wk 2 ($P > 0.05$; Table 2). The primary elimination half-life declined from 39 min in wk 1 to 19 min in wk 2 (Table 2). The terminal elimination rate constant was 0.0098/min in both weeks (Table 2). The average terminal elimination half-life of 98 min did not change between weeks (Table 2). $AUC_{0-\infty}$ did not differ between wk 1 (1305 $\mu\text{g min/ml}$) and wk 2 (1286 $\mu\text{g min/ml}$).

DISCUSSION

Intravenous Infusion. Lambs dosed intravenously with 1,8-cineole stopped feeding sooner than controls in all three periods. This finding supports the satiety hypothesis, which predicts high plasma concentrations of secondary metabolites such as 1,8-cineole induce cessation of feeding (Provenza, 1995, 1996). The possibility that postabsorptive feedback from terpenes exerts serious effects on feeding behavior has been ignored, except in marsupials (Foley et al., 1987; Boyle et al., 2001; Boyle and Mclean, 2004). Nevertheless, feedback from cells and organs in response to nutrients and toxins influences which foods and how much of those foods are eaten by an animal (Provenza, 1995; Provenza and Villalba, 2006). While some authors suggest sagebrush monoterpenes are expelled quickly or degraded in the rumen (Cluff et al., 1982; White et al., 1982; Welch et al., 1983), there have been no definitive analyses of their potential postabsorptive effects (Meyer and Karasov, 1991). Rather, most work has focused on the effects of terpenes on rumen microbes (Nagy et al., 1964; Oh et al., 1968; Ngugi et al., 1995). Our results show that 1,8-cineole exerts significant physiological influences that cause cessation of feeding.

The time lambs fed declined over the three periods (Figure 1), suggesting increased sensitivity to infusions due to progressively stronger physiological effects (Jaffe, 1990; Rozman and Klaassen, 2001), which were conditioning an aversion to the food (Provenza and Villalba, 2006). The sharp decline in plasma concentration of 1,8-cineole during alpha elimination apparently caused these responses. Rapid alpha elimination occurs as a compound is distributed into other

tissues and is thus out of the central circulation; it is then redistributed to maintain equilibrium between blood and tissue concentrations. Rapid alpha elimination is likely critical in limiting intake and conditioning aversions. To protect the body from overingesting a toxin, feedback from cells and organs must be rapid, and in our studies rapid absorption and alpha elimination were better correlated with cessation of feeding than were plasma concentrations during excretion (beta elimination) (see also discussion on rumen infusions). With the toxin LiCl, cessation of feeding and food aversions occur as the toxin is being absorbed into the body, not during the much longer elimination phase (Provenza et al., 1993). Thus, terpenes cause lambs to satiate quickly, and when alternative foods are available, food-specific satiety mediated by toxins causes animals to eat other foods (Provenza, 1995, 1996; Provenza et al., 2003; Villalba et al., 2004).

The time to return to feeding postdosing declined from trial 1 (18 min) to trials 2 (8 min) and 3 (7 min). Apparently, cessation of dosing and lower plasma concentrations allowed lambs to resume feeding sooner. Secondary elimination was slow, and plasma concentrations did not change much from 3 to 8 hr after dosing. Under natural foraging conditions, slow secondary elimination may prolong the effects of 1,8-cineole on eating by influencing the duration and frequency of feeding bouts (Dziba and Provenza, 2006), and if suitable alternatives are available, animals eat them in preference to plants high in terpenes during beta elimination (Villalba et al., 2004). Thus, absorption and alpha elimination are likely responsible for initiating satiety, whereas beta elimination is likely responsible for the duration of the satiety effect.

The efficiency of elimination of toxins can increase through induction of liver enzymes including various CYP450 enzymes (Cheeke, 1998; Pass et al., 1999). Conversely, these enzymes can also be inhibited by the activity of toxins, decreasing rates of elimination (Pass et al., 1999; Pass and Mclean, 2002). Thus, feeding behavior may be positively or negatively influenced as metabolism and elimination of toxins is enhanced or depressed, respectively, during repeated exposure to toxins. In the case of intravenous infusions of 1,8-cineole, there was no evidence of induction or inhibition following initial exposure. Primary elimination rates did not differ among trials, yet *in vitro* studies have shown the induction of certain cytochrome P450 enzymes by 1,8-cineole in marsupials (Pass et al., 1999). In retrospect, our study of induction or inhibition was compromised by several days to weeks of no exposure to 1,8-cineole between periods. Daily exposure of animals would yield a more accurate account of whether 1,8-cineole induces or inhibits elimination rates.

Production of hydrogen ions can reduce pH in the central circulation and alter the acid–base balance (Foley et al., 1995; Illius and Jessop, 1997; Loudon et al., 1999). However, in our study, 1,8-cineole did not induce measurable changes in acid–base balance—blood pH was maintained within its narrow

range of 7.35–7.45—indicating a capacity in the acid–base balance to cope with the increase in acidic metabolites (Foley et al., 1995). Urinal excretion of hydrogen ions is one way animals can effectively maintain acid–base balance (Foley et al., 1995; Guyton and Hall, 2000). In addition, liberation of CO₂, as a means of regulating acid–base balance, could explain the observed tachypnea in lambs (Mudge and Weiner, 1990). Measuring urine pH or using other sensitive methods for monitoring urine output and chemistry might have given better information about possible challenges to acid–base balance for lambs fed terpene-containing foods (Dearing et al., 2001, 2002).

The effect of 1,8-cineole on various target organs is unknown, but observed clinical effects suggest neural affects on feeding behavior. In humans, 1,8-cineole affects the central nervous system, and common symptoms include respiratory depression and hypoventilation, depression of reflexes, convulsions, and loss of consciousness (Tibballs, 1995). Drowsiness occurs following ingestion of 1,8-cineole (Craig, 1953), which is consistent with our observations that lambs were sleepy and mildly ataxic. In humans, 1,8-cineole also causes abdominal pain, nausea and vomiting, and diarrhea.

Most of these clinical signs do not occur in herbivores, as they regulate intake of terpenes below levels that induce these symptoms (Foley et al., 1999; Dziba and Provenza, 2006). Animals experience mild versions of these and other clinical effects that lead to loss of appetite and nausea, which decrease intake by causing mild (transient) to strong (persistent) food aversions, which in turn are conditioned by aversive postingestive feedback from toxins, not by drowsiness, heavy breathing, and ataxia (Garcia et al., 1985; Garcia, 1989; Provenza et al., 1993; Provenza, 1995, 1996).

Rumen Dosing. Feeding behavior was influenced by rumen administration of 1,8-cineole. Lambs dosed with 1,8-cineole stopped feeding sooner than controls, which provides additional evidence of satiation (Provenza, 1995, 1996). Lambs also adapted to repeated dosing with 1,8-cineole. They fed longer and ate more during wk 2 than wk 1. The absorption rate constant increased from 0.035 to 0.076/min from wk 1 to 2, while the absorption half-life declined from 24 to 10 min from wk 1 to 2. The maximum plasma concentrations and the time to reach maximum plasma concentrations were no greater in wk 2 than in wk 1, but the primary elimination rate constant was 2.3 times higher in wk 2 (0.058/min) than wk 1 (0.025/min). Previous studies have also shown that animals steadily and dramatically increase their intake of sagebrush over time (Banner et al., 2000; Villalba et al., 2002; Dziba et al., 2006). These data suggest lambs adapted to 1,8-cineole in the rumen-dose study but not in the intravenous-dose study.

Several mechanisms may increase feeding time and intake. Primary elimination was much faster after the initial exposure to 1,8-cineole, although Foley et al. (1999) suggest elimination rates may have to increase as much as threefold to change feeding behavior. First-pass rates of metabolism may also

increase (Benet et al., 1990; Cheeke, 1998). Finally, rumen microbes adapt to monoterpenes (Nagy et al., 1964; Oh et al., 1968; Johnson et al., 1976), and given time, larger amounts of 1,8-cineole may be degraded in the rumen, resulting in lower levels of plasma concentrations, reduced toxic effects of 1,8-cineole, and increased food intake. Results of the rumen dosing trials, compared with the intravenous infusions trials, suggest that rumen degradation, absorption, and first-pass metabolism of 1,8-cineole all may have played important roles in detoxification and adaptation of lambs to 1,8-cineole.

In humans, 1,8-cineole quickly elicits toxic effects, suggesting rapid absorption and distribution (Patel and Wiggins, 1980). Our study also confirmed rapid absorption and immediate postabsorption effects that influenced feeding behavior. As mentioned, rates of absorption are likely critical for setting limits on intake, and in the rumen dosing studies the absorption phase was better correlated with cessation of feeding than were plasma concentrations. This makes sense; to protect the body against a toxin threat, feedback from cells and organs must be rapid. Rumen dosed lambs had a more rapid alpha elimination–distribution during the second period, yet lambs spent more time feeding and ate more during this second period. Rapid alpha elimination–distribution occurs as a compound is distributed into other tissues and later redistributed to maintain equilibrium between blood and tissue. This distribution effect likely enabled lambs to eat more after a single rumen dose of terpenes, since the infusion dosing resulted in higher plasma concentrations that likely resulted in much higher tissue concentrations. Thus, the lambs appear to more easily acclimate to the lower tissue concentrations and the resultant negative feedback on ingestion.

In addition, the increased plasma concentrations during the second period may not have been enough to change feeding behavior, particularly because the total dose was the same. Tolerance—reduced response to a toxic compound following prior exposure—of 1,8-cineole might also explain the increased feeding time and food intake (Jaffe, 1990; Rall, 1990). While all lambs licked their lips during the first rumen dosing, no lambs did during the second week. The absence of other clinical signs during rumen dosing suggests a more subdued effect of 1,8-cineole with rumen dosing than with the intravenous infusions.

Finally, our results have implications for managing rangelands dominated by sagebrush. Because 1,8-cineole limits intake by causing satiation, any mechanism that increases elimination rates or enhances ruminal adaptation might enhance use of sagebrush by sheep. Thus, prior exposure to a terpene-containing diet, training animals to mix sagebrush with other nutritious forages, and strategic supplementation to enhance detoxification processes are all likely to enhance use of sagebrush and biodiversity of landscapes dominated by sagebrush (Provenza et al., 2003).

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ANALYSIS OF INSECT CUTICULAR HYDROCARBONS USING MATRIX-ASSISTED LASER DESORPTION/ IONIZATION MASS SPECTROMETRY

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Abstract—Insect cuticular hydrocarbons (CHCs) were probed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry with a lithium 2,5-dihydroxybenzoate matrix. CHC profiles were obtained for 12 species of diverse insect taxa (termites, ants, a cockroach, and a flesh fly). MALDI spectra revealed the presence of high molecular weight CHCs on the insect cuticle. Hydrocarbons with more than 70 carbon atoms, both saturated and unsaturated, were detected. When compared with gas chromatography/mass spectrometry (GC/MS), MALDI-TOF covered a wider range of CHCs and enabled CHCs of considerably higher molecular weight to be detected. Good congruity between GC/MS and MALDI-TOF was observed in the overlapping region of molecular weights. Moreover, a number of previously undiscovered hydrocarbons were detected in the high mass range beyond the analytical capabilities of current GC/MS instruments. MALDI was shown to hold potential to become an alternative analytical method for insect CHC analyses. The ability of MALDI to discriminate among species varying in the degree of their relatedness was found to be similar to GC/MS. However, neither MALDI-MS nor GC/MS data were able to describe the phylogenetic relationships.

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Key Words—Ants, cockroach, cuticular hydrocarbons, lithium 2,5-dihydroxybenzoate, Nei distance, flesh fly, MALDI-TOF, mass spectrometry, termites.

INTRODUCTION

Hydrocarbons represent universal constituents of the insect cuticle (Wigglesworth, 1965; Blomquist and Dillwith, 1985; Hadley, 1985; Chapman, 1998). Cuticular hydrocarbons (CHCs) are considered to be stable end products of genetically controlled metabolic pathways (Ross et al., 1987; Grunshawn et al., 1990); insects are known to synthesize most hydrocarbons *de novo* by elongation–decarboxylation pathway (review, Blomquist and Dillwith, 1985; Lockey, 1985, 1988; Howard and Blomquist, 2005). The common function of CHCs is to protect against desiccation. In social insects, CHCs are regarded as the main signals responsible for nestmate recognition (review, Blomquist et al., 1998; Howard and Blomquist, 2005; ants, see Lenoir et al., 2001; termites, see Clément and Bagnères, 1998), although this premise has been confirmed only in a few cases (e.g., Takahashi and Gassa, 1995; Lahav et al., 1999). As evidenced by gas chromatography (GC), the hydrocarbon mixture usually contains up to 100 different hydrocarbons (Nelson et al., 1981). The length of hydrocarbon chains usually varies from 23 to 47 carbon atoms (Blomquist and Dillwith, 1985). Species living in dry conditions generally contain longer hydrocarbon chains in comparison to their relatives living in wet conditions (review, Blomquist and Dillwith, 1985; Lockey, 1988). Insect species usually possess complex mixtures of hydrocarbons including *n*-alkanes, branched mono-, di-, or trimethylalkanes, and others (Jackson and Blomquist, 1976). Unsaturated CHCs are also present and the position of the double bond varies within the chain (Howard and Blomquist, 1982; Blomquist and Dillwith, 1985; Lockey, 1985, 1988). Hydrocarbon profiles may serve as fingerprints defining particular species. The composition of CHCs has been extensively studied in social insects, and in termites it is often used in taxonomic discrimination (Watson et al., 1989; Kaib et al., 1991; Bagine et al., 1994; Haverty et al., 1996; Takematsu and Yamaoka, 1997; Haverty and Nelson, 1997) and sibling species recognition (Haverty and Nelson, 1997).

To date, coupled gas chromatography-mass spectrometry (GC/MS) has been the primary tool for CHC analysis. However, both GC and electron ionization MS (EI-MS) have limitations for high molecular weight compounds. For these compounds, special high-temperature GC columns have to be used to withstand the temperatures needed. However, high temperatures can cause samples to decompose. Moreover, EI-MS identification of high-mass hydrocarbons is problematic because the molecular ion is virtually absent. Thus, GC/MS enables detection of only some of the hydrocarbons present on the insect

cuticle. To obtain a more complete picture of the composition of the cuticular waxy layer, other techniques have to be employed. Laser desorption/ionization (LDI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry have been shown to be suitable methods for analyzing hydrocarbons and hydrocarbon polymers. Published methods (Kuhn et al., 1996; Yalcin et al., 1997; Dutta and Harayama, 2001; Pruns et al., 2002) used silver or copper ionization to produce ions that were subsequently detected. Recently, a new method utilizing lithium ions was described (Cvačka and Svatoš, 2003). A simple isotopic pattern of lithium adduct ions greatly simplifies spectrum interpretation. Accurate masses measured by time-of-flight (TOF) mass spectrometry have been used for the assignment of molecular structure.

In this work, the utility of MALDI-MS for characterizing CHCs was demonstrated especially for those of high molecular weight. Our objectives were to learn (1) the range of CHCs' molecular weights, (2) the types of hydrocarbons that form the high molecular weight portion of CHCs, and (3) whether MALDI mass spectra can be used for discriminating among species and for chemotaxonomical purposes. Results obtained by MALDI-MS were compared with data from GC/MS, a well-established method for CHC studies. Most work on CHCs has been done on social insects; therefore, termites were selected as our model group. Species representing different ecological strategies were chosen. In addition to termites, two ant species, the American cockroach, and a flesh fly were selected to represent related and nonrelated, social and nonsocial insects.

METHODS AND MATERIALS

Chemicals and Materials. Reagent grade standards of saturated hydrocarbons (triacontane, hexatriacontane, and tetracontane; $\geq 97\%$ pure) were obtained from Aldrich; tetratetracontane, pentacontane, and hexacontane from Fluka. Apolane-87 (24,24-diethyl-19,29-dioctadecylheptatetracontane) was from Alltech Associates (Deerfield, MA, USA). Squalane (2,6,10,15,19,23-hexamethyltetracosane) C grade was purchased from the California Corporation for Biochemical Research (Los Angeles, CA, USA), and squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene, min. 98%) from Sigma. Polyethylene glycol (PEG) oligomers (PEG 300, PEG 600, and PEG 1000) were purchased from Aldrich. Other hydrocarbon standards, 9-methylheptacosane, nonadecylcyclohexane, and icosylcyclopentane were synthesized and provided by Dr. Karel Stránský (IOCB, Prague, Czech Republic). Solvents used for sample and matrix preparation (chloroform, diethyl ether, hexane, and methanol) were distilled in glass from analytical-grade solvents.

Preparation of Lithium 2,5-Dihydroxybenzoate. Lithium-6 hydroxide monohydrate (0.55 g, 95 at.% ^6Li ; Aldrich) or lithium-7 hydroxide

monohydrate (0.55 g, 97 at.% ^7Li ; Aldrich) was dissolved in 6 ml ultrapure water and treated with freshly recrystallized 2,5-dihydroxybenzoic acid (1.50 g, Aldrich) at room temperature. The solution was cooled in a refrigerator, and the resulting white needles were washed with CHCl_3 (10 ml) and dried under vacuum. Elemental analyses revealed that both salts ($^6\text{LiDHB}$ and $^7\text{LiDHB}$) crystallize from water as tetrahydrates. For $\text{C}_7\text{H}_5\text{O}_4^6\text{Li}\cdot 4\text{H}_2\text{O}$ calc. 36.36% C, 5.68% H; Found 36.41% C, 5.59% H; for $\text{C}_7\text{H}_5\text{O}_4^7\text{Li}\cdot 4\text{H}_2\text{O}$, calc. 36.20% C, 5.65% H; Found 36.29% C, 5.65% H, respectively. A MALDI matrix solution was prepared fresh daily in CHCl_3 /acetone mixture (1:2, v/v) in a concentration of 7.5 mg/ml.

Insects. Subterranean and dry wood termite species (Isoptera: Rhinotermitidae and Kalotermitidae) used in this study originated from laboratory colonies where they were kept at $24 \pm 2^\circ\text{C}$ after being collected. Samples included 20 workers of *Reticulitermes lucifugus* (Rossi, 1792), abbreviated RL, collected in Crocemoschito, 70 km SE of Rome, Italy, 1998; 20 workers of *Reticulitermes (lucifugus) grassei* (Clément, 1981), abbreviated RG, collected in Forêt de la Coubre, near Bordeaux, France, 1998; 20 workers of *Reticulitermes santonensis* (de Feytaud, 1928), abbreviated RS, collected in Forêt des Saumonards, Ill D'Oleron, France, 1998; 20 workers of *Reticulitermes virginicus* (Banks, 1907), abbreviated RV, collected in Fort Lauderdale, FL, USA, 2003; 20 workers of *Coptotermes formosanus* (Shiraki, 1909), abbreviated CF, from a colony established in 1992 from alates from a colony collected in Hsin-hui, Guandong, China, 1963; 20 pseudergates of *Neotermes castaneus* (Burmeister, 1839), abbreviated NC, collected in Caimito, La Habana, Cuba, 1978; 20 pseudergates of *Neotermes cubanus* (Snyder, 1922), abbreviated NCub, collected in Topes de Collantes, Cuba; and 20 pseudergates of *Cryptotermes declivis* (Tsai and Chen, 1963), abbreviated CD, a colony obtained from the Guandong Institute of Entomology, Guangzhon, China, 1992. Flesh fly, cockroach, and ant species also originated from laboratory cultures where they were reared after being collected: 10 or 100 imagos of gray flesh fly *Neobellieria bullata* (Parker, 1916) (Diptera: Sarcophagidae), abbreviated NB; 1 or 10 males of the American cockroach, *Periplaneta americana* (Linnaeus, 1758) (Blattaria: Blattidae), abbreviated PA, from laboratory breedings of the Research Institute of Crop Production, Prague, Czech Republic; 7 individuals of the leaf-cutting ant *Acromyrmex echinator* (Forel, 1899), abbreviated AE, collected in a Paracou field station in French Guiana, 2001; and 6 individuals of *Atta colombica* (Guerin-Meneville, 1844) (Hymenoptera: Formicidae), abbreviated AC, collected in Barro Colorado Island, Panama, 1998. The relatively high number of insect individuals in this study was used to optimize experiments; routine measurements require at most a few individuals for successful MALDI experiments.

Sample Preparation. Insects were immobilized in the cold and stored frozen at -18°C . Prior to extraction, they were placed in a desiccator for 1 hr to get rid of surface moisture. CHCs were extracted with CHCl_3 (3–6 ml, elution time ca. 5 min) in a small glass column sealed on the bottom with precleaned cotton wool. Solvent from the extract was evaporated almost to dryness under a stream of argon. The crude extract was fractionated on precleaned glass thin layer chromatography plates (TLC, 36×76 mm) coated with Adsorbosil-Plus [Applied Science Labs; layer thickness 0.2 mm with gypsum (12%)] using hexane as a mobile phase. Spots were visualized by spraying Rhodamine 6G solution (0.05% in ethanol). To isolate both saturated and unsaturated hydrocarbons, the silica corresponding to a “relative to front” (R_F) value of 0.22 and 1.0 was scraped off the plate and extracted with 10 ml freshly distilled diethyl ether. The solvent was evaporated to dryness under a stream of argon, and the residues were weighed. The residues were reconstituted in CHCl_3 to a concentration of 1% and stored in sealed glass ampoules at -18°C . Silver ion chromatography was carried out using the same size TLC plates with a silica gel stationary phase impregnated with AgNO_3 (20%), and hexane as a mobile phase. To isolate the saturated hydrocarbons, silica around the solvent front was scraped off the plate; unsaturated hydrocarbons were isolated from all the rest. Simultaneously with preparative TLC, an analytical TLC (both normal-phase and silver ion) was run for each sample. Spots were visualized by heating the plates after spraying with conc. sulfuric acid containing ethyl vanillin. A stainless steel MALDI sample target (100 positions, Micromass) was cleaned stepwise with water, acetone, and dichloromethane (sonication for 15 min) to remove possible contamination. Chloroform extract (0.5 μL) was spotted onto a MALDI target, and, after drying, the sample was covered with 0.75 μL LiDHB matrix solution and allowed to dry in air; isotopically pure $^7\text{LiDHB}$ was used for routine measurement. For internal calibrations, 0.2 μL of a mixture of PEG oligomers (PEG 300, PEG 600, and PEG 1000, 0.1 mM each in acetone) was added to a sample. Plastic equipment was avoided during all manipulations with the samples and solvents to prevent possible contamination.

Infrared Spectroscopy and Nuclear Magnetic Resonance. IR spectra were taken as KBr pellets using a Bruker Equinox 55/S FT NIR spectrometer. ^1H NMR and ^{13}C NMR spectra were recorded using a Bruker AVANCE-500 (500.1 MHz for ^1H and 125.8 MHz for ^{13}C) FT NMR spectrometer in CDCl_3 , using Me_4Si as an internal standard at 300 K.

Gas Chromatography. GC/MS experiments were performed using a Fisons 8000 series GC equipped with MD800 quadrupole mass detector. A nonpolar DB-5 MS column (30 m \times 0.25 mm i.d., phase thickness 0.25 μm ; J&W Scientific, Folsom, CA, USA) was used to separate CHCs. The injector was operated in splitless mode at 220°C . The detector temperature was set to 200°C ;

standard 70 eV spectra were recorded at 1 scan/sec. The temperature of the GC oven was programmed as follows: 50°C/min, 10°C/min to 320°C, hold at 320°C for 10 min. Helium was used as a carrier gas at 1 ml/min. The data were analyzed using the MassLab (ver. 1.4) program. CHCs were quantified from chromatograms reconstructed from the m/z 57 ion; all hydrocarbons eluting after 15 min were integrated.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Matrix-assisted laser desorption/ionization experiments were performed on a ToFSpec 2E instrument (Micromass, Manchester, UK) operated in a reflectron mode using delayed extraction (Cvačka and Svatoš, 2003). Desorption and ionization were accomplished using a nitrogen UV laser. Matrix ions were suppressed with a low mass (300 Da or less) cutoff. Scans were accumulated using 20 laser shots and the spectra were averaged from at least 20 consecutive scans. Baseline subtracted data were smoothed, centered, and calibrated with lithium adducts of PEG. Data were collected and analyzed with MassLynx 3.2 software running on a PC workstation.

Statistical Analyses of CHCs. Statistical analysis of data from 12 insect species was carried out to compare the ability of MALDI-MS and GC/MS to discriminate among species. The Nei distances (or the Nei indices of identity) between all possible pairs of species were computed by using their CHC profiles as proposed by Nei (1972) and applied by Gush et al. (1985), Nowbahari et al. (1990), and Kaib et al. (1991). The relative ratios of the 63 most prominent peaks of GC/MS chromatograms and 492 peaks of MALDI-MS spectra were selected for the analysis. The matrices of Nei distances were submitted to cluster analyses using single linkage and UPGMA clustering methods. Hierarchical trees based on this clustering were drawn. The clustering was performed using the Statistica 6.0 program (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Hexane is the most commonly used solvent for extraction of insect CHCs. It dissolves hydrocarbons of low and medium molecular weight, i.e., hydrocarbons that can be analyzed by gas chromatography. However, its ability to extract long-chain hydrocarbons from complex matrices is somewhat limited. Based on our laboratory experiences, we selected CHCl_3 for hydrocarbon extractions. It can efficiently dissolve the entire wax layer, including long-chain hydrocarbons and more polar compounds present on the insect cuticle. The hydrocarbon fraction was isolated from the whole extract by TLC on silica, eluting with hexane. Although retention behavior of hydrocarbons and related compounds bearing functional group(s) in TLC is commonly known, we conducted experiments to ensure that very long hydrocarbon chain(s) do not affect

retention of polar compounds considerably. We observed that even very long-chain triacylglycerols, fatty acids, and alcohols stayed at the origin. Wax esters and long-chain ethers were also retained [R_F of behenyl behenate ($C_{44}H_{88}O_2$) was 0.05 and R_F of dioctadecyl ether ($C_{36}H_{74}O$) was 0.08]. Thus, when CHC extracts were separated on silica TLC plates, polar compounds remained at or close to the origin, whereas one to three spots were observed close to the solvent front, with R_F values of 0.85–1.00. These were due to hydrocarbons with various structural features, such as branching, double bonds, or rings. For selected samples, the hydrocarbon fraction was further separated on silver ion TLC. Saturated CHCs migrated with or close to the solvent front, whereas unsaturated CHCs formed zone(s) close to the origin ($R_F = 0.10$ –0.26).

Cuticular Hydrocarbons Analyzed by FT-IR, 1H NMR, and ^{13}C NMR. To obtain basic information on CHC mixtures extracted using $CHCl_3$, a TLC-separated hydrocarbon fraction was examined by IR and NMR spectroscopy. Because these methods require relatively large amounts of samples, the experiments were performed only with the extract of *N. bullata*, which was available in sufficient quantity. The goal of these experiments was to learn more about the structural features of hydrocarbons present in the sample and to confirm that no compounds containing polar functionalities remained in the hydrocarbon fraction. The IR spectrum showed intense methyl and methylene C–H stretching and bend absorptions, methylene ($-(CH_2)_n-$) rocking absorptions, and bend absorption of methyl group. Alkene C–H stretching absorption gave weak signals in the spectrum. No frequency that can be attributed to polar functional groups was found in the spectrum. In the 1H NMR spectrum, signals of hydrogens in methylene groups and in terminal methyls were recorded. Allylic protons were also observed. ^{13}C NMR revealed the presence of methylene groups of long-hydrocarbon chains and terminal and nonterminal methyls. Carbons of methylene groups next to methyl branching or next to quaternary carbon atoms were also detected. As in 1H NMR, signals indicating the presence of isolated double bonds on a long hydrocarbon chain were observed. No signals that would suggest the presence of six-membered rings or saturated polycyclic structures were found. Based on the IR and NMR spectra, it was concluded that *N. bullata* CHC fractions contained only saturated and unsaturated long-chain hydrocarbons, some of which were branched.

Cuticular Hydrocarbons Analyzed by Gas Chromatography. CHC fractions of $CHCl_3$ extracts were analyzed by GC/MS using a conventional nonpolar capillary column, and individual hydrocarbons were identified based on their mass spectra (Doolittle et al., 1995) and retention behavior (Katritzky et al., 2000). In the samples, *n*-alkanes, methylalkanes, or alkadienes (*P. americana*) were the main components; di- and trimethylalkanes and alkenes were also present (Table 1). The hydrocarbon profile was noticeably different in the *A. colombica* sample, in which long-chain CHCs (>30 carbon atoms) formed

TABLE 1. INSECT CUTICULAR HYDROCARBONS IDENTIFIED BY GC/MS^a

Compound	CN	<i>lucifugus</i>	<i>virginicus</i>	<i>grassei</i>	<i>santonensis</i>	<i>formosanus</i>	<i>cubanus</i>	<i>castaneus</i>	<i>declivis</i>	<i>americana</i>	<i>bullata</i>	<i>colombica</i>	<i>echinator</i>
	R.	R.	R.	R.	R.	R.	N.	N.	C.	P.	N.	A.	A.
Pentadecane	15	0.6	—	—	—	—	0.1	0.2	—	—	—	1.1	7.7
Hexadecane	16	0.3	—	—	—	0.4	0.2	0.2	—	—	—	1.6	8.7
Heptadecane	17	1.2	0.3	0.4	0.4	1.3	0.4	0.4	—	—	—	2.6	14.6
Octadecane	18	0.7	—	0.2	0.2	0.8	0.2	0.2	—	—	—	1.4	6.2
Unknown	0.5	—	0.2	—	—	0.5	0.1	0.2	—	—	—	0.6	4.0
Nonadecene	19	—	—	—	0.1	—	—	—	—	—	—	1.2	—
Nonadecane	19	0.5	—	0.2	0.2	1.4	0.2	0.2	—	—	—	0.9	5.8
Eicosane	20	0.7	0.2	0.3	0.2	1.5	0.2	0.2	—	—	—	0.8	3.9
Heneicosane	21	0.9	0.4	0.4	0.3	1.7	0.2	0.8	—	—	—	0.8	4.3
Docosane	22	1.6	0.6	0.6	0.5	1.9	0.4	0.8	—	—	—	—	3.5
Tricosane	23	2.2	11.5	0.8	7.5	2.1	14.4	41.9	0.1	—	0.1	0.9	12.0
11-Methyltricosane	24	0.6	0.8	—	1.0	0.5	9.2	0.4	—	—	0.1	—	0.7
2-Methyltricosane	24	—	1.6	—	2.2	—	4.6	2.2	0.3	—	—	—	0.4
3-Methyltricosane	24	—	0.9	—	0.3	—	0.3	2.4	—	—	—	—	0.6
Tetracosane	24	1.5	4.7	1.0	5.9	2.6	3.1	3.0	—	—	0.2	—	1.9
Unresolved 11-Methyltetracosane and 12-Methyltetracosane	25	0.4	0.9	—	1.9	—	—	—	—	—	—	—	—
2-Methyltetracosane	25	—	17.4	—	25.8	—	2.3	7.6	1.4	—	—	—	—
3-Methyltetracosane	25	—	0.3	—	0.6	—	0.3	0.9	—	—	—	—	—
Pentacosane	25	9.0	22.5	1.8	24.7	4.0	12.9	31.7	23.7	11.9	16.6	5.9	12.1
Unresolved 13-Methyl and 9-Methylpentacosane	26	—	—	—	—	—	—	—	—	—	—	—	—
5-Methylpentacosane	26	4.5	—	0.1	1.6	—	39.6	—	—	—	4.1	—	—
2-Methylpentacosane	26	0.9	3.7	0.3	7.6	10.6	2.1	—	3.4	—	2.1	—	—
3-Methylpentacosane	26	—	7.6	—	13.0	2.0	3.6	3.4	6.5	16.9	1.4	—	—

Hexacosane	26	2.5	1.2	2.7	1.2	3.8	0.6	0.6	5.4	—	1.1	—	—
Unknown	27	0.4	—	2.1	0.3	—	1.4	—	—	—	—	—	—
2-Methylhexacosane	27	2.8	0.6	0.8	0.6	2.7	—	—	7.7	—	—	—	—
6,9-Heptacosadiene	27	—	—	—	—	—	—	71.3	—	—	—	—	—
Heptacosane	27	0.3	—	0.5	0.2	0.4	—	—	10.1	—	0.3	—	—
Heptacosane	27	26.8	3.1	29.5	1.6	7.2	2.8	2.7	33.2	—	26.4	7.1	8.7
Unresolved	28	4.9	3.1	14.5	0.3	23.9	0.6	—	2.2	—	2.3	—	—
13-Methyl and													
9-Methylheptacosane													
7-Methylheptacosane	28	—	—	—	—	—	—	—	—	—	1.8	—	—
5-Methylheptacosane	28	7.6	—	2.1	—	—	—	—	1.1	—	0.4	—	—
2-Methylheptacosane	28	—	3.0	15.8	—	13.6	—	—	—	—	—	—	—
3-Methylheptacosane	28	2.2	1.3	—	—	6.9	—	—	0.7	—	5.1	—	—
Octacosane	28	3.0	0.5	4.8	0.5	2.1	0.3	—	0.9	—	0.8	1.0	1.2
Unknown	—	—	—	4.5	—	—	—	—	0.5	—	—	—	—
Nonacosane	29	4.4	—	7.1	—	2.3	—	—	—	—	14.0	11.4	3.7
Unknown	—	—	0.9	—	0.7	—	—	—	1.9	—	—	—	—
Unresolved	30	1.9	0.8	3.4	—	5.6	—	—	—	—	8.0	—	—
13-Methyl-,													
9-Methyl-, and													
7-Methylnonacosane													
5-Methylnonacosane	30	7.3	1.5	—	—	—	—	—	—	—	0.8	—	—
2-Methylnonacosane	30	3.8	—	—	—	—	—	—	—	—	—	—	—
3-Methylnonacosane	30	—	—	—	—	—	—	—	—	—	9.0	—	—
Unknown	—	—	—	—	—	—	—	—	—	—	1.1	—	—
Unknown	—	0.4	—	—	0.3	—	—	—	0.8	—	1.0	0.8	—
Unresolved	31	—	—	—	—	—	—	—	—	—	2.0	—	—
13-Methyl-,													
9-Methyl- and													
7-Methylhentriacontane													
3-Methylhentriacontane	31	—	—	—	—	—	—	—	—	—	1.3	—	—

TABLE 1. (CONTINUED)

Compound	R.	R.	R.	R.	R.	C.	N.	N.	C.	P.	N.	A.	A.
	CN	<i>lucifugus</i>	<i>virginicus</i>	<i>grassei</i>	<i>santonensis</i>	<i>formosanus</i>	<i>cubanus</i>	<i>castaneus</i>	<i>declivis</i>	<i>americana</i>	<i>bullata</i>	<i>colombica</i>	<i>echinator</i>
11,18-Dimethyl triacontane	32	2.2	1.9	1.6	-	-	-	-	-	-	-	-	-
11,16-Dimethyl hentriacontane	33	1.5	2.5	2.2	-	-	-	-	-	-	-	-	-
8,12-Dimethyl dotriacontane	34	1.8	2.8	1.1	-	-	-	-	-	-	-	-	-
Unknown		-	0.8	-	-	-	-	-	-	-	-	-	-
11-Methyltrtriacontane	34	-	2.5	0.9	0.4	-	-	-	-	-	-	-	-
3,7,11-Trimethyl hentriacontane	34	-	-	-	-	-	-	-	-	-	-	12.9	-
4,8,12-Trimethyl dotriacontane	35	-	-	-	-	-	-	-	-	-	-	8.3	-
Unknown		-	-	-	-	-	-	-	-	-	-	4.8	-
3,7,11-Trimethyl triacontane	36	-	-	-	-	-	-	-	-	-	-	17.8	-
Unknown		-	-	-	-	-	-	-	-	-	-	0.6	-
Unknown		-	-	-	-	-	-	-	-	-	-	2.2	-
4,8,12-Trimethyl tetatriacontane	37	-	-	-	-	-	-	-	-	-	-	7.3	-
Unknown		-	-	-	-	-	-	-	-	-	-	0.9	-
Unknown		-	-	-	-	-	-	-	-	-	-	2.9	-
3,7,11-Trimethyl pentatriacontane	38	-	-	-	-	-	-	-	-	-	-	2.3	-
Unknown		-	-	-	-	-	-	-	-	-	-	1.0	-
Unknown		-	-	-	-	-	-	-	-	-	-	1.0	-

^aRelative composition in percent based on the peak areas integrated in reconstructed chromatograms based on the *m/z* 57 ion. CN = carbon number.

about 60% of the total peak area. We compared our GC/MS results with literature data; reports for *R. grassei*, *R. santonensis*, *C. formosanus*, *A. colombica*, *P. americana*, and *N. bullata* were available (Martin and MacConnell, 1970; Jackson, 1972; Jackson et al., 1974; Arnold and Regnier, 1975; Haverty et al., 1990, 2000; Vauchot et al., 1998). We found a good correlation between published data and our results with all the main compounds being detected. Minor differences were observed for unsaturated CHCs, where some alkenes were not found, probably due to coelution with other hydrocarbons. The integration of reconstructed chromatograms based on the m/z 57 ion also underestimated unsaturated hydrocarbons. Although direct comparison of the extraction using CHCl_3 and the common method with hexane was not performed, the agreement with literature data suggests that both extraction methods are comparable. As expected, silver ion TLC efficiently separated saturated CHCs from unsaturated CHCs. In *N. bullata*, almost all CHCs detected by GC/MS were saturated, with only a few tiny peaks due to unsaturated compounds being detected. Using GC/MS, we observed CHCs with up to 35 carbons. Higher injector and analysis temperatures would probably shift the CHC profile toward the hydrocarbons of slightly higher molecular weights, but it is clear that GC/MS discriminates against higher molecular weight components, if they are detected at all.

Cuticular Hydrocarbons Analyzed by MALDI-TOF. When irradiated with a laser pulse, the LiDHB matrix produced a mixture of matrix ions. Spectra of $^7\text{LiDHB}$ measured without ion suppression showed intense lithium cation signals at m/z 7.0 and an intense ion series at m/z 161.0, 167.1, and 173.1, corresponding to protonated $^7\text{LiDHB}$ and to protonated $^7\text{LiDHB}$, with one and two hydrogen atoms replaced with ^7Li , respectively. An ion at m/z 143.0 was formed from loss of water from protonated $^7\text{LiDHB}$. For a short time at the beginning of irradiation, we observed two more ion series representing clusters of two and three molecules of LiDHB with a various number of hydrogens replaced by lithium. These ions had the general structures $\text{C}_{14}\text{H}_{10-n}\text{Li}_{2+n}\text{O}_8$ and $\text{C}_{21}\text{H}_{15-n}\text{Li}_{2+n}\text{O}_{12}$, where $N = 0-4$; they disappeared quickly after several laser shots. Another lithiated ion series separated by 28 mass units at m/z 409.3, 437.3, and 465.3 was observed in the spectra. These ions were probably not related to the matrix and came from contamination; however, we were not able to trace the source of the contamination. The relatively small number of matrix ions enabled spectra to be recorded starting from m/z 200. However, because compounds with low molecular weights are somewhat volatile, compounds with molecular weights of <400 Daltons were not analyzed. Thus, with the exception of the matrix and contamination peaks mentioned above, clean, featureless blank matrix spectra were routinely obtained.

Using the $^7\text{LiDHB}$ matrix, lithium adduct ion formation was studied for several hydrocarbon standards. Representatives of diverse hydrocarbon classes were analyzed, including long-chain saturated hydrocarbons (triacontane, hexa-

triacontane, tetracontane, tetratetracontane, pentacontane, and hexacontane), branched saturated hydrocarbons (9-methylheptacosane, squalane, Apolane-87), and unsaturated and cyclic hydrocarbons (squalene, nonadecylcyclohexane, icosylcyclopentane). The sample (0.5 μg) was spotted onto a MALDI target. For all these compounds, single signals at m/z $[M + {}^7\text{Li}]^+$ with the expected isotope pattern were observed in the spectra. No cluster ions (e.g., $[2M + \text{Li}]^+$) were observed for any of the compounds or for mixtures. At this concentration range, no dehydrogenation was observed; however, at 10 times the concentration, a minor peak two mass units lower than that of $[M + {}^7\text{Li}]^+$ was detected. In no case did the relative intensity of this peak exceed 10% of the adduct ion. The mechanism of formation of these dehydrogenated ions is unclear. However, a tentative explanation could be laser-induced hydrocarbon dehydrogenation, which may be more pronounced at low matrix/hydrocarbon ratios. The alkenes formed likely have more affinity for complexing with the Li ion and therefore appear more abundant. Different ionization efficiencies of diverse hydrocarbon classes (saturated vs. unsaturated) were observed but not quantified because of the lack of sufficiently nonvolatile standards.

When CHC samples were subjected to MALDI-TOF analysis, distinctive profiles were observed for individual insect species (Figures 1–3). The most intense ions were observed roughly between m/z 500 and 700. In some samples, a second series of hydrocarbons also appeared around m/z 800–850. The largest hydrocarbons observed in the spectra had more than 70 carbon atoms. In the low-mass region of MALDI-TOF spectra, ions that correspond to hydrocarbons measured by GC/MS were observed. An example of the low-mass region of *R. lucifugus* spectrum is shown in Figure 4. When compared with GC/MS results (see Table 1), the peaks of high intensity in the MALDI-TOF spectrum can be assigned as follows: m/z 387.4 corresponds to the sum of heptacosane and 2-methylhexacosane; m/z 401.4 can be explained by a sum of methylheptacosanes and octacosane; m/z 415.4 represents nonacosane; and m/z 429.4 is the sum of methylnonacosanes. In the MALDI-TOF spectrum, there were also peaks that were not seen in the GC/MS traces. These ions might be unsaturated or cyclic derivatives of heptacosane (m/z 383.4) and nonacosane (m/z 409.4; m/z 411.4; and m/z 413.4). There are two possible explanations for why these compounds were observed in MALDI but not in GC/MS traces. First, these compounds form cationic adducts efficiently in MALDI experiments. Second, these compounds may decompose in GC injectors. In order to characterize ions observed in the MALDI spectra more precisely, the samples were mixed with an internal calibrant (PEG) and mass spectra were recorded. Because the nominal mass of methylene group is the same as the nominal mass of 14 hydrogens, one can easily confuse a hydrocarbon having seven double bonds or rings with a hydrocarbon that is one methylene group shorter. The difference (CH_2 -14H) is 0.094 u, which can be reliably measured with instruments having a resolution in

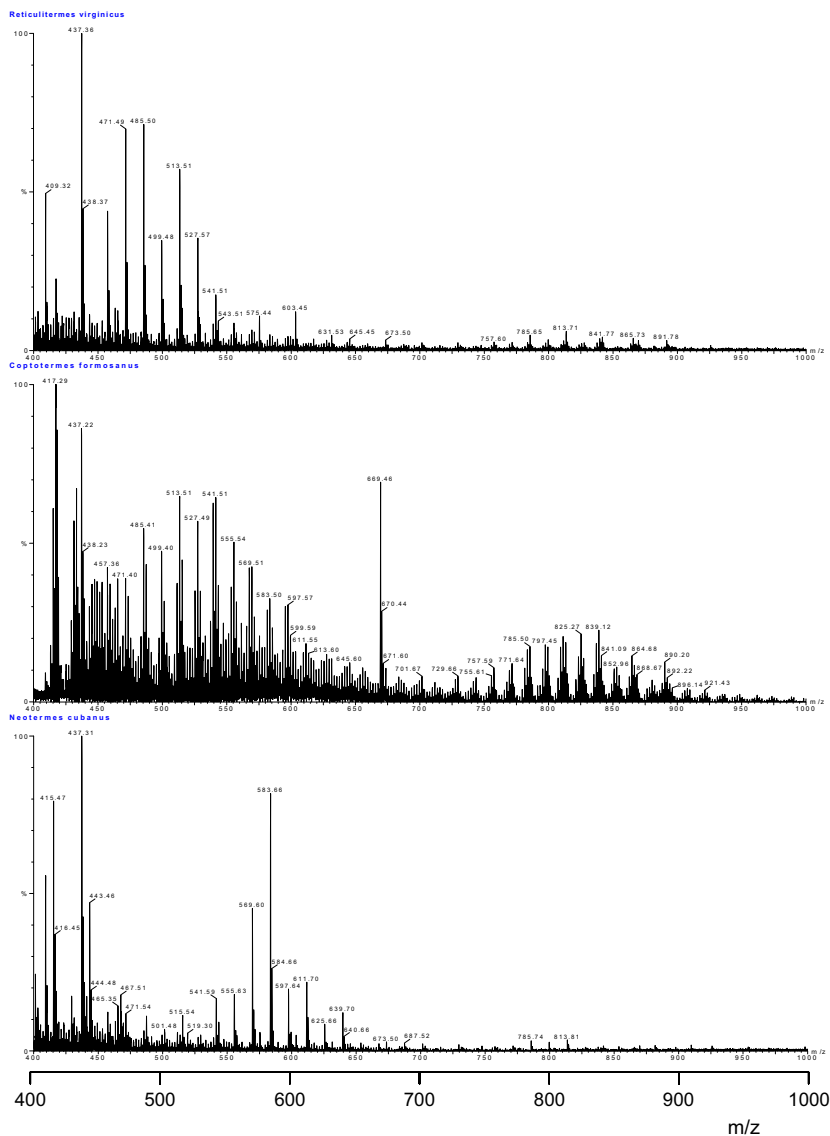


FIG. 1. MALDI-TOF mass spectrum of cuticular hydrocarbons of (a) *Reticulitermes virginicus*, (b) *Coptotermes formosanus*, and (c) *Neotermes cubanus*. $^7\text{LiDHB}$ matrix ions were eliminated by using a low mass (300 Da) cutoff, scans were accumulated from 20 laser shots, and spectra shown are averages of at least 20 consecutive scans. Baseline subtracted data were smoothed, centered, and m/z scale calibrated using lithium ions of polyethylene glycol standards.

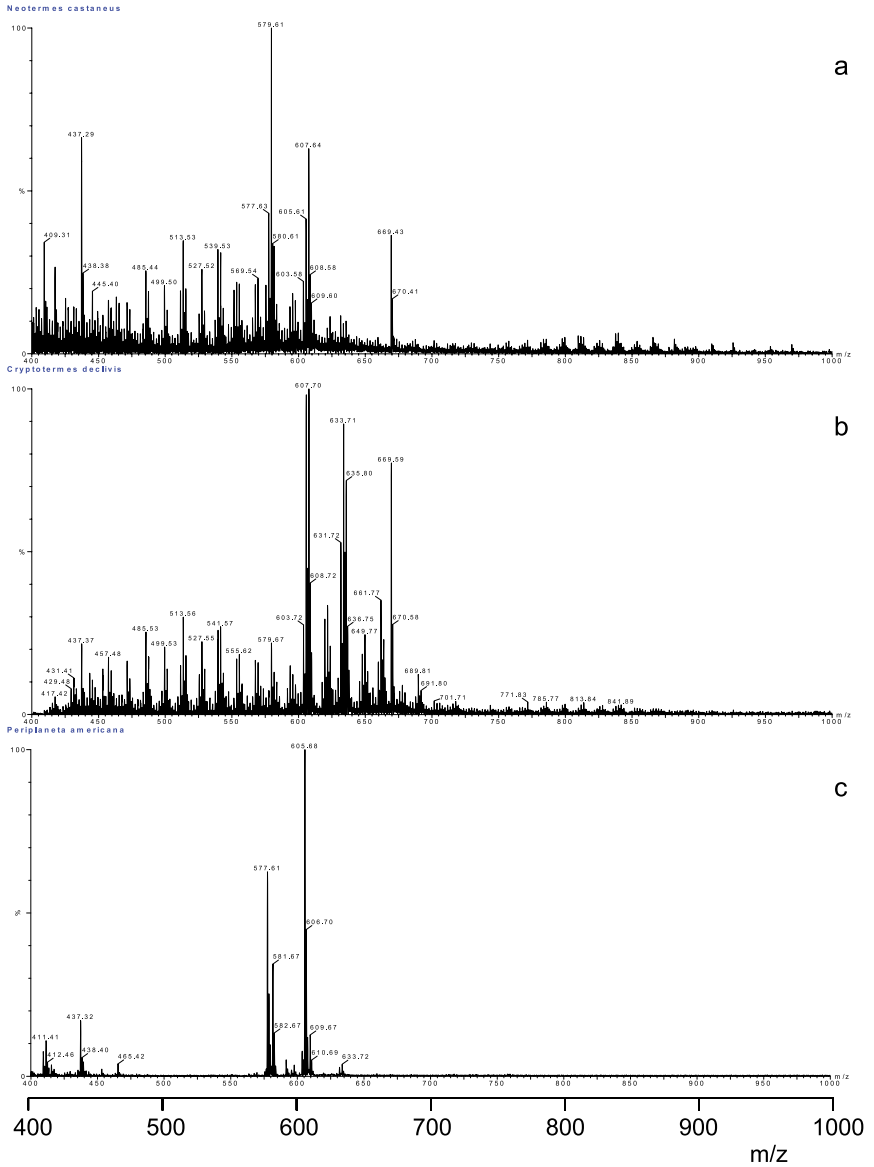


FIG. 2. MALDI-TOF mass spectrum of cuticular hydrocarbons of (a) *Neotermes castaneus*, (b) *Cryptotermes declivis*, and (c) *Periplaneta americana*. For details, see Fig. 1.

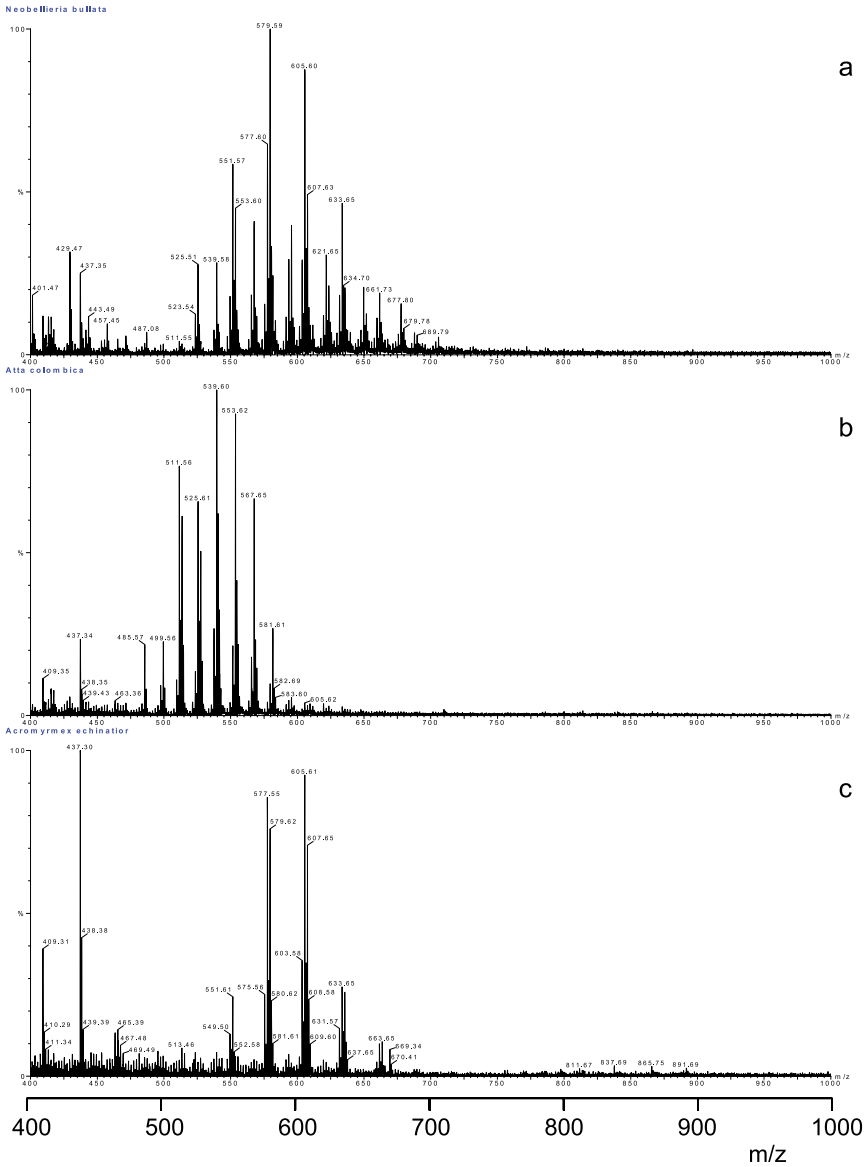


FIG. 3. MALDI-TOF mass spectrum of cuticular hydrocarbons of (a) *Neobellieria bullata*, (b) *Atta colombica*, and (c) *Acromyrmex echinator*. For details, see Fig. 1.

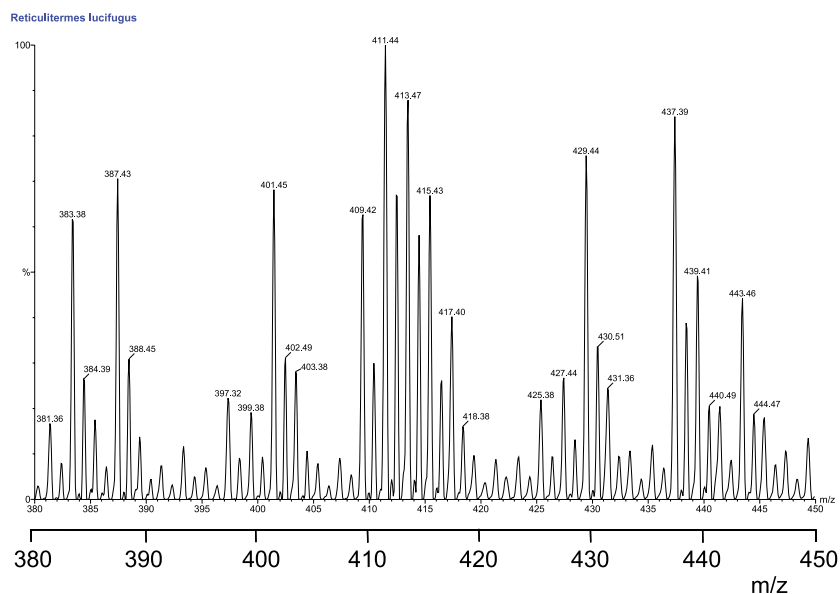


FIG. 4. Low mass region of MALDI-TOF mass spectrum of *Reticulitermes lucifugus*. For details, see Fig. 1.

the range of 5000–10,000. Such resolution is possible among current TOF analyzers. The mass precision achieved with our instrument was affected by a relatively low sampling rate (500 MHz) giving roughly 8 data points per spectrum peak, but internal calibration provided mass precision of measurements to about 75 ppm. The ions observed in the spectra were identified with the help of a software (available upon request) that calculates the possible hydrocarbon adduct ions of the general structure C_xH_yLi and scores the hits based on the mass error. Hits with the best matches were considered positively identified.

The mass spectra of *Reticulitermes* termite samples indicated the presence of saturated acyclic hydrocarbons up to roughly C45 (e.g. *R. virginicus*, Figure 1a). These compounds were the main constituents and were found in all samples of this genus studied in this work. Differences between species were in the intensity of particular hydrocarbons and in the presence of other hydrocarbons. *Reticulitermes* species also showed a second series of lithium adduct ions corresponding to highly unsaturated or cyclic C55–C65 hydrocarbons with 18–19 sites of unsaturation. The CHCs of *C. formosanus* (Figure 1b) were quite complex. The first series reached its maximum around C36–C38, and the second around C60. A surprisingly high number of sites of unsaturation (27) was

calculated for an ion at m/z 669.43 (C51). However, the presence of such large numbers of double bonds or rings in both the *Reticulitermes* species and *C. formosanus* remains to be confirmed by other methods. Mass spectra of CHCs from two *Neotermes* species differed greatly from one another. The *N. cubanus* sample (Figure 1c) contained mainly saturated acyclic hydrocarbons up to C46. A few peaks of highly unsaturated or cyclic C55–C65 CHCs were also present. The mass spectrum of *N. castaneus* CHCs (Figure 2a) showed groups of ions with 0–4 sites of unsaturation. As with *C. formosanus*, a distinct peak at m/z 669.45 as well as a second series around C60–C70 was observed. A MALDI mass spectrum of *C. declivis* is shown in Figure 2b. Internal calibration for this sample was not performed because of insufficient sample amounts. Thus, the interpretation was done based on external calibration data only. Both saturated and unsaturated or cyclic hydrocarbons were identified. The spectrum showed distinct intense peaks interpreted as C43 and C45 with 3–4 sites of unsaturation. The MALDI spectrum of *P. americana* (Figure 2c) showed two groups of hydrocarbons (C41 and C43) with 1 and 3 sites of unsaturation. A mass spectrum of flesh fly *N. bullata* CHCs (Figure 3a) showed a distribution of C36–C50 hydrocarbons. Lithium adduct ions formed groups corresponding to acyclic saturated hydrocarbons and unsaturated or cyclic CHCs with 1–4 sites of unsaturation. Hydrocarbons with an odd number of carbon atoms were more intense compared to those with an even number of carbons. In the mass spectrum of a leaf-cutting ant *A. colombica* (Figure 3b), a distribution of C34–C42 CHCs was found. Adduct ions formed groups corresponding to saturated hydrocarbons and those having 1–2 sites of unsaturation. The sample from another leaf-cutting ant, *A. echinator* (Figure 3c), contained almost exclusively odd-numbered CHCs (C41–C49). Lithium adduct ions in the spectra corresponded to compounds with 1–4 sites of unsaturation.

To obtain more in-depth insight into CHCs, fractions of saturated and unsaturated hydrocarbons were analyzed separately. Figure 5 shows the MALDI spectrum of saturated (Figure 5a) and unsaturated (Figure 5b) hydrocarbons of *P. americana*. The most intense signals in the spectrum of saturated CHCs give alkanes with 25 and 26 carbons; they were identified by GC as pentacosane and 3-methylpentacosane (Table 1, Jackson, 1972). Less abundant higher hydrocarbons, C27 and C42, were previously characterized as heptacosane and 3-methylhentetracontane (Jackson, 1972). Few other saturated CHCs (C29, C40, and C44) were observed in the MALDI spectrum. The spectrum of unsaturated hydrocarbons from *P. americana* (Figure 5b) showed alkenes with 1–3 sites of unsaturation. An ion at m/z 383.43 is a lithium adduct of (*Z,Z*)-6,9-heptacosadiene, previously identified from this cockroach (Jackson, 1972; Suiter et al., 1996). Two clusters of CHCs with 41 and 43 carbons probably contain trienes, dienes, and monoenes. Trienes had not previously been described, whereas monoenes were identified as *cis*-15-hentetracontene and

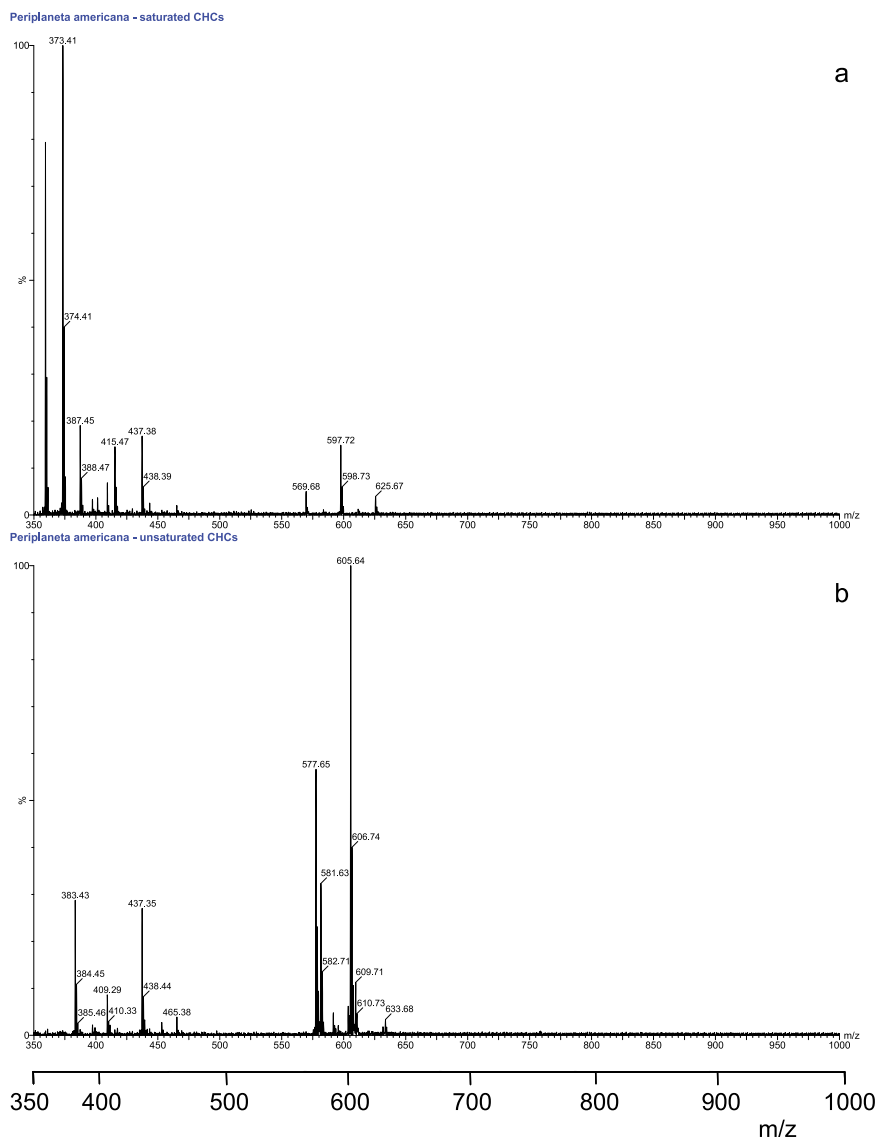


FIG. 5. MALDI-TOF mass spectrum of (a) saturated and (b) unsaturated CHCs of *Periplaneta americana*. For details, see Fig. 1.

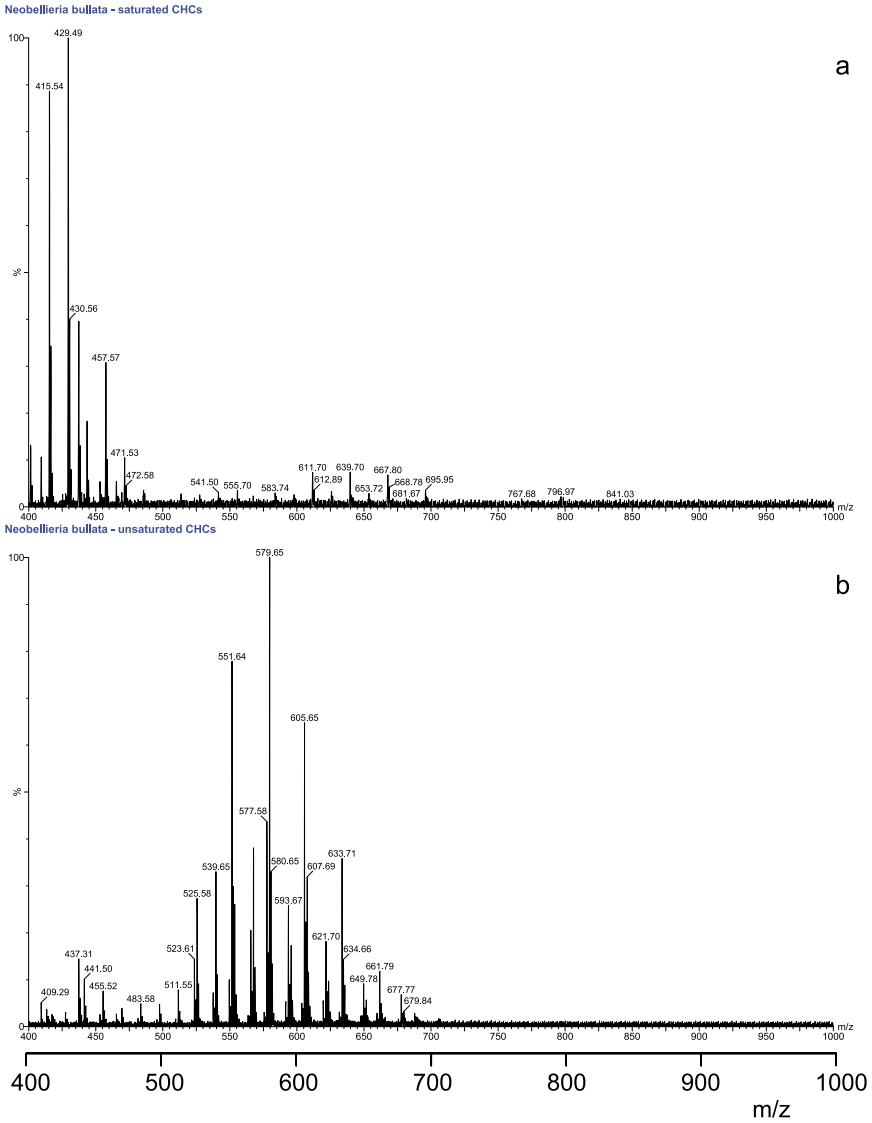


FIG. 6. MALDI-TOF mass spectrum of (a) saturated and (b) unsaturated CHCs of *Neobellieria bullata*. For details, see Fig. 1.

cis-15-tritetracontene (Jackson, 1972). In the MALDI spectra, low-intensity signals of CHCs with 42 and 45 carbons and 3 sites of unsaturation were also detected. Thus, our MALDI spectra was in agreement with published data. In addition, hydrocarbons with 3 sites of unsaturation, which are present in significant amounts, were newly revealed. Another example of separately analyzed saturated and unsaturated CHCs (*N. bullata*) is given in Figure 6a and b. The spectrum of saturated CHCs shows alkanes with up to 47 carbons. The literature (Jackson et al., 1974; Arnold and Regnier, 1975) and our results from GC/MS identified these peaks as nonacosane (C29), methyl nonacosanes (C30), hentriacontane (C31), methyl hentriacontane and dotriacontane (C32), and tritriacontane (C33), whereas the others had not been described previously. When Figure 6b is compared with Figure 3a, it is apparent that almost all MALDI peaks in *N. bullata* are due to the unsaturated CHCs. As described above, CHCs with up to 4 sites of unsaturation were detected, with those having an odd number of carbons being most abundant. We regard the presence of rings as unlikely (no evidence exists for rings from NMR experiments) and consider these structures to be mono-, di-, tri-, and tetraenes.

The spectra of all CHCs were a simple sum of the spectra of saturated and unsaturated CHCs because unsaturated compounds more effectively compete for lithium ions, resulting in saturated CHCs being less pronounced in the composite spectra. Therefore, crude extracts should be fractionated into saturated and unsaturated hydrocarbons by silver ion chromatography before analysis of complex hydrocarbon mixtures. Importantly, silver ions do not appear to contaminate CHC fractions during separation, because no silver ion adducts were observed in the MALDI spectra. To summarize, distinctive CHC profiles were recorded for all tested species using MALDI-TOF analysis. Saturated hydrocarbons, as well as hydrocarbons with varying degrees of unsaturation, were found. It is likely that polyunsaturated CHCs readily ionize, increasing their intensities in the spectra. Also, the exact structures of the unsaturated hydrocarbons could not be determined from the MALDI-TOF spectra.

The MALDI technique was also tested for reproducibility and capacity for quantitatively measuring CHCs, for its possible use in chemotaxonomy. Although LDI and MALDI are not techniques generally considered suitable for quantitation, their usefulness in this field has been demonstrated (Dutta and Harayama, 2001). Pruns et al. (2003) applied principal component analysis to LDI-TOF mass spectra to characterize paraffin oils and petrolatum samples. In our experiment, spectra of a model sample (paraffinum liquidum, 5.0 µg) spotted in triplicates onto two MALDI targets were recorded. Averaged spectra from 20 to 120 scans were compared, and similar-looking spectra differing slightly in peak intensities were obtained. To quantify the reproducibility of the measurement, relative standard deviations (RSDs) were calculated for ratios of selected peaks. Low RSDs (<10%) were calculated for ratios of peaks differing

in 14 mass units (adjacent alkanes). However, the reproducibility of ratios of peaks differing by more than 100 mass units was worse, with RSDs between 20 and 50% being obtained. Several factors affect the reproducibility of the signals in MALDI experiments. First, the homogeneity of sample deposition and uniform incorporation of analyte molecules into the matrix is critical. The LiDHB matrix is polar and finding a suitable solvent system that allows the dissolution of both the sample and the matrix is not straightforward. Nonuniform samples with variable concentrations of sample components across the sample well on the MALDI target can result. Uniform samples can be achieved using solvent-free preparation methods, but a substantially larger amount of sample is needed. Another method to obtain homogenous MALDI samples is electrospray sample deposition (Hanton et al., 2004). Second, lower molecular weight hydrocarbons under the low pressure in the ion source may tend to volatilize off the target. Therefore, the time between the introduction of the target plate into the vacuum and sample measurement should be controlled. Third, a substance-dependent time delay between the beginning of the irradiation of the sample and the appearance of the signal in the spectrum was observed, with long-chain saturated hydrocarbons taking a longer time (or laser shots) to appear in the spectrum. Therefore, the relative intensities of peaks in the spectra depend on the acquisition time. Thus, precise control of experimental parameters will increase reproducibility. The reproducibility that exists in GC/MS measurements cannot be achieved but the quality of the MALDI data is sufficient for statistical processing and discriminating among species. The work described below summarizes results from two sets of experiments (including extraction steps) that were performed consecutively over 6 months. Comparable CHC profiles were obtained for all model species.

Statistical Analysis. Nei distances proved to be an appropriate tool for evaluating phenotype distances within CHCs among studied species. The clustering of Nei distances among CHC profiles (Figure 7) showed a high potential for discriminating with both GC/MS and MALDI-MS data. The consistency between hierarchical classifications calculated by two different methods (UPGMA and single linkage) was similar for both MALDI-MS and GC/MS. The position of a single species (*C. formosanus* in MALDI-MS and *N. cubanus* in GC/MS) was the only difference between the two statistical methods used (the interchange of *P. americana* and *C. declivis* in Figure 7d and e is due to the different insertion of *C. formosanus*). When compared with GC/MS, MALDI-MS data showed similar efficiency in discriminating among particular species (compare Figure 7a–d). With both techniques, some related species were repeatedly grouped together (e.g., *R. santonensis* and *R. virginicus* in GC/MS, the whole genus *Reticulitermes* in MALDI), whereas others were consistently separated (both ant species, both *Neotermes* species). The CHC profile of an unrelated species, the cockroach *P. americana*, measured by GC/

MS, differed quite notably from all others. It consisted primarily of three CHCs (Table 1). Moreover, the major compound (6,9-heptacosadiene, 71%) was unique to this species, which set it apart from all others (Figure 7a,b). In the MALDI spectrum of *P. americana* CHCs, all 25 peaks (the average number is 199 for the other species studied) had the same mass as peaks in other species, and therefore the cockroach was placed deeply inside the tree (Figure 7c,d).

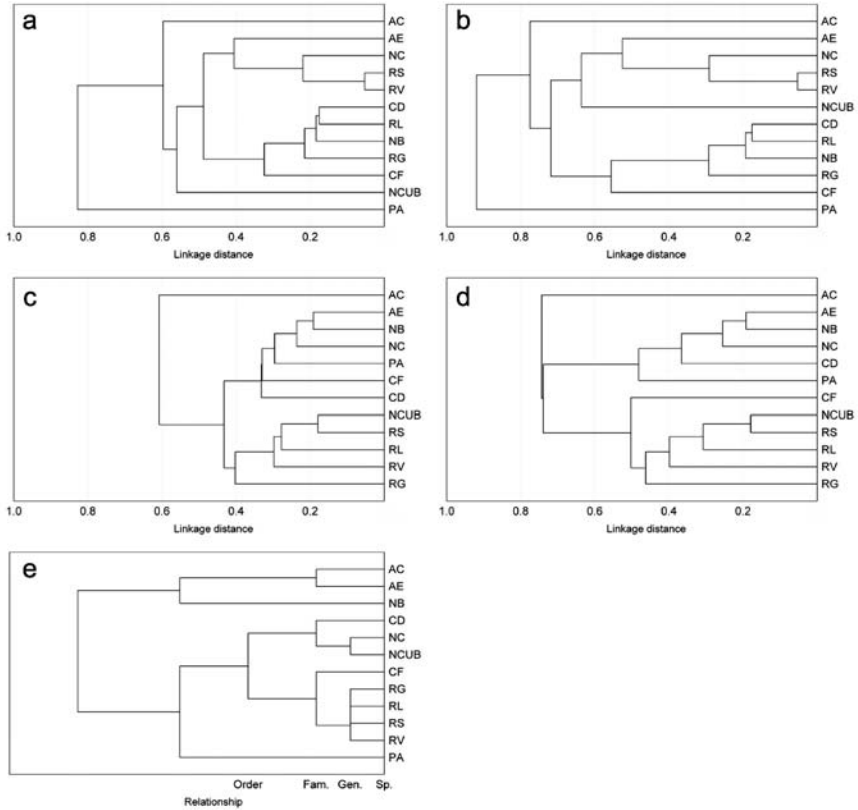


FIG. 7. Hierarchical tree made by single linkage clustering of Nei distances among cuticular hydrocarbon profiles analyzed by GC/MS (a), made by UPGMA clustering of Nei distances among cuticular hydrocarbon profiles analyzed by GC/MS (b), made by single linkage clustering of Nei distances among cuticular hydrocarbon profiles analyzed by MALDI MS (c), made by UPGMA clustering of Nei distances among cuticular hydrocarbon profiles analyzed by MALDI MS (d), hierarchical tree representing the general phylogenetic relationships among the 12 analyzed species (e). Abbreviations of insect species are listed in [Insects](#); Sp.: species, Gen.: genus, Fam.: family.

Conversely, because few peaks in both the GC/MS and MALDI spectra of *A. colombica* CHCs are shared with other species, this ant species was always placed apart from the others (Figures 7a–d).

The primary function of CHC, water regulation, may be largely independent of their composition. Therefore, CHCs may be good candidates for phylogenetic characters. However, this selection neutrality may also enable rapid changes in CHC profiles during evolution, which could conceal the real relatedness even in closely related species. Support for this hypothesis is provided by the considerable differences found in CHC profiles of many closely related species (Watson et al., 1989; Kaib et al., 1991; Golden et al., 1992; Bagine et al., 1994; Haverty et al., 1996; Haverty and Nelson, 1997; Takematsu and Yamaoka, 1997). On the other hand, CHCs were shown to reliably indicate the relatedness on the lowest levels, i.e., at the (sub)species, population, or colony level (Kutnik et al., 2004). Not surprisingly, neither GC/MS nor MALDI-MS was able to figure out the phylogeny of the studied species. Contrary to our expectations, none of the groups of closely related species were separated from nonrelative species in any of the tests. On the other hand, MALDI-MS data grouped together all four species from the genus *Reticulitermes*, but, unfortunately, also with *N. cubanus*. Nevertheless, MALDI-MS of CHCs may have some utility in phylogenetic and chemotaxonomic studies.

In conclusion, MALDI-TOF mass spectrometry with a lithium 2,5-dihydroxybenzoate matrix revealed the presence of high molecular weight hydrocarbons on insect cuticles, including saturated hydrocarbons, and highly unsaturated and/or cyclic compounds. A detailed investigation of *N. bullata* CHC extracts using IR and NMR confirmed the presence of structural features commonly observed in GC/MS analyses, such as long hydrocarbon chains with some methyl branching and double bonds, but no evidence of rings. MALDI-MS spectra for the same samples corroborated the presence of hydrocarbons with 0–4 double bonds. The samples of other species, however, gave composite spectra possibly consistent with a considerably higher number of double bonds and/or rings. However, no independent proof of such hydrocarbons has been obtained yet. Both MALDI mass spectra and GC/MS chromatograms were able to discriminate among the species studied. Therefore, MALDI-MS has the potential to become an alternative or complementary analytical technique for chemotaxonomic studies, enabling us to extend the range of molecular weights of compounds studied. Further instrumentation developments may provide even more information about analyzed samples, for example, by interfacing of MALDI with high-performance liquid chromatography (Esser et al., 2000) or supercritical fluid chromatography (Planeta et al., 2002). Furthermore, excellent quality data (mass accuracy, resolution, and sensitivity) can be obtained with MALDI Fourier-transform ion cyclotron resonance mass spectrometry, and MS experiments might help to elucidate structures of unknown CHCs. Further research is needed

to elucidate complete structures of the high molecular weight hydrocarbons found in this study, and subsequently, to determine their possible biological activity.

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(Z)-9-NONACOSENE—MAJOR COMPONENT OF THE
CONTACT SEX PHEROMONE OF THE BEETLE
Megacyllene caryae

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Abstract—Male *Megacyllene caryae* (Gahan) (Coleoptera: Cerambycidae) respond to females only after touching them with their antennae, indicating that mate recognition is mediated by a contact sex pheromone. Gas chromatography-mass spectrometry analyses of whole-body solvent extracts of male and female *M. caryae* revealed substantial differences in hydrocarbon profiles, with nearly half of the compounds in the extracts from females being absent from those of males. Biological activities of fractions of crude extracts of females, and reconstructed blends of the most abundant straight-chain (nC_{27} , nC_{28} , nC_{29}), methyl-branched (2Me- C_{26} , 9Me- C_{29} , 11, 13, 15Me- C_{29}), and unsaturated (Z9: C_{29} , Z13: C_{29} , Z14: C_{29} , Z13: C_{31} , Z14: C_{31} , Z15: C_{31}) compounds in extracts of females were tested in arena bioassays, assessing four steps in the mating behavior sequence of males (orientation, arrestment, body alignment, mounting and attempting to couple the genitalia). Males showed limited response to dead females treated with fractions of the crude extract or blends of synthetic straight-chain and methyl-branched alkanes, but responded strongly to the blend of synthetic monoenes. Further trials determined that the complete sequence of mating behaviors, up to and including coupling the genitalia, was elicited by Z9: C_{29} alone. Z9: C_{29} is a homolog of the contact pheromone (Z9: C_{25}) of the congener *M. robiniae* (Förster). Previous work with *M. robiniae* suggested that wipe sampling of cuticular

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hydrocarbons of females by solid phase microextraction yielded a more representative profile of components actually encountered by a male's antennae, and so provided a more readily interpretable profile of potential semiochemicals present in the wax layer than does solvent extraction. We tested this hypothesis by comparing hydrocarbon profiles of female *M. caryae* by the two sampling methods. Z9:C₂₉ was the only compound among the dominant hydrocarbons that was present in higher abundance in SPME than in solvent extracts (~12% vs. ~8%, respectively), supporting this hypothesis.

Key Words—Cerambycidae, mating behavior, cuticular hydrocarbon, solid-phase microextraction, contact sex pheromone.

INTRODUCTION

The wax layer on the insect cuticle is a complex mixture of long-chain hydrocarbons, fatty acids, alcohols, esters, aldehydes, and ketones that protects insects from desiccation (Gibbs, 1998). Some of these compounds also have secondary roles as contact pheromones (Blomquist et al., 1993). Contact pheromones mediate mate recognition in several species of cerambycid beetles (Kim et al., 1993; Fukaya et al., 1996, 1997, 2000; Wang, 1998; Ginzl and Hanks, 2003; Ginzl et al., 2003a,b). We report here the results of studies on a cuticular sex pheromone of female *Megacyllene caryae* (Gahan) (Coleoptera: Cerambycidae).

M. caryae is endemic to north-central North America (Linsley, 1964). Adults are diurnal, aposematically colored wasp mimics, ~2 cm long, and are active in spring. Adults of both sexes are mutually attracted to volatiles emanating from their larval hosts, stressed or weakened hickory trees (Ginzl and Hanks, 2005). Males orient to females only after contacting them with their antennae, and mate recognition is cued by contact chemoreception (Ginzl and Hanks, 2003). After touching a live or freeze-killed female with his antennae, a male *M. caryae* aligns his body with that of the female and attempts to copulate, bending his abdomen to couple with the female's genitalia (Ginzl and Hanks, 2003). Males did not display any of these behaviors when presented with solvent-extracted, freeze-killed females, but reapplying crude solvent extract to solvent-washed female carcasses restored activity, demonstrating that mate recognition and subsequent mating behaviors are cued by extractable chemicals in the cuticular wax layer (Ginzl and Hanks, 2003).

We report here the identification of an important component of the female-produced contact sex pheromone for *M. caryae*. Two methods of sampling were used: the traditional whole-body solvent extraction and solid phase microextraction (SPME) wipe sampling. SPME has been suggested as an alternative to solvent extraction of insect cuticular hydrocarbons (e.g., Turillazzi et al.,

1998; Peeters et al., 1999; Liebig et al., 2000; Sledge et al., 2000; Roux et al., 2002) and reportedly yields samples that are qualitatively and quantitatively similar to those obtained by solvent extraction (Moneti et al., 1997; Monnin et al., 1998; Bland et al., 2001; Tentschert et al., 2002). However, study on the contact pheromones of a congener of *M. caryae*, *M. robiniae* (Förster) revealed that whole-body solvent extraction and SPME wipe sampling of female elytra, the most likely surfaces contacted by antennae of males, yielded markedly different hydrocarbon profiles (Ginzel et al., 2003b). The contact pheromone of female *M. robiniae*, Z9:C₂₅, comprised ~16% of the total hydrocarbons in hexane extracts of females and was codominant with two other hydrocarbons that were not active. In contrast, in SPME wipe samples of several areas of the cuticle, Z9:C₂₅ appeared as the single dominant peak, comprising ~35% of the sampled hydrocarbons (Ginzel et al., 2003b). This finding suggested that hydrocarbons that cue mate recognition are more abundant on the surface of the cuticular wax layer in females where they are readily accessible to the antennae of males. We concluded that wipe sampling by SPME may yield a more representative profile of cuticular components actually encountered by the antennae of male insects, and so may provide a more representative profile of potential semiochemicals present on the body surface than does solvent extraction. We tested this hypothesis in the present study by sampling cuticular hydrocarbons of female *M. caryae* by both methods, predicting that compounds which cue mate recognition would be present in higher relative proportions in SPME wipe samples of the female cuticle than in whole-body extracts.

METHODS AND MATERIALS

Source of Beetles. The trunk of a shagbark hickory that was wind-thrown during spring 2002 at Brownfield Woods (Champaign County, IL) and infested with immature *M. caryae* was sectioned in March 2003 and the bolts stored in a 4°C walk-in cooler to prolong prepupal diapause. As adult beetles were needed for bioassays in May through August 2003, we moved logs to an indoor cage at ambient temperature to induce emergence. As adult beetles emerged, we housed individual males and females separately in 0.3 m³ cages of aluminum window screen held under ambient laboratory lighting and temperature conditions. Caged beetles were provided feeder vials of 10% sucrose solution (glass vial with a cotton dental roll; Patterson Dental Supply, South Edina, MN, USA), and feeders were replaced every 2–3 d. Beetles used in preparation of extracts and in bioassays were vigorous and active in cages. Thirty adult male and female *M. caryae* that had emerged in 2003 were caged with shagbark hickory bolts (~30 cm diam, ~45 cm long) from a tree collected

at Allerton Park (Piatt County, IL, USA). Following oviposition, the bolts were stored at ambient temperature, and adults that emerged the following May were used in bioassays during 2004.

Preparation of Whole-body Extracts and Identification of Cuticular Hydrocarbons. Cuticular chemicals were extracted from 10 freeze-killed virgin female and male *M. caryae* by individually immersing each beetle in two 1-ml aliquots of hexane for 2 min each. The two aliquots were combined and concentrated to 1 ml under nitrogen. Extracts were initially analyzed at the University of Illinois at Urbana–Champaign (UIUC) by coupled gas chromatography-mass spectrometry (GC-MS) with electron impact ionization (EI, 70 eV) using a Hewlett-Packard (HP) 6890 GC (Hewlett-Packard, Sunnyvale, CA, USA) equipped with a DB-5MS capillary column (30 m \times 0.25 mm \times 0.25 μ m film; J&W Scientific, Folsom, CA, USA) in splitless mode, interfaced to an HP 5973 mass selective detector (MSD), with helium carrier gas. The column was programmed from 50°C/1 min, 10°C/min to 240°C, then 240 to 300°C at 2°C/min with a 5 min hold at 300°C. Injector and transfer line temperatures were 280°C. Quantitative data presented in Table 1 and Figure 1 were produced by these analyses.

Compounds in whole-body extracts were identified in the laboratory of J.G.M. with an HP 6890 GC equipped with a DB-5MS column (30 m \times 0.25 mm \times 0.25 μ m film) and interfaced to an HP 5973 MSD. The temperature program was 100°C/1 min, 10°C/min to 280°C, with a hold at 280°C for 20 min. Injector and transfer line temperatures were 280°C. To determine the positions of double bonds in alkenes in the extracts, the crude extracts were epoxidized by treating an aliquot of an extract with a few drops of *m*-chloroperbenzoic acid in CH₂Cl₂ (25 μ l of a 2 mg/ml solution). After 2 hr at room temperature, the mixture was extracted with 1 M aqueous NaOH, and the hexane layer was dried over anhydrous Na₂SO₄, and then analyzed as described above.

Solid Phase Microextraction. We sampled cuticular components of five adult female *M. caryae* by gently rubbing the dorsal surface of the elytra with a 100 μ m (film thickness) polydimethylsiloxane SPME fiber (Supelco Inc., Bellefonte, PA, USA) for 30 sec. The same beetles also were sampled by whole-body hexane extraction, allowing us to compare profiles produced by the two sampling methods. Samples were analyzed at UIUC by GC-MS by desorbing the SPME fiber for 1 min in the injection port (250°C) with the same temperature settings that were used in analyzing extracts (see above).

Fractionation of Whole-Body Extracts. A column prepared from a Pasteur pipette plugged with a small plug of glass wool was loaded with 400 mg silica gel impregnated with AgNO₃ (10% w/w) and oven-dried overnight at 120°C. After cooling, the column was rinsed with hexane, then loaded with a concentrated composite extract of the whole-body extracts from six females in hexane (~0.5 ml total volume). The column was eluted sequentially with 4 ml hexane

(saturated hydrocarbons fraction), 2 ml 20% cyclohexene in hexane (alkenes fraction), and 3 ml ether (polar compounds). One half of the saturated alkanes fraction was concentrated to dryness under a stream of N₂, then 0.5 ml isooctane was added, and the extract was concentrated to dryness again to remove traces of hexane. The residue was taken up in 2 ml isooctane and stirred overnight with 50 mg oven-dried powdered 4 Å molecular sieve. The resulting slurry was filtered through a glass wool plug, yielding a branched alkanes fraction. The various fractions were tested for contact pheromone activity in bioassays.

Chemical Standards. C₂₇–C₂₉ straight-chain hydrocarbons were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Lancaster Synthesis (Pelham, NH, USA). The syntheses and spectral data of other standards, including Z9:C₂₉, Z13:C₂₉, Z14:C₂₉, Z13:C₃₁, Z14:C₃₁, Z15:C₃₁, 7Me-C₂₅, 11Me-C₂₅, 2Me-C₂₆, 9Me-C₂₉, 11Me-C₂₉, 13Me-C₂₉, 15Me-C₂₉, 13Me-C₃₁, and 15Me-C₃₁, are described in the online Supplementary Information available for this article, at <http://dx.doi.org/10.1007/s10886-005-9010-y> and is accessible for authorized users.

Bioassays with Fractions and Hydrocarbon Standards. Hexane solutions of hydrocarbon standards were prepared in concentrations that approximated the original extracts of female *M. caryae* (quantified by comparing peak areas from integrated total ion chromatograms with that of an internal standard). We tested the activity of the crude hexane extract of females, then the saturated hydrocarbon, branched alkane, and alkene fractions of the crude hexane extract, and then reconstructed the blends of the most abundant compounds based on chemical class. We then tested for synergistic responses of males to the monoenes, the most bioactive functional group (see Results), and the straight-chain alkanes. Next, we tested the bioactivity of the monoenes blend without Z9:C₂₉, the most abundant alkene in the hexane extract and SPME samples of the female cuticle (see Results). We then tested Z9:C₂₉ alone and in combination with the straight-chain alkanes and methyl-branched alkanes (see Results). The bioactivities of these blends and individual compounds were tested with the following bioassay (see Ginzel et al., 2003a,b) in May–June 2003 and 2004:

- (1) A female was freeze-killed (−4°C for 20 min), then the carcass was warmed to room temperature (~15 min), and presented to a male in a clean glass Petri dish lined with filter paper. An attempt to mate was taken as evidence that recognition cues were intact and that a behavioral response by the female was not necessary for mate recognition.
- (2) We removed cuticular components from the dead female by immersing her in two sequential 1-ml aliquots of analytical-grade hexane for 2 min each.
- (3) The solvent-washed female carcass was air-dried for 30 min to allow the solvent to evaporate and then presented to the same male. Lack of a

TABLE 1. CUTICULAR HYDROCARBONS OF FEMALE AND MALE *Megacyllene caryae*

Peak number	Retention time	Hydrocarbon	Percent of total hydrocarbons $\pm 1 SE^a$				Diagnostic Ions
			Hexane extracts		SPME		
			Male	Female	Female	Male	
1	27.79	2Me-C ₂₆	0.19 \pm 0.001	13.2 \pm 1.41	12.0 \pm 1.19	380 (M ⁺), 365, 337	
2	28.51	nC ₂₇	0.39 \pm 0.002	7.46 \pm 0.84	5.49 \pm 0.62	380 (M ⁺)	
3	29.15	11Me-C ₂₇	nd	2.75 \pm 0.60	2.60 \pm 0.96	379 (M ⁺ -15), 168/252	
		13Me-C ₂₇				379 (M ⁺ -15), 196/224	
4	29.22	9Me-C ₂₇	nd	1.07 \pm 0.37	1.74 \pm 0.65	379 (M ⁺ -15), 140/280	
5	29.73	2Me-C ₂₇	nd	0.91 \pm 0.10	1.38 \pm 0.28	394 (M ⁺), 379, 351	
6	30	3Me-C ₂₇	1.09 \pm 0.003	2.52 \pm 0.35	2.86 \pm 0.36	394 (M ⁺), 379, 365	
7	30.56	nC ₂₈	nd	1.15 \pm 0.05	0.59 \pm 0.06	394 (M ⁺)	
8	31.92	2Me-C ₂₈	6.30 \pm 0.006	17.3 \pm 1.32	12.5 \pm 0.75	408 (M ⁺), 393, 365	
9	32.05	Z13:C ₂₉ ^b	nd	3.36 \pm 0.47	5.11 \pm 0.56	406 (M ⁺), 83, 97, 111	
		Z14:C ₂₉ ^b				406 (M ⁺), 83, 97, 111	
10	32.26	Z9:C ₂₉ ^b	nd	7.73 \pm 0.69	11.6 \pm 1.11	406 (M ⁺), 83, 97, 111	
11	32.77	nC ₂₉	2.16 \pm 0.003	9.57 \pm 0.70	6.38 \pm 0.66	408 (M ⁺)	
12	33.48	11Me-C ₂₉	0.09 \pm 0.001	11.2 \pm 1.16	12.1 \pm 1.35	422 (M ⁺), 168/280	
		13Me-C ₂₉				422 (M ⁺), 196/252	
		15Me-C ₂₉				422 (M ⁺), 224	
13	33.59	9Me-C ₂₉	nd	3.45 \pm 0.23	4.13 \pm 0.27	422 (M ⁺), 140/308	
14	34.11	13,17-, 11,15-, 9,13-dimethyl C ₂₉	nd	1.76 \pm 0.31	1.90 \pm 0.18	421 (M ⁺ -15), 196/267; 168/295, 224/239; 140/323, 252/211	
15	34.51	3Me-C ₂₉	8.66 \pm 0.2	0.67 \pm 0.25	0.35 \pm 0.17	407 (M ⁺ -15), 393	
16	36.29	C ₃₁ diene	nd	0.35 \pm 0.16	0.63 \pm 0.19	432 (M ⁺), 82, 96, 110, 124, 138	
17	36.42	C ₃₁ diene	nd	0.59 \pm 0.11	1.01 \pm 0.12	432 (M ⁺), 82, 96, 110, 124, 138	

18	36.63	2Me-C ₃₀	15.7 ± 0.02	4.27 ± 1.60	2.01 ± 0.35	436 (M ⁺), 421, 393
19	36.76	Z13:C ₃₁ ^b Z14:C ₃₁ ^b Z15:C ₃₁ ^b	nd	2.51 ± 0.32	3.75 ± 0.27	434 (M ⁺), 83, 97, 111 434 (M ⁺), 83, 97, 111 434 (M ⁺), 83, 97, 111
20	37.04	Z9:C ₃₁ ^b	15.7 ± 0.03	1.81 ± 0.25	2.90 ± 0.33	434 (M ⁺), 83, 97, 111
21	37.52	nC ₃₁	4.34 ± 0.004	0.93 ± 0.15	0.90 ± 0.10	436 (M ⁺)
22	38.29	13Me-C ₃₁	6.57 ± 0.003	4.14 ± 0.29	5.32 ± 0.54	435 (M ⁺ -15), 196/280
23	38.94	15Me-C ₃₁ 13,17-Dimethyl C ₃₁	nd	1.35 ± 0.22	1.84 ± 0.23	435 (M ⁺ -15), 224/252 451 (M ⁺ -15), 196/295, 224/267
24	39.59	3Me-C ₃₁	3.08 ± 0.003	nd	nd	435 (M ⁺ -15), 435, 421
25	41.07	C _{33:1} ^b	3.30 ± 0.007	nd	nd	462 (M ⁺), 83, 97, 111
26	42.02	C _{33:1} ^b	4.58 ± 0.008	nd	nd	462 (M ⁺), 83, 97, 111
27	43.56	13Me-C ₃₃ 15Me-C ₃₃	7.91 ± 0.004	nd	nd	463 (M ⁺ -15), 196, 308 463 (M ⁺ -15), 224, 280
28	44.17	17Me-C ₃₃ 13,17- & 15, 19-dimethyl C ₃₃	10.3 ± 0.02	nd	nd	463 (M ⁺ -15), 252 477 (M ⁺ -15), 196/323, 252/267
29	47.04	C ₃₅ diene	5.58 ± 0.007	nd	nd	477 (M ⁺ -15), 224/295 488 (M ⁺), 82, 96, 110, 124, 138

^aPeak numbers correspond to those in Figure 1. DB-5 MS capillary column, 50°C/1 min, 10°C/min to 240°C at, then 240–300°C at 2°C/min, 5 min hold at 300°C. Percent of total hydrocarbons represent means for five individuals. "nd" = Not detected.

^bDouble bond position and stereochemistry determined from mass spectra and retention times of the corresponding epoxides, see Results section.

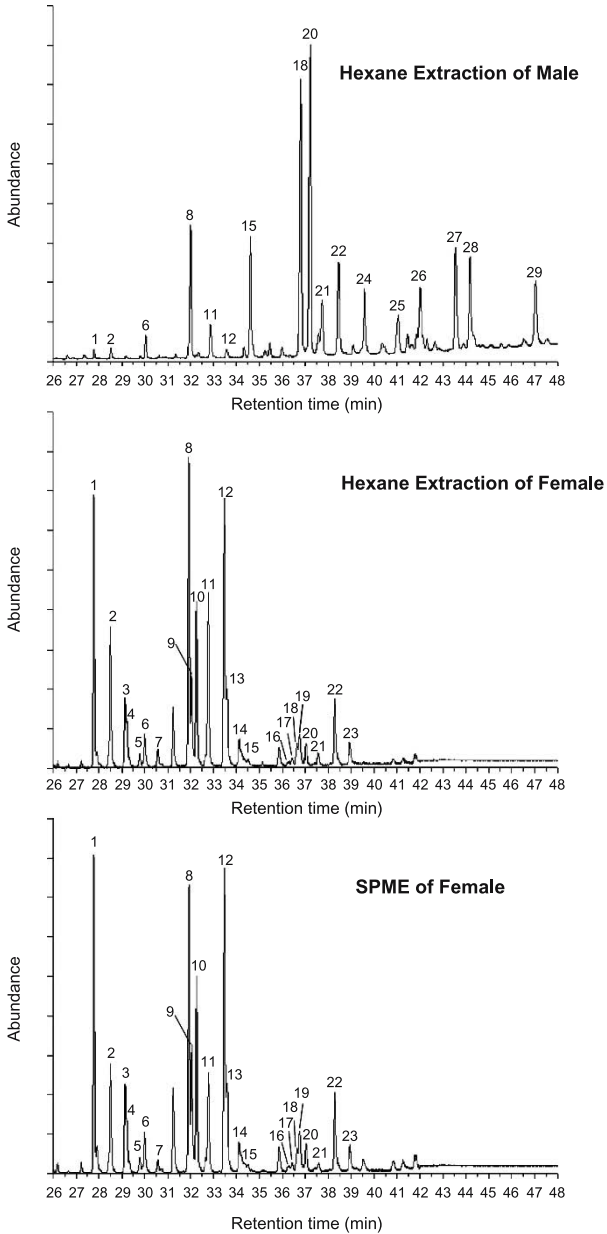


FIG. 1. Representative total ion chromatograms of whole-body hexane extracts of an adult male (top) and female (middle) *Megacyllene caryae*, and SPME wipe sample of female cuticle (bottom). Analysis conditions are described in [Methods and Materials](#).

response by the male was evidence that chemical recognition cues had been eliminated.

- (4) We tested the bioactivity of extracts, fractions of extracts, and individual or blends of hydrocarbon standards by pipetting solutions onto the body of the solvent-washed female, allowing the solvent to evaporate, and presenting the treated carcass again to the same male. We used 0.8 female equivalents (FE) in 20 μ l hexane, because males responded best to this dosage of crude extract (Ginzl and Hanks, 2003). Test solution was applied to the solvent-washed female carcass and 20 μ l hexane were applied to another solvent-washed female carcass as a control. The two carcasses were then presented to individual males simultaneously, on opposite sides of a Petri dish arena, with their positions randomized to control for location effects.

For all bioassays, we presented each of at least five dead females to two or three different males ($N = 10$ –20 males). We videotaped responses of males in the Petri dish arenas. Males used in bioassays had not mated for 24 hr prior to bioassays and were used in bioassays only once per day. After a male *M. caryae* contacted a living female with his antennae, a clear progression of behaviors led to copulation: (1) the male oriented to (turned toward) the female, (2) the male stopped walking (= arrestment), (3) the male aligned his body with the female, and (4) the male mounted the female and attempted to couple the genitalia. Thus, our assessment of behavioral response was cumulative: males must perform steps 1 through 3 to reach step 4.

A trial was scored as a “response” if the test male displayed at least the first behavior (turning toward female), within 5 min after first contacting a treated or control female carcass with his antennae. A trial was scored as “no response” if the male showed none of these behaviors within 5 min of initial antennal contact, but rather continued to walk after contacting either a treatment or a control with his antennae. Statistical significance of the response of males to treatments was tested with Fisher’s exact test (Sokal and Rohlf, 1995), (a) comparing numbers responding to crude extract (i.e., reaching behavioral step 1 at least) vs. solvent controls, (b) comparing numbers responding to treatments vs. crude extract, and (c) comparing numbers showing a complete mating response (i.e., reaching behavioral step 4) to treatments vs. crude extract.

RESULTS

Preliminary Bioassays. As a prelude to further investigation, we determined that male *M. caryae* could discriminate between female and male conspecifics by contact chemoreception, by pairing males with females and males with males in Petri dish arenas ($N = 10$ for each combination of sexes). Every

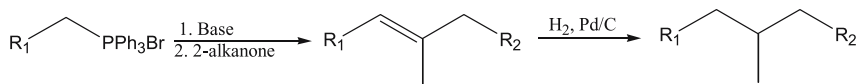
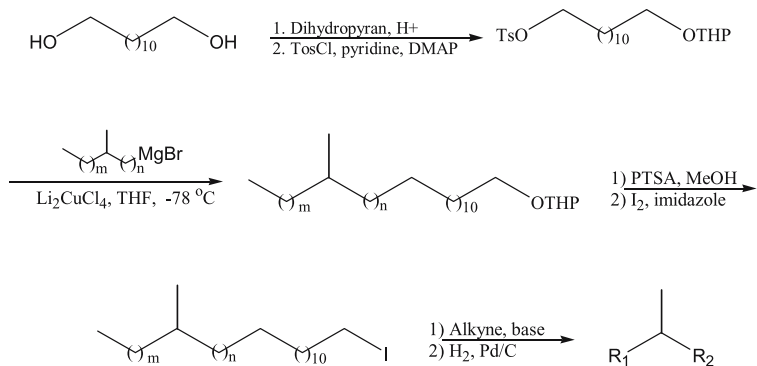
male attempted to mate with the female immediately after first antennal contact, but males only briefly antennated other males, then subsequently avoided them. The responses of male *M. caryae* to females and other males were consistent with earlier laboratory studies (Ginzel and Hanks, 2003; M.D. Ginzel, personal observation). These findings suggested that the chemical cues that males use to recognize females are absent in males, or that males have other compounds that inhibit the mating response.

Identification of Cuticular Hydrocarbons. Hexane extracts of female and male *M. caryae* contained saturated, branched, and unsaturated hydrocarbons (Table 1, Figure 1). Cuticular hydrocarbon components were identified by comparison of mass spectra and retention times with those of standards, or from the parent M^+ ions and the corresponding molecular formulae, retention times relative to straight-chain compounds, and diagnostic mass spectral fragments that unequivocally demonstrated the position of methyl branches, as thoroughly documented by previous researchers (e.g., Nelson, 1993; Nelson and Blomquist, 1995). Double bond locations and geometries of monoenes were determined by epoxidation of extracts followed by GC-MS analysis of the resulting derivatives. The epoxides gave large diagnostic fragments from cleavage on either side of the epoxide, unequivocally demonstrating the position of the double bond in the parent molecule. GC-MS analysis of the epoxides of *E* and *Z* isomers determined that the *E* isomers eluted before the *Z* isomers on the DB-5MS column.

There were consistent, sex-specific differences in the hydrocarbon profiles of males and females, with the dominant compounds in solvent extracts of females including 2Me-C₂₆ (peak 1), 2Me-C₂₈ (peak 8), and coeluting 11-, 13-, and 15Me-C₂₉ (peak 12). Extracts of males contained some of the compounds present in females as minor components, but also several longer chain hydrocarbons that were specific to males, primarily alkenes and mono- and dimethyl-branched alkanes.

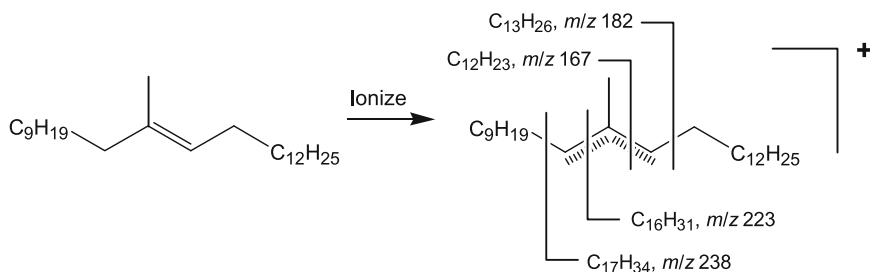
Solid Phase Microextraction. Samples of cuticular hydrocarbons from female *M. caryae* taken by SPME wipe sampling yielded a profile of hydrocarbons that was similar to that of the whole-body solvent extracts (Table 1; Figure 1 middle and bottom). All components present in SPME samples were also present in solvent extracts. The most conspicuous difference between profiles from solvent extraction and SPME sampling was a 50% increase in the relative amount of Z9:C₂₉ in SPME samples (peak 10, Table 1; Figure 1, middle and bottom; means for two sampling methods significantly different, ANOVA: $F_{1,9} = 8.85$, $P < 0.018$). Z9:C₂₉ was the only one of the dominant hydrocarbons that showed this increase in relative abundance, suggesting that it might be an important component of the contact pheromone of females.

Synthesis of Cuticular Hydrocarbons. Two strategies were used for the synthesis of monomethyl-branched hydrocarbons. The first strategy used Wittig

Route A:**Route B: Building blocks synthesis**

SCHEME 1. Synthetic routes for the preparation of monomethyl branched hydrocarbons.

reaction between a 2-alkanone and the appropriate phosphonium salt to generate methylalkenes, followed by catalytic hydrogenation (Scheme 1, route A). The commercial availability of many 2-alkanones (varying from acetone to 2-octadecanone) and *n*-alkylphosphonium salts rendered this two-step methodology simple and straightforward. Eight methyl-branched hydrocarbons were obtained using route A (Scheme 1), with overall yields varying from 38% to 65% (for details, see online Supplementary Information available for this article, at <http://dx.doi.org/10.1007/s10886-005-9010-y> and is accessible for authorized users). In the first step, the Wittig reaction was nonselective, producing the methylalkenes as mixtures of the *Z* and *E* isomers (ratios, 54:46 to 64:36). The EI mass spectra of these alkenes were characterized by distinct molecular ions (ca. 5–12% of the base peak) and two pairs of enhanced fragment ions, with ions in each pair separated by 15 mass units (Scheme 2). These ions indicated the position of the methyl group in the parent compound, but did not indicate which side the double bond was on. Catalytic hydrogenation of the methylalkenes then produced the desired methyl-branched alkanes, the structures of which can be unambiguously determined by EI mass spectrometry from the small molecular ions, and the enhanced even-mass ions from cleavage



SCHEME 2. EI-mass spectral fragmentation trends for monomethyl branched alkenes. Fragments from 11-methyl-11-pentacosene are illustrated.

on either side of the methyl group with a hydrogen transfer. A small M-15 fragment also was usually observed in the spectra, helping to confirm the M^+ ion.

The second synthetic route was designed to provide a flexible synthesis of compounds for which intermediates were not commercially available (Scheme 1, route B). This strategy is illustrated by the synthesis of 2Me-C₂₆. Thus, a double-ended building block was used, with introduction of the methyl branch at the desired position at one end by coupling a branched alkylmagnesium bromide and an O-protected tosylate with dilithium tetrachlorocuprate catalysis (Burns et al., 1997; Krause and Gerold, 1997). This was followed by deprotection of the other end of the building block and elongation of the chain to the desired length. Our first attempt at chain elongation by oxidation of 15-methylhexadecan-1-ol to the corresponding aldehyde, to be followed by Wittig reaction, failed due to polymerization of the aldehyde. Instead, the alcohol was converted to the iodide, followed by coupling with an alkyne (Corey et al., 1983) and reduction (Scheme 1, route B). Overall, this general strategy is amenable to production of a branched alkane with the branch in any desired position, and any desired chain length. It would also be amenable to the production of chiral hydrocarbons by attachment of a chiral rather than a racemic fragment in the first coupling step.

Bioassays with Fractions and Hydrocarbon Standards. All male *M. caryae* (100% of 200 trials) attempted to mate with freshly freeze-killed females, confirming that mate recognition cues were intact on female carcasses. Males did not respond to dead females after solvent extraction, consistent with earlier studies (Ginzal and Hanks, 2003; Ginzal et al., 2003a,b) and demonstrating that the contact sex pheromone(s) had been removed. None of the males (0 of 200 trials) showed any response to solvent-extracted dead female carcasses treated with pure solvent (controls) that were paired with hydrocarbon-treated females (Fisher's exact test of comparison with response to crude extract, $P < 0.001$).

TABLE 2. RESPONSE OF MALE *Megacyllene caryae* TO SOLVENT-WASHED DEAD FEMALES TO WHICH HYDROCARBON EXTRACTS OR STANDARDS HAD BEEN APPLIED IN DOSAGES OF 0.8 FE

Compound(s)	No. of males tested	Percent of males responding per step in behavioral sequence ^a			
		Step 1 ^b	Step 2	Step 3	Step 4 ^c
Crude extract	20	100	100	65	40
Saturated hydrocarbons fraction	10	60**	50	50	30
Branched alkanes fraction	20	40***	20	10	10*
Alkenes fraction	20	60**	60	30	10*
Straight-chain alkanes (<i>n</i> C ₂₇ , <i>n</i> C ₂₈ , <i>n</i> C ₂₉)	20	75*	65	15	0**
Branched alkanes (2Me-C ₂₆ , 9, 11, 13, 15Me-C ₂₉)	10	80	70	0	0*
Monoenes (Z9:C ₂₉ , Z13:C ₂₉ , Z14:C ₂₉ , Z13:C ₃₁ , Z14:C ₃₁ , Z15:C ₃₁)	20	100	95	25	25
Monoenes + straight-chain alkanes	10	80	80	30	20
Monoenes minus Z9-C ₂₉	20	80	75	10	0**
Z9:C ₂₉	20	95	90	35	25
Z9:C ₂₉ + straight-chain alkanes	20	100	95	40	15
Z9:C ₂₉ + branched alkanes	20	100	85	20	10*

Behavior of responding males was categorized according to the natural sequence of mating behavior (see [Methods and Materials](#)). Males did not respond to any of the solvent-washed controls.

^aResponses of males to treatments vs. crude extracts were tested with Fisher's exact tests (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

^bNumber of males responding to treatments as measured by reaching behavioral step 1 was compared to responses to crude extract (H_0 = treatment and crude extract do not differ in activity).

^cNumber of males reaching behavioral step 4 in response to a treatment was compared to the response to crude extract (H_0 = treatment and crude extract do not differ in activity).

All of the males ($N = 20$) responded to females treated with 0.8 FE of their own crude extract, although only 40% reached behavioral step 4 (mounting the female and attempting to couple the genitalia; Table 2). Males showed a weaker response in behavioral step 1 to fractions (saturated hydrocarbons, branched alkanes, alkenes) of crude solvent extract of female cuticle when compared to their response to crude extract (Table 2), but the proportion of males that reached behavioral step 4 was similar for the saturated fraction and crude extract (Table 2).

We further evaluated the activity of structural classes by testing blends of synthetic components (Table 2). In these bioassays, the number of males re-

sponding (behavioral step 1) to a reconstructed blend of straight-chain alkanes was lower than the response to crude extract, as was the number of males reaching behavioral step 4 (Table 2). A reconstructed blend of branched alkanes did not differ from crude extract in bioactivity for the first step of the behavioral sequence, but no males reached behavioral step 4 (Table 2). Males responded most strongly to the reconstructed blend of the dominant monoenes, and addition of the straight-chain alkanes did not significantly affect activity (Table 2). Similar numbers of males reached behavioral step 4 in response to the blend of monoenes compared to crude extract (Table 2). However, activity of the alkene blend was reduced (no males reaching step 4) when Z9:C₂₉ was eliminated. When tested separately, Z9:C₂₉ elicited a response from males that was equivalent to that elicited by the complete blend of monoenes. Combining Z9:C₂₉ with the blend of alkanes again did not affect the level of response compared to that elicited by Z9:C₂₉ alone (Table 2). Finally, combining Z9:C₂₉ with the dominant methyl-branched alkanes reduced its activity with respect to behavioral step 4 (Table 2).

DISCUSSION

Identification and Bioassay of Cuticular Hydrocarbons. Recent research on several longhorned beetle species in the subfamily Cerambycinae has suggested that mate location and recognition involves three distinct behavioral stages (Ginzel and Hanks, 2005): (1) the sexes are mutually attracted to volatiles emanating from host plants, (2) males on host plants produce volatile pheromones that draw females into their proximity over short or moderate distances (reviewed by Lacey et al., 2004), and (3) males recognize females by contact chemoreception with the antennae (see Ginzel and Hanks, 2003; Ginzel et al., 2003a,b). In the study reported here, the lack of response by male *M. caryae* to solvent-extracted female carcasses, or dead females treated with solvent alone, indicated that contact pheromones were the primary signals used for mate recognition, with tactile or visual cues possibly playing a subordinate role. The activity of Z9:C₂₉ in bioassays indicated that it is an important component of the contact sex pheromone of female *M. caryae*, if not the sole component. Combinations of this compound with other cuticular chemicals failed to identify any synergists. Furthermore, Z9:C₂₉, is a homolog of Z9:C₂₅, the contact sex pheromone of the congener *M. robiniae*. The structural similarity in their contact pheromones is not unexpected given their close phylogenetic relationship. In fact, females of the two species share 13 cuticular hydrocarbons, including Z9:C₂₉ (representing ~1% of the total hydrocarbons of female *M. robiniae*), and another seven of the 30 cuticular hydrocarbons of female *M. caryae* have chain-length analogs in *M. robiniae* (Ginzel et al.,

2003b). Other insect congeners, such as species in the *Drosophila melanogaster* group (Coyne, 1996), are known to share cuticular hydrocarbon components.

The role of Z9:C₂₉ in mate recognition was further supported by its complete absence in males. In fact, extracts from male and female *M. caryae* were qualitatively very different, with sex-specific compounds representing almost half of the hydrocarbons of females and a third of the hydrocarbons of males (Table 1, Figure 1). Extracts from males contained a greater percentage of higher molecular weight, longer chain compounds, as appears to be true for some longhorned beetle species (Ginzel et al., 2003a,b). However, in other cerambycids, including *M. robiniae*, hydrocarbon profiles of males and females are qualitatively more similar, with most components being present in extracts from both sexes (Ginzel et al., 2003a,b).

The male-specific compounds of *M. caryae* may serve as abstinons (chemical deterrents to mating), although this function has yet to be investigated in cerambycids. Other types of insects, such as tsetse fly (Nelson and Carlson, 1986) and the bark beetle *Ips lecontei* Swaine (Page et al., 1997), have sex-based differences in alkyl chain lengths of cuticular hydrocarbons, but with higher molecular weight compounds predominating in females rather than males.

The fact that male *M. caryae* frequently did not exhibit mating behaviors toward solvent-extracted female carcasses that had been treated with their own crude extracts was unexpected, because males of other cerambycid species typically show strong responses to female carcasses treated with extracts of females in identical bioassays (e.g., Ginzel et al., 2003a,b). Males of a congener, *M. robiniae*, may respond more strongly to reconstituted wax layers of females than male *M. caryae* because the contact pheromone was a dominant hydrocarbon in crude extracts (Ginzel et al., 2003b), while that of *M. caryae* was considerably less abundant.

Z9:C₂₉ was not as dominant in hydrocarbon profiles determined by SPME of female *M. caryae* as was the contact pheromone of female *M. robiniae* (Ginzel et al., 2003b). Nevertheless, examination of Figure 1 revealed that Z9:C₂₉ was the only compound among the dominant hydrocarbons that was present in higher abundance in SPME wipe samples than in solvent extracts. These findings support the hypothesis that identification of contact pheromones may be facilitated by comparing hydrocarbon profiles of solvent extracts and SPME, to determine which compounds are present in higher abundance on the surface of the wax layer (SPME) as opposed to throughout the wax layer (solvent extracts).

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IDENTIFICATION OF QUEEN SEX PHEROMONE COMPONENTS OF THE BUMBLEBEE *Bombus terrestris*

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Abstract—We investigated the origin and chemical composition of the queen sex pheromone of the primitively eusocial bumblebee, *Bombus terrestris* (Apidae). Physiologically and behaviorally active compounds were identified by coupled gas chromatography electroantennography (GC-EAD), gas chromatography-mass spectrometry (GC-MS), and laboratory behavioral tests. In the behavioral assays, virgin queens frozen previously at -20°C were highly attractive to males. Dummies impregnated with surface and cephalic extracts obtained from virgin queens that had been frozen at -50°C were more attractive to males than odorless dummies. Male mating behavior was stimulated by components of cephalic secretions that are smeared onto the cuticle surface by the queen. Overall, 21 compounds present in surface and cephalic extracts evoked electroantennographic responses in male antennae. These included saturated and unsaturated fatty acids, ethyl- and methyl esters of the fatty acids, heptacosene, 2-nonanone, and geranyl geraniol. A blend of synthetic versions of these compounds elicited typical male mating behavior. Since solvent-impregnated dummies were approached by the males, but did not release copulatory behavior, visual cues may be important in the initial

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step of stimulating male mating behavior. Close-range olfactory signals are more important for releasing male mating behavior as well as for species recognition. In further behavioral assays, the attractiveness of a frozen virgin queen decreased as the storage time at -20°C increased from 2 hr to 1 d. Therefore, the chemical composition of the sex pheromone may change during freezing as behaviorally active compounds may decompose.

Key Words—Hymenoptera, Apoidea, Apidae, *Bombus terrestris* L., queen sex pheromone, chemical communication, mating behavior, chemical analysis, electroantennography

INTRODUCTION

Chemical communication has reached an advanced and sophisticated form in social insects (Vander Meer et al., 1998). Insect pheromones are often produced by exocrine glands that are modifications of epidermal cells of the integument (Ayasse et al., 2001). Exocrine glands generate an array of biologically active compounds, mediating almost every facet of social behavior in bees (Hymenoptera: Apidae) (Vander Meer et al., 1998). Sex pheromones of bees stimulate one or more behavioral reactions in the opposite sex, bringing the sexes together for the purpose of mating (Shorey, 1973).

In general, little is known about the chemical composition of sex pheromones in bees (Ayasse et al., 2001). Compounds that elicit the copulatory behavior in males have rarely been identified. The first sex pheromone identified in bees was 9-keto-(2*E*)-decanoic acid (9-ODA), a compound produced in the mandibular glands of the queen honey bee, *Apis mellifera* L. (Apidae), and which stimulates mating behavior in drones (Butler, 1971; Gary, 1962). In many bee species, the female sex pheromone is located on the cuticle surface (Ayasse et al., 2001). Isopentenyl esters of unsaturated fatty acids identified in the surface extracts of *Lasioglossum malachurum* (Halictidae) females play a key role in eliciting mating behavior of males (Ayasse et al., 1999). However, the site of production remains unknown. Since bees frequently produce an array of volatiles (Duffield et al., 1984), electrophysiological investigations provide important tools for the identification of potential pheromones in multi-component mixtures. For example, in the solitary bee *Andrena nigroaenea* (Andrenidae), saturated and unsaturated hydrocarbons triggered electroantennographic responses in male antennae (Schiestl et al., 1999, 2000). In field experiments, synthetic forms of these compounds induced copulatory attempts by males.

Bees have evolved a remarkable variety of mating strategies (Eickwort and Ginsberg, 1980). In the genus *Bombus*, mating behavior varies even between species (Haas, 1949). Volatile signals acting at long and/or short distances seem

to play a key role in mating behavior and are produced by either males or females depending on the species' mating strategy (Ayasse et al., 2001). Newman (1851) and, later, Darwin (1886), first described the flight routes of *Bombus* males. In addition, Sladen (1912) noticed that males patrol along more or less fixed flight routes and deposit scent on landmarks. Unmated conspecific queens are attracted by the long-range signals and remain at the marked place of the rendezvous site, until the patrolling male reappears (Bergman, 1997), as is typical for many non-resource-based mating strategies (Alcock et al., 1978). However, many investigations have shown that the male mating signal does not elicit copulation behavior (Free, 1971; Kullenberg, 1973; Tengö, 1979; Borg-Karlson and Tengö, 1986). Female-produced pheromones acting proximally may be responsible for species and sex recognition as well as for eliciting copulatory behavior (Djegham et al., 1994).

Krüger (1951) and Free (1971) were the first to perform behavioral experiments with the objective of localizing the source of sex pheromone production in virgin bumblebee queens. Free (1971) found that isolated heads and thoraces offered to males elicited more copulation attempts than the amputated abdomen alone. Further experiments with a number of bumblebee species (*B. terrestris* L., *B. pratorum*, and *B. hypnorum*) indicated that the mandibular glands are the source of a species-specific queen sex pheromone (Van Honk et al., 1978). Living queens with extirpated mandibular glands stimulated relatively fewer mounting events and copulation attempts by males than did untreated living queens. A study on the chemical composition of glands from mated *B. terrestris* queens revealed that the mandibular glands contain more than 180 compounds (Hefetz et al., 1996). Cahlíková et al. (2004) provided a more detailed description of the composition of secretions of various glands from virgin *B. terrestris* queens. However, to date, no investigations have been undertaken with the objective of identifying the behaviorally active compounds.

B. terrestris, a frequently cultivated species for pollination in greenhouse crops, is one of the best studied bumblebee species with respect to its sociobiology (Röseler et al., 1990). This primitively eusocial species produces a single generation during the summer season in temperate regions (Westrich, 1989). In early spring (March), the fertilized solitary queens leave their hibernaculum and establish monogyne colonies. During the initial period of colony development, the queen first produces workers, whereas in a subsequent phase males and virgin queens are reared (Plowright and Lavery, 1984). Six days after emergence, the sexuals leave the nest for mating (Röseler et al., 1990). The queens normally mate only once (Estoup et al., 1995; Schmid-Hempel and Schmid-Hempel, 2000; Paxton et al., 2001) and hibernate in special chambers in the soil. The mated queens are the only survivors of the original colonies, whereas the workers and males do not overwinter (Wilson, 1971).

We performed behavioral experiments with synthetic compounds and solvent extracts of virgin *B. terrestris* queens; with living and dead frozen queens; and conducted electrophysiological and chemical analyses with these stimuli in order to identify chemical compounds that elicit mating behavior in the males. The objectives of this study were to answer the following questions: (1) Where does the queen sex pheromone originate? and (2) Which volatile compounds are active in stimulating mating behavior in the males? In a further series of behavioral tests, we also investigated whether the attractiveness of dead virgin queens is influenced by storage conditions.

METHODS AND MATERIALS

Sample Collection. Young virgin *B. terrestris* queens, provided by Koppert Biological Systems (The Netherlands), were freeze-killed at -50°C . Surface extracts were obtained by rinsing the whole bodies for 1 min in 0.5 ml diethyl ether (Uvasol, Merck). Subsequently, the heads were dissected from the thorax, the mandibles were gently opened to allow solvent penetration into cephalic glands, and the heads were extracted in 0.2 ml diethyl ether. Extraction lasted for at least 24 hr at room temperature (21°C). Thoraces and abdomens were extracted under the same conditions. Resulting extracts (natural samples) were concentrated to 100 μl /queen equivalent in a water bath (40°C). From each sample (5 queen equivalents), 90% of the total volume was used for behavioral tests and 10% was used for chemical and electrophysiological analyses by gas chromatography with simultaneous flame ionization detection (GC-FID) and electroantennographic detection (GC-EAD).

Attractiveness of Various Groups of Females. In a series of behavioral assays, the attractiveness of frozen virgin queens, frozen workers, and living virgin queens ($N = 8$) to males was investigated. Virgin queens were put in Eppendorf tubes and frozen at -20°C for either 2 hr ($N = 11$) or 1 d ($N = 10$) to investigate whether the attractiveness of dead virgin females is influenced by different storage conditions. An additional group of virgin queens was killed in liquid nitrogen by shock freezing for 3 sec ($N = 17$). Workers were frozen at -20°C for 2 hr ($N = 9$). Before they were offered to males, frozen individuals were kept for 8 min at room temperature and fixed on insect pins (through the thorax). Five types of male behavioral responses were recorded (modified after Van Honk et al., 1978; Djegham et al., 1994): (1) *no attention*—male shows no typical mating behavior and does not react to the female; (2) *approach*—male flies toward a female; (3) *touch*—male hovers in flight with antennal inspection of the female; (4) *mounting*—male mounts the female; and (5) *copulation*—male attempts to copulate with the female by placing his middle legs around her

abdomen and trying to insert his genitalia. To avoid habituation effects, the female position in the flight cage was changed after each test, and males were renewed at least once per day. Each female was only used once per day. To ensure comparable test conditions for the various experimental groups, frozen queens and natural samples were tested alternatively. Behavioral tests with shock-frozen queens were conducted in a glasshouse at the University of Vienna; the other tests were carried out in Utrecht.

Location of the Queen Sex Pheromone. Dead virgin *B. terrestris* queens were exhaustively Soxhlet-extracted with dichloromethane for 48 hr, dried at 45°C in an oven for 12 hr, and fixed on insect pins. During bioassays these dummies served as controls and as solid supports for testing natural or synthetic samples. In a first series of tests in Utrecht (Laboratory of Comparative Physiology, Social Ethology, Univ. of Utrecht), the behavioral activity elicited by dummies impregnated with queen extracts was assayed to locate the source of the sex pheromone in virgin *B. terrestris* queens. Males used for the tests were collected from rearing colonies. Behavioral tests were carried out from 9:00 A.M. to 12:00 noon in a flight cage (50 × 50 × 70 cm). A mercury lamp (HPL-N 250 W; Philips, Vienna) was fixed above the cage to improve light conditions, which in turn increased the flight activity of males (Ayasse, unpublished observation). In each test, a single dummy was treated with 100 µl of a test sample or dichloromethane (solvent control) and offered to seven males simultaneously for 10 min. The test mixture was applied to the body surface by using a microsyringe (Hamilton, 1710N, Bonaduz, Switzerland). Before the treated or control dummy was fixed in the flight cage, the solvent was allowed to evaporate for 1 min. The behavioral responses of males toward the dummies were recorded as described above. Each dummy was only used once per day. After each test, the dummy was Soxhlet-extracted with dichloromethane (200 ml) for 24 hr and dried at 45°C in an oven for 12 hr.

Attractiveness of Synthetic Compounds. Synthetic versions of GC-EAD-active compounds identified from samples that were attractive to males (e.g., surface extracts, cephalic extracts) were mixed in the relative proportions and total amounts as they occurred in natural samples (Table 1). Behavioral assays of these synthetic blends were carried out in a flight cage (60 × 60 × 60 cm) at the University of Vienna. Due to sufficient light conditions in the glasshouse, no mercury lamp was used. Since part of the solution might be trapped in the dummy (Schiestl and Ayasse, 2000), we applied five-female equivalents to each dummy. The attractiveness of each dummy was tested in the flight cage with 30 males simultaneously present for 10 min. Males were obtained from Koppert Biological Systems.

Electrophysiology (GC-EAD). For GC-EAD investigations, 1 µl of a behaviorally active extract was used. Separations were carried out by using an HP 6890 gas chromatograph, equipped with a DB5-MS column (30 m × 0.25 mm ×

TABLE 1 ELECTROPHYSIOLOGICALLY ACTIVE COMPOUNDS IN SURFACE ($N = 4$) AND CEPHALIC EXTRACTS ($N = 5$) OF VIRGIN *Bombus terrestris* QUEENS

No. ^a	Name	Surface	Head	Ratio head/surface
		(ng)	(ng)	
		Mean \pm s.e.	Mean \pm s.e.	
1	2-Nonanone ^b	54.77 \pm 13.44	1318.30 \pm 253.50	25
2	Methyl decanoate ^b	0.00 \pm 0.00	36.90 \pm 5.18	–
3	3-Hydroxydecanoic acid ^b (<i>S/R</i> = 99:1)	0.68 \pm 0.68	164.60 \pm 35.36	250
4	Methyl tetradecanoate ^b	5.20 \pm 1.96	89.30 \pm 10.65	17
5	Ethyl tetradecanoate ^b	25.24 \pm 6.04	687.40 \pm 260.10	20
6	Methyl hexadec-7-enoate	111.70 \pm 24.21	130.60 \pm 33.15	1
7	Methyl hexadec-11-enoate ^b	21.92 \pm 7.85	144.50 \pm 14.38	7
8	Methyl hexadecanoate ^b	35.64 \pm 9.57	343.90 \pm 64.02	10
9	(<i>Z</i>)-11-Hexadecenoic acid ^b	68.91 \pm 12.15	839.40 \pm 95.78	12
10	Hexadecanoic acid ^b	253.30 \pm 53.10	2494.70 \pm 251.80	10
11	Dodecyl hexanoate ^b	11.74 \pm 6.67	500.50 \pm 116.60	50
12	Ethyl hexadecanoate ^b	40.50 \pm 9.45	712.00 \pm 127.90	18
13	Methyl linoleate ^b	30.20 \pm 4.81	110.50 \pm 13.38	3
14	Methyl oleate ^b	3634.80 \pm 739.40	1863.70 \pm 207.70	0.5
15	Methyl octadec-11-enoate ^b	53.86 \pm 17.23	790.90 \pm 70.26	15
16	Linolenic acid ^b	67.35 \pm 26.76	479.20 \pm 70.32	7
17	Linoleic acid + Geranyl geraniol ^{b,c}	681.50 \pm 192.20	10,274.90 \pm 1393.10	1.5
18	Oleic acid	64.04 \pm 50.36	23.03 \pm 12.71	0.3
19	(<i>Z</i>)-11-Octadecenoic acid ^b	73.06 \pm 13.77	312.70 \pm 60.89	4
20	Octadecanoic acid ^b	258.00 \pm 119.00	1066.70 \pm 151.70	4
21	(<i>Z</i>)-13- + (<i>Z</i>)-12-Heptacosene ^c	11,623.70 \pm 2749.40	8026.10 \pm 183.00	1

^aNumbers are the same as in Figure 3.

^bSignificantly different in both extracts: Mann–Whitney *U* tests, $P < 0.05$.

^cSeparation was impossible.

0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). The following conditions were applied: splitless injection at 50°C for 1 min, temperature program 50–310°C at a rate of 10°C/min, 1.5 ml/min helium as carrier gas, injector and detector temperature 310°C. At the end of the column, a GC effluent splitter (press-fit connection; split ratio 1:1) was used to supply an FID and an EAD, whose signals were simultaneously recorded. To operate the EAD, effluent from the GC column was added to a purified and humidified air stream passed over the excised antenna from a *B. terrestris* male. The tip of the

antenna was cut off, and the antenna was mounted between two electrodes. Each end of the antenna was embedded in electrode gel (spectra 360, electrode gel; Parker Laboratories, Orange, NJ, USA) applied on electrode holders. The EAD signal was transferred via an interface board (IDAC; Syntech, Hilversum, The Netherlands) to a PC. The gel method allows the simultaneous use of several antennae to increase the EAD signal. To discriminate electroantennographic responses from noise, 10–15 GC-EAD runs were performed with each behaviorally active sample. The reproducibility of all responses was checked at the respective retention times. GC-EAD-active compounds were identified by performing GC-EAD runs using reference substances and GC-MS analysis.

Chemical Analyses. Surface extracts and cephalic extracts were analyzed with an HP 5970 GC (Hewlett-Packard, Palo Alto, CA, USA) equipped with a DB5 capillary column (30 m × 0.32 mm, 0.25 µm film thickness; J&W Scientific). The gas chromatograph was operated under the following conditions: splitless at 50°C for 30 sec, followed by a programmed increase to 280°C at 4°C/min, 1.5 ml/min helium as carrier gas, injector and detector temperature of 310°C. For quantitative analysis, 1 µg *n*-octadecane was added to each sample to serve as an internal standard. Structure elucidation of individual compounds was based on coupled gas chromatography-mass spectrometry (GC-MS) analysis (VG70/250 SE instrument; Vacuum Generators, Manchester, England). The MS was linked to an HP 5890 GC (gas chromatographic condition as mentioned above). Mass spectra were compared with those reported in the literature (McLafferty and Stauffer, 1989), and gas chromatographic retention times of unknowns were compared with those of authentic reference samples (by coinjection). Double-bond positions in unsaturated compounds were assigned according to Buser et al. (1983) and Dunkelblum et al. (1985). Structural confirmation was carried out with authentic reference compounds.

Synthetic Compounds. The following compounds were purchased from Aldrich (Taufkirchen, Germany): 2-nonanone, methyl decanoate, methyl tetradecanoate, ethyl tetradecanoate, methyl hexadecanoate, ethyl hexadecanoate, hexadecanoic acid, octadecanoic acid, methyl oleate, oleic acid, methyl linoleate, linoleic acid, linolenic acid. Alkenes and alkenoic acids were prepared by Lindlar hydrogenation (Lindlar catalyst from Lancaster, UK) of the corresponding alkynoic precursors. The latter were synthesized according to laboratory standards (Brandsma, 1988) and purified by column chromatography on silica (Merck 60, 120–400 mesh/hexane); final purity was higher than 95%. Esters were obtained from the corresponding acid chlorides and the appropriate alcohols according to standard laboratory methods. Mass spectra of synthetic compounds were in accord with expected patterns (Francke et al., 2000). The synthesis of optically pure 3-hydroxydecanoic acid has been described earlier (Hefetz et al., 1996).

Statistical Analyses. Since the flight activity of males during the test periods differed and test periods of different lengths were used, male reactions were standardized into a behavioral score (mean proportion of male reaction). The number of male reactions for each of the five behavioral categories was divided by the total number of male reactions and multiplied by 100. The data were analyzed with the SPSS 10.0 statistical system (SPSS Inc., 2000). Since the mean proportion of male reactions elicited by solvent application did not differ significantly (Mann–Whitney U test, $P > 0.05$; Sokal and Rohlf, 1995) between the test series performed either in Utrecht or in Vienna, solvent groups were pooled and used as one control group for all groups. The mean proportions of male reactions (no attention, approach, touch, mounting, and copulation) in the different test groups were compared with the mean proportion of reactions toward the control group with the Mann–Whitney U test (Sokal and Rohlf, 1995). An analysis of variance (ANOVA) followed by a multiple comparison test (LSD test) was used for comparisons among all treatment groups.

RESULTS

*Attractiveness of Various Groups of *B. terrestris* females.* In the test series with whole insects, living queens elicited more copulation attempts from the males compared to dead frozen queens (ANOVA, LSD test, $P < 0.001$; Figure 1). Furthermore, the attractiveness of a queen as a copulatory target was dependent on the freezing duration. Shock-frozen queens elicited the highest amount of copulatory events, whereas males rarely tried to copulate with queens that were frozen 1 d before.

During our behavioral assays, the “no attention” event only occurred with frozen workers and the control. In all test groups, males approached the scented dummies intensively, and there was no difference in this category of response between any of the experimental treatments and the control (Figure 1). The mean proportion of male touch events was lower for 1-d-frozen queens, higher for 2-hr-frozen queens, and highest for shock-frozen queens. The mean proportion of male mounting reactions for each of the queen groups and for the worker group were always significantly higher than that of the control. The result that living queens were seemingly less frequently mounted than frozen or shock-frozen queens can be explained by the higher number of copulation events with living queens. Similar to the touch event with frozen queens, the copulation attempts progressively increased from 1-d-frozen queens via 2-hr-frozen queens to shock-frozen queens (Figure 1). Consequently, under storage conditions of -20°C , the attractiveness of a queen is negatively correlated with the duration of freezing. Frozen workers were attractive to males, but they never stimulated copulatory events.

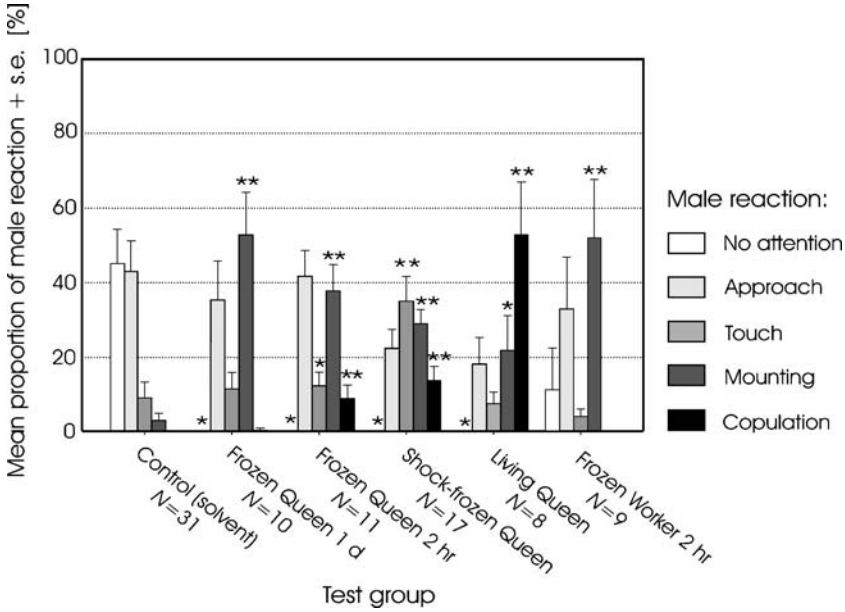


FIG. 1. Mean proportions of male reaction to virgin frozen and virgin living *Bombus terrestris* queens and workers. Various freezing durations were tested: 2 hr = 2 hr in freezer at -20°C (queens: $N = 11$, workers: $N = 9$); 1 d = 24 hr in freezer at -20°C ($N = 10$); shock = 3 sec in liquid nitrogen ($N = 17$). In the control test, 100 μl of solvent was used ($N = 31$) (* $P < 0.05$; ** $P < 0.001$, relative to control, Mann–Whitney U test).

Location of the Queen Sex Pheromone. Our behavioral tests showed that surface extracts, cephalic extracts, and abdominal extracts (i.e., natural extracts) of virgin queens were each more attractive than the control for several of the behavioral criteria (Figure 2). The cephalic extract stimulated significantly more touching reactions in the males compared to the control. Surface, cephalic, and abdominal extracts elicited a higher proportion of the more intensive male mounting reaction than the control group. Most males showed approaches toward all dummies tested, and there was no difference in approach reactions between various queen extracts tested (ANOVA, LSD test, $P > 0.05$). Males never tried to copulate with impregnated dummies from any treatment group.

Electrophysiologically Active Compounds in Surface and Cephalic Extracts. In GC-EAD experiments with surface and cephalic extracts, we found 21 peaks that elicited electroantennographic responses in male antennae (Table 1, Figure 3). Most of the electrophysiologically active compounds belong to two main chemical classes: fatty acids and various types of corresponding esters.

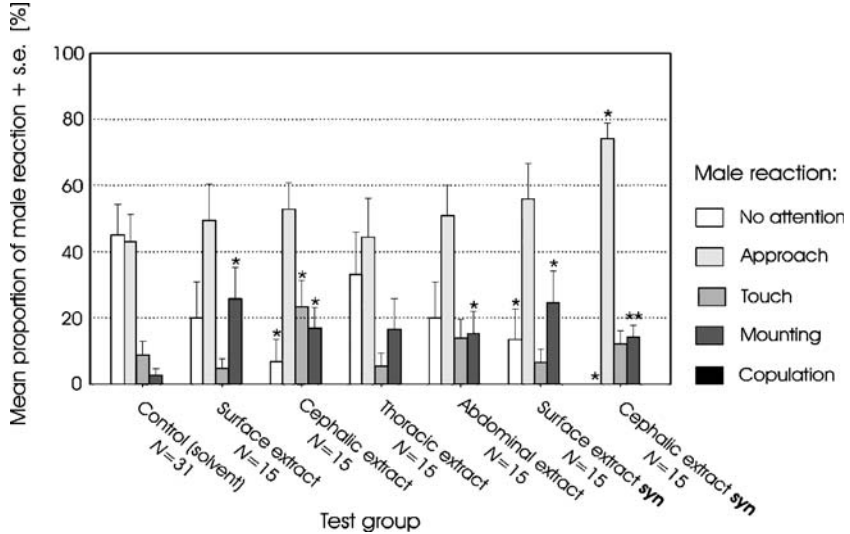


FIG. 2. Mean proportions of male reaction to natural extracts and mixtures of synthetic compounds from virgin *B. terrestris* queens. In the control test, 100 μ l of solvent was used ($*P < 0.05$; $**P < 0.001$, relative to control, Mann–Whitney *U* test; Syn: mixture of synthetic compounds).

Furthermore, heptacosene, 2-nonanone, and the diterpene geranyl geraniol were identified. The following pairs of compounds could not be separated under the GC conditions used: (*Z*)-13- and (*Z*)-12-heptacosene, as well as linoleic acid and geranyl geraniol. Most of the GC-EAD-active compounds occurred in both surface and cephalic extracts; however, the absolute amounts of single compounds differed. Methyl hexadec-7-enoate, methyl linoleate, and (*Z*)-13-heptacosene/(*Z*)-12-heptacosene were found in similar amounts in surface cephalic extracts (Table 1); all the other compounds differed significantly in quantity. While methyl oleate and oleic acid were found in higher amounts in surface extracts, most of the other compounds were higher in cephalic extracts. 3-Hydroxydecanoic acid was identified in a 250-fold higher amount on the cuticle surface. In some cases, it was difficult to quantify neighboring peaks, because of incomplete separation (Figure 3, peaks 16–20).

Attractiveness of Synthetic Compounds. Mixtures of 21 synthetic compounds (for compositions, see Table 1) identified in surface and cephalic extracts and applied to dummies elicited a higher mounting response than solvent-treated dummies did (Figure 2). The cephalic extract applied to dummies elicited a higher approach index than the solvent-treated dummies. This response exceeded that of all other treatments in this category. Synthetic

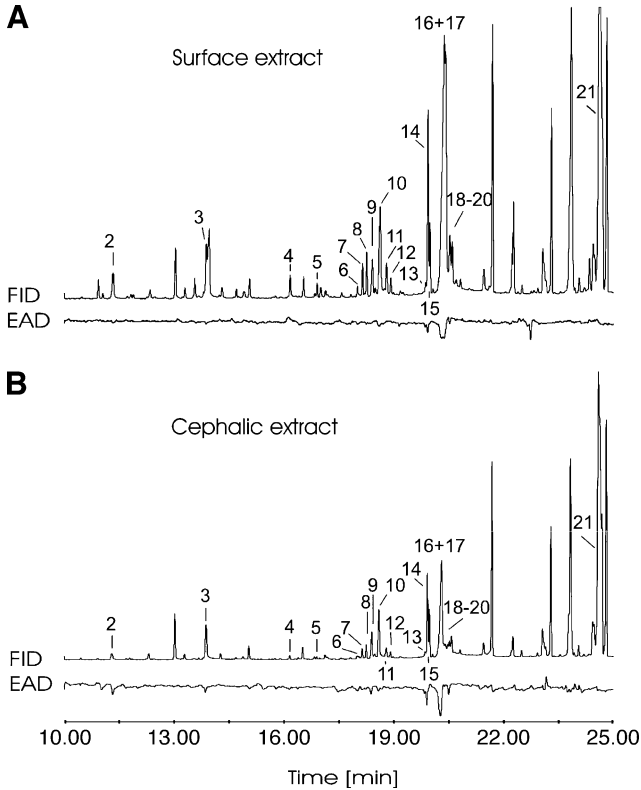


FIG. 3. Gas chromatographic analyses with flame ionization (FID) and electroantennographic (EAD) detection by male *Bombus terrestris* antennae to (A) surface extract of *B. terrestris* queens and (B) cephalic extract of *B. terrestris* queens. Numbered peaks correspond to compounds (Table 1) that elicit electroantennographic responses. To detect all “physiologically active” compounds and to discriminate electrophysiological responses from noise, 10–15 GC-EAD runs were performed for each type of sample to check the reproducibility of responses at the same retention times. Compound no. 1 has a retention time less than 10 min and is therefore missing in the figure.

blends of surface or cephalic extracts elicited smaller amounts of the “no attention” event as compared to the control.

DISCUSSION

How Important are Olfactory Cues in Mate Recognition by B. terrestris Males? In most species of bees, olfactory cues are essential in mate attraction and in eliciting mating behavior in the opposite sex (Ayasse et al., 2001).

However, in certain species, visual stimuli may also play a role for males locating females (Butler, 1965; Meyer-Holzapfel, 1984). Visual cues alone may be responsible for attracting a male toward a female (approaches), whereas volatiles elicit more intense male behavior (touching and mounting). Virgin *B. terrestris* queens seem most willing to mate 6 d after emergence (Gretenkord, 1997), at the time when chemical cues that stimulate premating behavior might be optimally established. Our data support a greater role for olfactory cues than visual stimuli in eliciting intense male mating behavior. Scentless *B. terrestris* dummies elicited significantly lower indices of the more intense categories of male mating behavior (touch, mounting, copulation) than living or dead gynes, or dummies impregnated with extracts of females. It is the shape, color, and size of female bumblebees, i.e., visual cues, which are most important in stimulating male approaches (Free, 1971). In bumblebees, body size might help the males distinguish queens from males and/or workers or other insects from a certain distance.

The importance of olfactory cues is indicated by experiments comparing the attractiveness of living queens with the attractiveness of queens whose mandibular glands had been extirpated (Van Honk et al., 1978). While a similar number of males approached and inspected both groups of queens, more males tried to copulate with untreated queens.

Where is the Sex Pheromone Located? Sex pheromones of female bees are produced in various glands and show a complex chemistry (Ayasse et al., 2001). In many bees, the sex pheromone is associated with the cephalic glands or more specifically with the mandibular glands as in *Bombus* (Free, 1971), *Eucera grisea* (Apidae) (Kullenberg, 1973), *Xylocopa sulcatipes* (Apidae) (Velthuis and Gerling, 1980), *Colletes cunicularius* (Colletidae) (Cane and Tengö, 1981), *Panurgus banksianus* and *P. calcaratus* (Andrenidae) (Tengö et al., 1988), *Nomia* (Apidae) (Wcislo, 1992), and in eusocial stingless bees (Engels et al., 1997). Van Honk et al. (1978) concluded that the compounds stimulating copulatory behavior are produced in the mandibular glands and are smeared onto the cuticle surface. Our results support this idea, since intense male responses were observed when the dummy bees were impregnated with surface and cephalic extracts.

Dispersion of the sex pheromone through grooming onto the cuticle of the queen may explain the attractiveness of the surface extracts (Figure 2). Attractiveness of abdominal extracts can be explained by the continued presence of behaviorally active compounds on the cuticle that were not entirely removed by solvent extraction of the surface. Cuticular lipids of insects have been thought to function primarily to prohibit loss of water (Hadley, 1984). Recent studies have shown, however, that in many insects they do more than prevent desiccation and regulate cuticular permeability. In bees, for example, specific cuticular lipids may function secondarily as sex pheromone components

(Ayasse and Dutzler, 1998; Ayasse et al., 1999, 2001; Schiestl et al., 1999). In most bee species, the site of sex pheromone production remains unclear, and a combination of the secretion from several glands might generate the behaviorally active bouquet on the cuticle surface.

Chemistry and Biology of the Sex Pheromone. In bees, chemical structures of female sex pheromones have been identified in only a few species (reviewed in Ayasse et al., 2001). In the stingless bee, *Scaptotrigona postica* (Apidae), 2-alcohols attracted males, and when combined with 2-ketones, they elicited male copulatory behavior (Engels et al., 1997). Macrocyclic lactones, which are produced in the Dufour's gland, stimulated mating behavior in the social sweat bee, *L. zephyrum* (Smith et al., 1985), and female-produced isopentenyl esters of unsaturated fatty acids play a key role in male mating behavior of *L. malachurum* (Ayasse et al., 1999).

This is the first report on the identification and composition of a female sex pheromone in bumblebees. It consists of fatty acids, various types of corresponding esters, heptacosene, 2-nonanone, and the diterpene geranyl geraniol. In electrophysiological analyses of surface and cephalic extracts, we demonstrated that all compounds triggered receptor potentials in male antennae (Figure 3). In behavioral experiments, synthetic mixtures of these GC-EAD compounds released typical mating behavior of the males. So far, mostly male marking pheromones have been identified (Kullenberg et al., 1970; Vinson et al., 1982; Bergman, 1997; Hovorka et al., 1998; Kindl et al., 1999). In a recent investigation, Cahlíková et al. (2004) gave a detailed description of the composition of secretions of virgin *B. terrestris* queens. They did not, however, perform behavioral experiments to identify the compounds that compose the sex pheromone. In the mandibular glands of virgin and breeding *B. terrestris* queens, about 180 chemical compounds have been identified (Hefetz et al., 1996; Cahlíková et al., 2004). Fatty acids as well as their methyl and ethyl esters are widespread and particularly abundant in bees (Wheeler and Duffield, 1988). They have already been identified as constituents of a sex pheromone in the solitary bee, *Osmia rufa* (Megachilidae) (Ayasse and Dutzler, 1998; Ayasse et al., unpublished data), and they also belong to the EAG-active compounds of *B. terrestris* queens. Many of the chemical compounds that trigger electrophysiological responses in the antennae of male *B. terrestris* antennae also have a pheromonal function in other Hymenoptera. For example, hexadecanoic acid is produced in the Dufour's gland of various *Colletes* bees and is used for nest cell lining (Albans et al., 1980). Ethyl tetradecanoate and ethyl hexadecanoate, which are produced in the female mandibular glands of several species of *Exoneura* (Allodapini), seem to have a defense function against intruders (Cane and Michener, 1983). Ethyl and methyl esters are found on the cuticle surface of honey bee, *A. mellifera*, larvae, and are reported to attract the bee mite, *Varroa jacobsoni* (LeConte et al., 1989).

Aliphatic hydrocarbons are ubiquitous in the Hymenoptera. Alkenes were found to have a function as a sex pheromone in the alfalfa leaf-cutter bee *Megachile rotundata* (Megachilidae) (Paulmier et al., 1999) and in *A. nigroaenea* (Schiestl et al., 2000). In *B. terrestris*, hydrocarbons produced in the tarsal glands, are deposited on rewarding food sources (Schmitt et al., 1991).

The identification of sex pheromones in bees has proven difficult, and in most cases the male reactions elicited by dummies impregnated with mixtures of synthetic compounds were found to be weak (Tengö, 1979; Cane and Tengö, 1981; Bergmark et al., 1984; Smith et al., 1985; Paulmier et al., 1999). There are several conceivable reasons for the diminished behavioral activity that we also registered in our experiments with *B. terrestris*: (1) Pheromone components may be thermally unstable. (2) The physicochemical properties of the dummy surface that accounts for adequate volatilization of the active compounds are not the same as those under natural conditions. Hydrocarbons represent the most abundant group of chemical compounds on the cuticular surface of *B. terrestris* females (Hefetz et al., 1996). Since most of them do not seem to have a communicative function, they may serve to solubilize and slowly release the active pheromone components (Ayasse et al., 2003). The surface of Soxhlet-extracted dummies that we used in our experiments is not covered with those hydrocarbons and does not possess the same physicochemical properties as it does on living or frozen queens. Thus, active compounds may not evaporate in the same proportions. (3) The dummies that were used do not provide important tactile cues. In the *Ophrys* pollination system, flowers not only imitate the females of their pollinators in scent and color, but also in the direction of the hairs on the flower surface in order to attract (and arrest) males for pollination (Ågren et al., 1984; Borg-Karlson, 1990). In behavioral experiments, the physicomachanical properties of the surface of the flower labellum or the female's cuticle are important cues that guide the landed male to a special position in order to initiate copulatory behavior. We cannot exclude the fact that the copulatory behavior of bumblebee males is stimulated by hairs on the cuticle surface of females. The hairs on the surface of the dummies often stick together during Soxhlet extraction and do not have the same arrangement as the hairs on a living female. Therefore, the tactile cues are probably missing. (4) The solvent extracts of the cuticle surface used in our bioassay may contain compounds that diminish the attractiveness of impregnated dummies. It has been shown that during copulation *B. terrestris* males transfer a sticky gelatinous product of their accessory glands, the so-called "mating plug," into the genital chamber of the queens (Duvoisin et al., 1999). Components of this mating plug are four fatty acids (palmitic acid, linoleic acid, oleic acid, stearic acid), which we also found in *B. terrestris* queen extracts (and which generated EAD responses in male antennae), as well as the 2,5-diketopiperazine made up of two proline molecules (Baer et al., 2000). Baer et al. (2001) performed behavioral assays showing that

linoleic acid in a queen's genital chamber reduced her willingness to mate and might repel mating attempts by *B. terrestris* males. Linoleic acid is a common natural product that can be found in the fat body of insects as well as on the cuticular surface (Lockey, 1988). Wasps use linoleic acid and oleic acid to repel ants (Dani et al., 1996). Since the queens that we used in our experiments were unmated and since linoleic acid is a compound that occurs in the hemolymph and fat body, a possible explanation for the lack of copulatory attempts in our experiments with cuticle extracts could be that linoleic acid was extracted from one of the cuticular layers of the queens. In future investigations, samples from the cuticle surface should be collected by solid phase microextraction (SPME) (Liebig et al., 2000), which permits sampling from the outer surface of the cuticle only. Chemical analyses of these samples may show the abundance of linoleic acid in various layers of the cuticle lipids.

Since *B. terrestris* males tried to copulate with frozen queens, the sex pheromone is still present and can be stabilized by freezing (Figure 1). Unlike the behavioral tests with frozen queens, no copulation attempts of males were recorded in tests with dummies impregnated with natural extracts. The temperatures and the duration of freezing may influence the chemical composition of the sex pheromone. While shock-frozen queens were highly attractive, queens stored at -20°C lost attractiveness with increasing freezing duration. In conclusion, elevated temperature combined with long freezing duration has a negative effect on the biological activity of the sex pheromone.

Questions concerning the loss of activity in natural extracts with time remain to be clarified. None of the compounds listed in Table 1 is supposed to rapidly decompose, nor should proportions of relevant components change under the storage conditions that we used. Assuming that the "active principle" is produced in the mandibular glands (see above), it is interesting to consider the relative ratios of electrophysiologically active components found in surface and cephalic extracts. Some compounds occurred preferentially in cephalic extracts in highly biased proportions (Table 1). Among these, methyl decanoate, 3-hydroxydecanoic acid, and 2-nonanone may be biogenically closely related: β -oxidation of a decanoyl precursor would yield the corresponding β -keto acyl compound, which could be easily transformed to 3-hydroxydecanoic acid or decarboxylated to 2-nonanone. The intermediate, 3-oxodecanoic acid or even its methyl ester would easily decompose on the injection port of a GC yielding 2-nonanone (W. Francke, unpublished data) and remain invisible upon analysis. Continuous production and release of β -ketoacids by a live organism may, however, generate a powerful chemical signal. We regard 3-oxodecanoic acid or a homolog as well as (*S*)-hydroxydecanoic acid (Hefetz et al., 1996) as reasonable candidates and are testing these compounds with respect to their behavior mediating capacity in sexual interactions of bumblebees.

Role of Sex Pheromones in the Mating Biology of B. terrestris. The function of sex pheromones in insects is to bring the sexes together for mating and to stimulate courtship behavior and copulation (Shorey, 1973). In *B. terrestris*, female- and male-produced compounds act as sex pheromones. Males establish flight routes that they patrol and along which they deposit a species-specific scent mark that acts as a premating, attractive signal to virgin queens (Bergman, 1997). After a virgin queen has landed in the patrolling area of a male, the pheromone produced by the female stimulates the mating behavior of males patrolling in close vicinity (Bergman, 1997). Our experiments with frozen females and dummies showed that visual cues attract males from a distance. At close range, olfactory cues are responsible for eliciting copulation behavior from a conspecific male. *B. terrestris* workers do not mate, and in our behavioral assays male reactions toward workers were intense, but males never attempted copulation. Free (1971) made similar observations with *B. pratorum*. Males reacted to workers but did not try to copulate. Djegham et al. (1994) suggested that the female sex pheromone does not elicit immediate copulation attempts. Alternatively, the sex pheromone, in addition to its role as a species-specific sex attractant, seems to have a contextual effect where it acts like an aphrodisiac.

Our understanding of how the sex pheromone operates should benefit from future investigations that focus on the role of temperature and its effect on the chemical composition of the sex pheromone. Future mating tests should examine the effect of single compounds on the whole sequence of precopulatory mating behaviors.

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RELIABLE SIGNALING BY CHEMICAL CUES OF MALE
TRAITS AND HEALTH STATE IN MALE LIZARDS,
Lacerta monticola

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Abstract—In spite of the importance of chemoreception in social organization and sexual selection of lizards, there is a lack of general knowledge on how the characteristics of chemical signals mediate these behaviors. Moreover, it is unknown which are the mechanisms that might confer honesty to the information provided by chemical signals. We analyzed here whether characteristics of the lipophilic fraction of femoral gland secretions of *Lacerta monticola* male lizards can be related to the morphology, physical condition, and health state of the sender. Our results indicated that some male traits, such as body size, number of blue spots, and number of femoral pores and their level of fluctuating asymmetry, were related to variability in the relative proportions of some lipophilic chemical compounds found in secretions. Thus, conspecifics could obtain reliable information on the producer of a scent mark based on chemicals alone, and this might be the basis of female choice observed in this lizard. Moreover, only males with a greater T-cell immune response had higher proportions of two steroids (ergosterol and dehydrocholesterol) in their femoral secretions, which might suggest that the signal is honest and costly to produce. We suggest that only high-quality males could divert these compounds from metabolism to secretions in order to produce an exaggerated and honest “chemical ornament.”

Key Words—Lizards, femoral glands, fatty acids, steroids, condition-dependent signaling, sexual selection.

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INTRODUCTION

While insect chemical ecology has received a great deal of interest in the last five decades, considerably less is known about many comparable aspects of vertebrate chemical ecology (Eisner and Meinwald, 1995; Wyatt, 2003). For example, despite the importance of chemoreception in social organization of many vertebrates, such as mammals or reptiles (Stoddart, 1980; Mason, 1992; Wyatt, 2003), there is a lack of general knowledge on how the characteristics of chemical signals mediate these behaviors. Chemical cues play an important role in the intraspecific communication of lizards (Mason, 1992). Several studies have shown pheromonal detection in different species, which is often based on precloacal and femoral gland secretions (e.g., Alberts, 1993; Labra and Niemeyer, 1999; Aragón et al., 2001a). The femoral pores are epidermal structures on the ventral surface of the thigh of many squamates connected to glands that produce copious amounts of holocrine secretion. The secretory activity of the femoral glands is greatest in the mating season, males produce more secretion than females, and androgens can influence their development and maintain their activity (reviewed in Mason, 1992; Alberts, 1993).

Studies have described the chemical composition of skin or gland secretions in a few species of lizards (Chauhan, 1986; Weldon and Bangall, 1987; Alberts, 1990; Mason and Gutzke, 1990; Weldon et al., 1990; Alberts et al., 1992; Escobar et al., 2001, 2003; López and Martín 2005a). Secretions are composed of both proteins and lipids, but the latter are thought to be the main compounds involved in communication (Mason, 1992). The presence and relative concentration of chemical components in femoral glands secretions seem to vary not only between sexes, but also consistently among individuals, which may convey information on the individual identity and serve a variety of functions (Alberts, 1993). For example, the ventral location of the femoral pores suggests that secretions are passively deposited on the substrate as lizards move through their home ranges. Moreover, behavioral experiments showed that scents of male lizards could advertise residence in a home range, and/or could convey information about the social status and competitive ability of the sender (Aragón et al., 2001a,b, 2003; López and Martín, 2002). Experiments have also suggested that secretion from the femoral pores might transmit chemical information about a male's traits or genetic compatibility, which may be used by female lizards in mate choice (Martín and López, 2000; López et al., 2002, 2003; Olsson et al., 2003). Therefore, chemoreception may be especially important in social organization, and play an important role in sexual selection processes in lizards (López and Martín, 2004). However, the role of specific chemical compounds as chemical signals in sexual selection of lizards remains largely unknown.

Sexual selection suggests that male sexual ornaments or signals may allow females or male rivals to evaluate a male's quality. However, if signaling were based on features that are not directly related to an individual quality, individuals might benefit from "cheating," that is, signaling at too high a level (Krebs and Dawkins, 1983). Thus, theoretical models have predicted that signals can only be evolutionarily stable if they are honest and condition-dependent, or costly to the signaler, and if the cost is correlated with the signaler's quality (Zahavi, 1975; Pomiankowski, 1988; Grafen, 1990; Zahavi and Zahavi, 1997). Although evidence for indicator models of sexual selection has been found in many empirical studies (see reviews in Jennions et al., 2001; Kotiaho, 2001), research has focused almost exclusively on visual or acoustic signals, often ignoring chemical signals (but see Penn and Potts, 1998; Rantala et al., 2003; Zala et al., 2004). Because parasitic infections and health state seem to affect the information conveyed by chemical signals (Penn and Potts, 1998; Zala et al., 2004), it is possible that pheromones also function as condition-dependent signals of mate quality. However, in vertebrates, it is unknown which are the mechanisms that might confer honesty to information provided by chemical signals.

The Iberian rock lizard (*Lacerta monticola*) is a small, diurnal lacertid lizard found mainly in rocky habitats of some high mountains in the Iberian Peninsula (Pérez-Mellado, 1998). Differential tongue flick rates to chemicals presented on cotton swabs and on substrate deposits have demonstrated that males can detect and discriminate between self-produced scents from the femoral pores and those of conspecific males, and between odors of familiar and unfamiliar males (Aragón et al., 2001a). Thus, scent-marked substrates may function in home range advertisement to other males (Aragón et al., 2001b), but also be used by females to choose mates (Martín and López, 2000; López et al., 2002, 2003).

In this paper, we examined whether characteristics of the lipophilic fraction (i.e., composition and relative proportions of each compound) of femoral secretions of males can be related to the morphology, physical condition, and health state of the sender. By examining the type of chemicals found in secretions, and how their proportions vary with the characteristics of males' traits and with their health state, we explored whether allocating specific compounds to femoral secretions can be condition-dependent. Thus, we aimed to understand how honesty of information contained in chemical signals could be achieved.

Specifically, we first examined whether chemical signals may give information on male traits known to play an important role in intrasexual selection and social organization between males. Thus, we measured morphological traits that reflect the social dominance of male lizards. Body and head size are known to determine the outcome of intrasexual agonistic contests, with males having larger body size and relatively larger heads being

more dominant (López et al., 2002). Also, many male lizards show a conspicuous row of small but distinctive blue spots that runs along the body side on the outer margin of the belly. The number of blue spots may function as a long-distance visual signal enhancing body size of older/dominant males (López et al., 2004).

Second, we examined whether chemical signals may give reliable information on male traits potentially used by females in intersexual selection processes (i.e., mate choice). Previous studies have suggested that female *L. monticola* preferred scents of males with more femoral pores and with a symmetric number of femoral pores in each leg (Martín and López, 2000; López et al., 2002). Symmetry presumably indicates the developmental stability of an individual and, thus, its ability to cope with genetic and environmental perturbations during development (Møller and Swaddle, 1997). Females may also select males based on their body condition and health state, which might affect the content of chemical signals (Penn and Potts, 1998; López et al., 2002; Rantala et al., 2003). During the mating season, the intensity of hemoparasite infection was greater in male than in female *L. monticola*, and parasite load had a negative effect on body condition (Amo et al., 2004). Thus, parasite load might be an indication of a lizard health state. Immune function can also be used as an indicator of health state of an animal (Sheldon and Verhulst, 1996; Westneat and Birkhead, 1998; Svensson et al., 2001), and females of other lizard species might base their mate choice on the quality of the males' immune system (López and Martín, 2005b).

METHODS AND MATERIALS

Lizards' Morphological Measurements. We captured (by noosing) 68 adult male *L. monticola* from May 17 to June 22, 2003, which coincided with the mating season of lizards, in different places over a 5-km² area ("Puerto de Navacerrada," Guadarrama Mountains, Central Spain). Only adult lizards with intact or fully regenerated tails were considered. We measured lizard body size with a ruler [snout-to-vent length (SVL), $\bar{x} \pm \text{SE} = 72 \pm 1$ mm; range = 63–78 mm) and their weight with a pesola spring scale ($\bar{x} \pm \text{SE} = 7.0 \pm 0.1$ g; range = 4.7–9.0 g). Head height ($\bar{x} \pm \text{SE} = 8.2 \pm 0.1$ mm; range = 7.0–9.5 mm) was measured with a digital caliper (to the nearest 0.01 mm) as the greatest vertical distance through the snout from the highest portion of the head to the bottom of the lower jaw. We removed the influence of body size on head height measurements by regressing each against SVL (all variables log-transformed) and used the residuals in further analyses. We also noted the number of blue spots on each side of the lizards and calculated an average number for both sides ($\bar{x} \pm \text{SE} = 6.0 \pm 0.3$ spots/side; range = 0–13.5 spots/side).

We counted under a magnifying glass the number of femoral pores on the right and left hindlimbs of males. Individuals with symmetric femoral pores are also more symmetric in several other meristic characters (P. López and J. Martín, unpublished data). We have previously shown that counts of femoral pores are highly repeatable, and that the absolute value of asymmetry of the femoral pores (asymmetry values ranged between -2 and $+2$), calculated as the unsigned right-minus-left number of pores, exhibits the properties of fluctuating asymmetry (FA) (Møller and Swaddle, 1997; see Martín and López, 2000, for statistical analysis of FA of femoral pores in this lizard species).

Lizard Condition and Health State. To assess differences in the physical condition of otherwise similar individuals, we calculated the body condition of each individual as the residuals from the regression equation of body mass (in g) on SVL (in mm), both variables log-transformed, which may represent an index of the relative amount of fat stored, and, hence, an estimation of individual physical condition or nutritional status (Bonnet and Naulleau, 1994).

To examine hemoparasite load, a smear was made on a microscope slide from blood taken from the lizards' postorbital sinus by using one 9- μ l heparinized hematocrit tube. Blood smears were air-dried. The smears were fixed in absolute methanol for 10 min and then stained in Giemsa diluted 1:9 with phosphate buffer (pH 7.2) for 40 min before their examination for parasites. The only hemoparasites found in this population were intraerythrocytic hemogregarines (Amo et al., 2004). Parasite intensity was estimated for each lizard by counting at $400 \times$ the number of infected red blood cells found in approximately 2000 cells ($\bar{x} \pm SE = 0.75 \pm 0.16$ infected cells in 2000 erythrocytes; range = 0–9.11).

To assess the T-cell-mediated immune (CMI) response, we used a delayed-type hypersensitivity test, the phytohemagglutinin (PHA) injection test. This is a reliable measure of T-cell-dependent immunocompetence *in vivo* (Lochmiller et al., 1993), which has been used in numerous studies on many animals including lizards (Svensson et al., 2001; Belliure et al., 2004). A subset ($N = 34$) of all captured lizards were individually housed at "El Ventorrillo" Field Station (Navacerrada, Madrid Province), 5 km from the capture site in outdoor opaque plastic cages (80 \times 50 cm) containing rocks for cover. We marked a point with permanent ink on the foot pad of both hindlimbs. We then measured the thickness at this point with a pressure-sensitive spessimeter (to the nearest 0.01 mm) to standardize pressure during measurements. Immediately after, we injected 0.02 mg PHA dissolved in 0.01 ml phosphate-buffered saline (PBS) water in the right foot pad, and the same volume of PBS in the left foot pad. Lizards were released in their terraria, and after 24 hr we measured again the foot pad thickness at the marked points. The CMI response was calculated as the difference between pre- and postinjection measures on the right foot pad (PHA injected) minus the same difference on the left foot pad (control, PBS injected)

(CMI, $\bar{x} \pm \text{SE} = 0.52 \pm 0.06$ mm; range = 0.10–0.93 mm) (Belliure et al., 2004). The only appreciable effect of the PHA injection was a slight swelling of the skin due to the immune response, which disappeared after 48 hr. No lizard showed any sign of stress or pain for these tests, and all looked healthy. The same day, lizards were released to their initial sighting location prior to the capture.

Extraction of Femoral Gland Secretions and Analyses of Chemicals. Immediately after capture, we gently pressed with forceps around the femoral pores of male lizards, and directly collected secretion in glass vials with Teflon-lined stoppers. Vials were stored at -20°C until analyses. We also used the same procedure on each sampling occasion, but without collecting secretion, to obtain blank control vials that were treated in the same manner to compare with the lizard samples, to exclude contaminants from the handling procedure or from the environment where lizards were found, and for further examining impurities in the solvent.

Samples were analyzed with a Finnigan-ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly(5% diphenyl/ 95% dimethylsiloxane) column (Supelco, Equity-5, 30 m length \times 0.25 mm ID, 0.25- μm film thickness) and a Finnigan-ThermoQuest Trace mass spectrometer (MS) as detector. We injected in the splitless mode 2 μl of each sample (dissolved immediately before the analyses in 250 μl *n*-hexane; Sigma, capillary GC grade). Column temperature was kept at 50°C for 10 min, then raised to 280°C at a rate of $5^{\circ}\text{C}/\text{min}$, and maintained at this temperature for 30 min. Initial identification of secretion components was done by comparison of mass spectra in the NIST/EPA/NIH 1998 computerized mass spectral library. Impurities identified in the solvent and/or the control vial samples were not considered. Identifications were confirmed by comparison of spectra and retention times with those of authentic standards (from Sigma-Aldrich Chemical Co.).

Statistical Analyses. In total, 41 chemical compounds were identified in femoral gland secretions (see López and Martín, 2005a). The relative amount of each component was determined as the percent of the total ion current (TIC). Then, we selected the peaks that represented $>0.1\%$ relative peak area and that were present in all individuals (which altogether represent 98% of the total TIC area) to reduce the number of variables to be used in multivariate statistical analysis (Aitchison, 1986; Dietemann et al., 2003). The relative areas of the 28 selected peaks were restandardized to 100% and 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 (Aitchison, 1986). The homogeneity of variance of these variables was tested with Levene's test, and Bonferroni's correction was applied. Only 26 variables had homogeneous variances and were considered. The transformed areas were used as variables in a principal component analysis (PCA). To examine whether we could use

proportions of chemicals to predict the characteristics of each of the variables describing the morphology, condition, and health state of lizards, we used forward stepwise general regression models (GRM) with the six principal components (PC) extracted as potential predictors. Because some morphological and health measures might be related to body size, we included SVL in some of the initial models as a potential predictor. The relationship between FA in femoral pores and PC scores describing chemical compounds was estimated by using generalized linear models (GLZM), with the absolute value of femoral pores symmetry following a binomial distribution ($\chi^2 = 0.11$, $P = 0.74$).

RESULTS

Chemicals in Femoral Secretions. The lipophilic fraction of femoral secretion of *L. monticola* is a mixture of steroids (mainly cholesterol) and carboxylic acids that range between $n\text{-C}_6$ and $n\text{-C}_{22}$, and their esters, but we also found alcohols, ranging from 16 to 22 carbons, and squalene (Table 1). On average, the five most abundant chemicals were cholesterol (71.4% of TIC), campesterol (5.9%), dimethyl cholesta-5,7-dien-3-ol (5.0%), hexadecanoic acid (3.9%), and octadecenoic acid (2.4%). The PCA for relative proportions of chemical compounds in femoral gland secretions produced six components [principal components (PCs)] with eigenvalues greater than 1 that together accounted for 77.3% of the variance (Table 1).

Lizard Morphology and Chemicals. Stepwise general regression modeling (GRM) showed that (snout-to-vent-length SVL) of lizards was positively correlated with PC-1 and PC-3 ($R^2 = 0.14$, $F = 5.28$, $df = 2,65$, $P = 0.007$) (Table 2). A similar relationship was found for body mass ($R^2 = 0.16$, $F = 6.22$, $df = 2,65$, $P = 0.003$). Therefore, secretions of lizards of different size/age were different. For example, larger lizards had secretions with relatively higher proportions of steroids such as cholesterol, campesterol, or sitosterol, and of nonanoic, dodecanoic, and octadecanoic acids, among others, and lower proportions of cholesta-5,7-dien-3-ol, and of octadecadienoic, hexadecenoic, and octanoic acids (see Table 1).

With respect to the traits that may be indicators of social status between males, relative head height was not significantly correlated with any PC, but the number of blue spots was positively correlated with PC-4 and negatively correlated with PC-5 ($R^2 = 0.12$, $F = 4.59$, $df = 2,65$, $P = 0.01$) (Table 2). Thus, lizards with a higher number of blue spots have lower proportions of chemicals such as docosanol, dimethyl-cholest-en-ol, or ergosta-dien-ol, and higher proportions of tetradecanoic and eicosanoic acids.

Femoral Pores and Chemicals. Lizards had an average (\pm SE) of 18.7 ± 0.2 (range = 16–23) femoral pores on each leg. The number of femoral pores was

TABLE 1. PRINCIPAL COMPONENTS ANALYSIS FOR RELATIVE PROPORTION OF CHEMICALS IN FEMORAL GLAND SECRETIONS OF MALE LIZARDS

Compound ^a	PC-1	PC-2	PC-3	PC-4	PC-5	PC-6
Octanoic acid	-0.27	-0.17	-0.63	0.24	-0.05	0.06
Nonanoic acid	0.77	0.02	-0.15	0.45	0.17	0.10
Decanoic acid	0.22	-0.02	-0.59	0.17	-0.13	0.48
Dodecanoic acid	0.74	0.09	-0.16	0.42	0.21	0.11
Tetradecanoic acid	-0.25	0.51	-0.35	-0.22	-0.52	-0.04
Hexadecenoic acid	-0.56	0.49	-0.12	0.19	-0.24	-0.16
Hexadecanoic acid	0.03	0.78	0.17	0.42	0.15	0.05
9,12-Octadecadienoic acid	-0.84	0.14	0.09	0.11	0.12	0.21
Octadecenoic acid	-0.01	0.71	0.25	0.37	0.07	0.23
Octadecanoic acid	-0.26	0.05	0.70	0.41	0.02	0.10
Eicosanoic acid	-0.30	0.00	0.48	-0.13	-0.53	0.21
Docosanol	-0.33	-0.07	-0.03	-0.80	0.01	-0.06
Squalene	0.50	-0.18	0.52	0.13	-0.11	0.22
Cholesta-3,5-diene	0.04	-0.88	0.13	0.15	-0.21	-0.04
Cholesta-4,6-dien-3-ol	-0.18	-0.79	-0.10	-0.18	0.34	0.22
Cholesterol	0.89	0.04	-0.11	0.30	0.20	-0.04
Cholesta-5,7-dien-3-ol	-0.68	0.01	-0.05	-0.05	0.12	-0.73
Ergosterol	0.06	-0.02	-0.04	-0.04	-0.04	-0.90
Stigmasta-5,24(28)-dien-3-ol	0.82	0.24	0.05	0.08	0.16	0.01
Campesterol	0.93	0.04	-0.05	0.16	0.15	0.01
Ergosta-5,8-dien-3-ol	0.28	0.11	0.08	-0.08	0.88	0.06
Lanost-8-en-3-ol	0.78	-0.12	0.06	0.27	0.11	-0.14
γ -Sitosterol	0.87	-0.13	0.05	0.25	0.14	-0.10
24-Propylidene-cholest-5-en-3-ol	-0.17	-0.36	0.08	-0.62	-0.38	0.23
4,4-Dimethyl-cholest-7-en-3-ol	-0.26	-0.22	0.03	-0.78	0.18	-0.13
4,4-Dimethyl-cholesta-5,7-dien-3-ol	0.84	0.14	0.16	-0.09	0.18	-0.01
Eigenvalue	8.98	3.97	2.14	1.92	1.69	1.39
% Variance	34.54	15.29	8.25	7.37	6.49	5.34

^aMarked correlations are significant at $P < 0.001$.

not related to body length ($R^2 = 0.01$, $F = 0.69$, $df = 1,66$, $P = 0.41$). The number of femoral pores was negatively correlated with PC-1 ($R^2 = 0.11$, $F = 8.42$, $df = 1,66$, $P = 0.005$) (Table 2). Thus, lizards with more femoral pores had secretions with relatively higher proportions of cholesta-5,7-dien-3-ol, and octadecadienoic and hexadecenoic acids, but lower proportions of steroids such as cholesterol or campesterol, and nonanoic and dodecanoic acids (Table 1).

The value of fluctuating asymmetry (FA) in femoral pores was negatively correlated with PC-2 (GLZ model, intercept: Wald's $\chi^2 = 44.18$, $P < 0.001$; PC-2: Wald's $\chi^2 = 5.26$, $P = 0.02$) (Figure 1). Thus, more symmetric lizards have relatively higher proportions of hexadecanoic and octadecenoic acids,

TABLE 2. FORWARD STEPWISE GENERAL REGRESSION MODELS (GRM) RELATING MORPHOLOGICAL TRAITS AND HEALTH STATE OF MALE LIZARDS WITH THE PC SCORES FOR RELATIVE PROPORTIONS OF CHEMICALS IN THEIR FEMORAL PORES SECRETIONS

<i>a</i>	$\beta \pm SE$	<i>B</i> \pm SE	<i>t</i>	<i>P</i>
Snout-to-vent length (SVL)				
Intercept		1.86 \pm 0.01	758.77	<0.001
PC-1	0.26 \pm 0.11	0.01 \pm 0.01	2.23	0.03
PC-3	0.27 \pm 0.11	0.01 \pm 0.01	2.36	0.02
Number of blue spots				
Intercept		3.33 \pm 0.12	28.78	<0.001
PC-4	0.24 \pm 0.12	0.24 \pm 0.12	2.07	0.04
PC-5	-0.26 \pm 0.12	-0.26 \pm 0.12	-2.21	0.03
Number of femoral pores				
Intercept		6.12 \pm 0.03	233.57	<0.001
PC-1	-0.34 \pm 0.12	-0.01 \pm 0.03	-2.90	0.005
T-cell-mediated immune response (CMI)				
Intercept		0.54 \pm 0.03	17.23	<0.001
PC-6	-0.59 \pm 0.14	-0.08 \pm 0.02	-4.18	<0.001

^aThe table provides standardized (β) and nonstandardized (*B*) regression coefficients and their standard errors, and results from *t*-tests (*t*) and associated probability (*P*) levels.

and lower proportions of cholesta-3,5-diene, and cholesta-4,6-dien-3-ol (Table 1).

Lizard Health and Chemicals. Body condition was not significantly correlated with any PC, but the CMI immune response was negatively

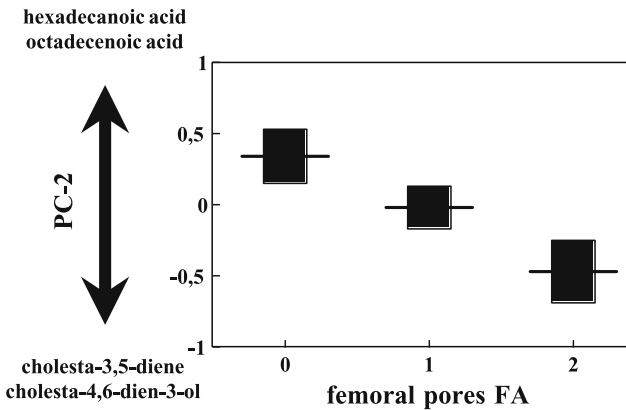


FIG. 1. Principal component analysis (PCA). PC scores (mean \pm SE) of male lizards with different fluctuating asymmetry (FA) values in their femoral pores, in the PC-2 axis extracted in a PCA for relative proportion of chemicals in their femoral gland secretions.

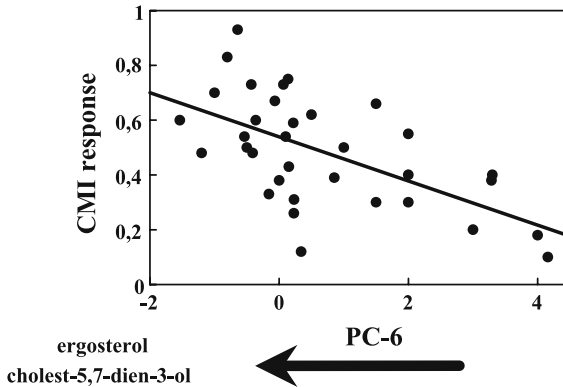


FIG. 2. Principal component analysis (PCA). Relationship between the PC-6 axis, extracted in a PCA for relative proportion of chemicals in femoral gland secretions of male lizards, and their T-cell-mediated immune (CMI) response.

correlated with PC-6 ($R^2 = 0.35$, $F = 17.48$, $df = 1,32$, $P < 0.001$) (Table 2). Thus, lizards with relatively higher proportions of ergosterol and cholesta-5,7-dien-3-ol in their femoral secretions have a better health state, as indicated by their greater CMI immune response (Figure 2). Hemoparasite load was not significantly correlated with any PC, although it was negatively related to CMI immune response ($R^2 = 0.19$, $F = 7.73$, $df = 1,32$, $P = 0.009$). Thus, males with a higher CMI response had a lower parasite load.

DISCUSSION

Our results indicate that some of the morphological traits of male *L. monticola* lizards, and also their health state, can be inferred from variability in the relative proportions of lipophilic chemical compounds found in their femoral secretions. Thus, conspecific lizards could obtain reliable information on the producer of a scent mark by assessing the information provided by chemicals contained in the signal. Lizards have a well-developed vomeronasal system and are able to make discriminations of many different scents, from prey, conspecifics, or predators, based on chemicals alone (Mason, 1992; Cooper, 1994). However, only a few studies have examined whether lizards can discriminate between different types of chemical compounds (Cooper and Pérez-Mellado, 2001; Cooper et al., 2001, 2002). These studies showed that the lacertid lizard, *Podarcis lilfordi*, can discriminate between lipids, proteins, and carbohydrates (Cooper et al., 2001), and also among different lipids such as

glycerol, cholesterol, and oleic and hexadecanoic acids (Cooper et al., 2002). It is likely that lizards may discriminate between many other similar chemicals found in femoral secretions and used in social contexts.

Behavioral experiments made with *L. monticola* lizards had previously indicated that lizards may discriminate among different individuals according to their morphological traits based on their scent alone. For example, the responses of males to scent marks of other unfamiliar males depended on the body size difference between both males (Aragón et al., 2001b), which suggested that males can assess body size and associated competitive ability of unknown males. The results of the current study indicate that body size could be inferred from the variability in chemicals found in femoral secretions. The number of lateral blue spots also correlated with the proportions of some specific chemicals. The number of spots seems to be a character showing ontogenetic change, as older males (territorial and dominant) showed more blue spots than younger ones (sneakers and subordinate) (López et al., 2003, 2004). These chemicals may be actually related to age and, thus, indirectly to the potential social status of a male. In contrast, we did not find any direct relationship between chemicals and relative head size or body condition, two morphological traits directly related to the social status of males of similar age (López et al., 2002). Females preferred to associate with substrates scent marked by older males (López et al., 2003). Females may increase their opportunities to mate with better males, because viability selection leads to older males of higher genotypic quality than younger males. Alternatively, females may select territorial old males to avoid harassment by sneaking young males. If the variation in compounds present in femoral secretions were directly related to age-related differences in, for example, hormonal levels, then chemical signals would be a reliable indicator of male age/size.

Previous experiments showed the following results. When we offered the scents of two male *L. monticola* of similar age and body size, females associated preferentially with scents of males with low FA in their femoral pores, and also with scents of males with a higher number of femoral pores (Martín and López, 2000; López et al., 2002). This suggested that chemical signals enabled the females to assess the males' ability to produce femoral secretions. The results of the current study indicated that the proportion of some chemical compounds are related to the number of femoral pores, and, moreover, to FA in this character. Particularly, lizards with more pores and more symmetry had relatively higher proportions of cholesta-5,7-dien-3-ol and unsaturated fatty acids, such as octadecadienoic, hexadecenoic, and octadecenoic acids, but lower proportions of steroids, such as cholesterol or campesterol, and short-chain saturated fatty acids. However, at this stage, we cannot prove (but see below) that there is a direct relationship between the proportions of these compounds in secretions and the "quality" of a male.

Also, there was a clear relationship between the CMI immune response of a male and femoral secretions with higher proportions of ergosterol and cholesta-5,7-dien-3-ol (=dehydrocholesterol). Ergosterol was also occasionally found in some individuals of the iguanid lizard *Liolaemus fabiani* (Escobar et al., 2003), but all male *L. monticola* examined presented these two steroids. Interestingly, proportions of these varied greatly among individuals (Ergosterol, range = 0.11–2.51% of TIC, coefficient of variation = 61.2%; Dehydrocholesterol, range = 0.05–0.99% of TIC, coefficient of variation = 90.5%). This suggests that their variability may be a good indicator of variability in the male immune system characteristics, which might be used by females to select mates. In fact, female *Podarcis hispanica* lizards prefer male scents that signal a better CMI response (López and Martín, 2005b). Our data also suggest that the signal might be honest and costly to produce, as only males with a greater CMI response had higher proportions of these two steroids in their femoral secretions.

Signals used to indicate the quality of a male should be costly to produce or to maintain, and should be condition-dependent, in order to be honest and evolutionarily stable (Zahavi, 1975; Pomiankowski, 1988; Grafen, 1990; Zahavi and Zahavi, 1997; Kotiaho, 2001). Knowledge of the biosynthesis and metabolic function of the specific chemical compounds of lizards will enable us to understand the mechanisms that relate “quality” of a male with these compounds. For example, cholesta-5,7-dien-3-ol present in the skin is transformed into vitamin D₃ after exposition to sun UV-B irradiation (Fraser, 1995; Carman et al., 2000). Vitamin D₃ is essential in calcium metabolism of lizards and other vertebrates (Allen et al., 1994; Laing and Fraser, 1999). Therefore, by allocating this precursor of vitamin D₃ to femoral secretions, males are diverting it from other metabolism. Such a divergence might be especially costly for low-quality individuals if they do not have enough body reserves of this steroid. Moreover, as hypervitaminosis D produces soft tissue calcification, the excess of its precursor might be partly eliminated in femoral secretions. In any case, its presence in high proportions in femoral secretions would indicate honestly the quality of metabolism in a male. In addition, vitamin D₂ has to be acquired from the diet, as only fungus can convert it from ergosterol under UV irradiation (Hay and Watson, 1977). It remains possible that ergosterol and vitamin D₂ were associated in invertebrate prey eaten by these lizards, and that the amount of ergosterol in femoral secretions was an indicator of the amount of vitamin D₂ that the male was able to acquire from the diet.

Similarly, the octadecadienoic (=linoleic) acid, found in higher proportions in secretions of males with more femoral pores, is an essential fatty acid that lizards must obtain through their dietary intake, as desaturation of oleic acid to linoleic acid occurs only in plants. Linoleic acid is involved in the maintenance of the epidermal water barrier (Hansen and Jensen, 1985). Animals require this acid for the production of n-6 and n-3 polyene acids, which are precursors of

prostaglandins, prostacyclin, and thromboxanes that mediate a wide range of physiological responses (Ziboh et al., 2000). Its presence in high proportions in femoral secretions might be costly and indicates the ability of a male to obtain it from the diet in sufficient quantity for maintaining both metabolism and the scent signal.

In other systems of sexual signals, the costs of producing and maintaining components of the immune system may have a major effect on condition, thus creating a link between immune system and condition-dependent sexual advertisement (Wedekind, 1992; Wedekind and Folstad, 1994). A trade-off is expected if sexual advertisement and immune responses use the same resources (Kotiaho, 2001). Only individuals in good condition apparently can mount a strong immune defense and produce an extravagant sexual ornament (Sheldon and Verhulst, 1996; Westneat and Birkhead, 1998). Our results show a relationship between immune response and the presence of cholesta-5,7-dien-3-ol and ergosterol in secretions. Thus, it seems that only males with a high CMI immune response might afford to allocate high proportions of these potentially costly chemicals to femoral secretions to produce an exaggerated and honest “ornament.”

We conclude that chemical compounds in femoral secretions of lizards may provide reliable information on the morphological traits and health state of a male. Further experimental studies are needed to test whether chemical signaling is costly and honest, which would clarify the basis of the mechanisms of sexual selection processes based on chemoreception observed in this and other lizard species.

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THE INHIBITORY EFFECTS OF COUMARIN
ON THE GERMINATION OF DURUM WHEAT
(*Triticum turgidum* ssp. *durum*, CV. SIMETO) SEEDS

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Abstract—The event chain leading to germination blockage in durum wheat (*Triticum turgidum* ssp. *durum* Desf.) seeds exposed to the allelochemical coumarin (2*H*-chromen-2-one) was studied. The physiological and biochemical aspects thought to be critical for a successful seed germination were measured. At concentrations above 200 μM , coumarin inhibited seed germination in a concentration-dependent manner. Inhibition occurred early during seed imbibition (phase I), was rapid, and irreversible. During phase I, coumarin inhibited water uptake, electrolyte retention capacity, and O₂ consumption. Later on, coumarin delayed the reactivation of peroxidases, enhanced the activity of superoxide dismutase, decreased the activities of selected marker enzymes for metabolic resumption, and repressed the transcription of molecular chaperons involved in secretory pathways. Insufficient and/or late seed rehydration caused by coumarin could have delayed membrane stabilization or decreased respiratory O₂ consumption, both of which are conducive to an overproduction of reactive O₂ species. Being unbalanced by an adequate upsurge of antioxidant defense systems, the resulting oxidative stress might have ultimately interfered with the germination program.

Key Words—Allelochemicals, coumarin, seed germination, antioxidants, molecular chaperons, durum wheat, *Triticum turgidum* ssp. *durum* Desf.

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Abbreviations: α -AMY = α -amylase; β -AMY = β -amylase; APX = ascorbate peroxidase; AsA = ascorbic acid; BiP = luminal binding protein; CAT = catalase; DHAR = dehydroascorbate reductase; DHAsA = dehydroascorbic acid; ER = endoplasmic reticulum; G6PDH = glucose-6-phosphate dehydrogenase; GluK = glucokinase; GPX = hydrogen-donor aspecific (guaiacol) peroxidase; GSH = reduced glutathione; GS-SG = glutathione disulfide; MDH = malate dehydrogenase; MDHAR = monodehydroascorbate reductase; OPPP = oxidative pentose phosphate pathway; PCR = polymerase chain reaction; PDI = protein disulfide isomerase; PyrK = pyruvate kinase; ROS = reactive oxygen species; RT = reverse transcription; SOD = superoxide dismutase; TRX = thioredoxin *h*.

INTRODUCTION

Coumarins form a large class of allelochemicals widely distributed in both natural plant communities and crops (Zobel and Brown, 1995). Being localized on the leaf, seed surface, and pollen wall, and released into the environment by living plants or by decomposing plant material, coumarins are involved in ecological interactions in both managed and natural plant communities (Rice, 1984; Zobel et al., 1991; Bertin et al., 2003; Bais et al., 2004).

Coumarin (2*H*-chromen-2-one), the simplest compound of this class, affects root form and function (Abenavoli et al., 2001, 2004), decreases respiration and photosynthesis (Moreland and Novitzky, 1987), and influences nitrogen uptake and metabolism (Abenavoli et al., 2001, 2003). Coumarin is a strong inhibitor of seed germination. This may confer an advantage to the producing plant species by reducing competition in its immediate environment, and/or by delaying germination of its own seeds under unfavorable conditions (Zobel and Brown, 1995).

To explain this inhibitory action, coumarin has been hypothesized to be either a blocker of the cell cycle (Zobel and Brown, 1995), uncoupler of oxidative phosphorylation (Ulitzer and Poljakoff-Mayber, 1963; Khan and Zeng, 1985), or to interfere with amino acid transport and protein synthesis (Van Sumere et al., 1972). Aliotta et al. (1992, 1993) reported that coumarin is an inducer of coat-imposed dormancy through the inhibition of water uptake during seed imbibition. Such a multiplicity of proposed roles suggests that the key mechanism(s) by which coumarin affects seed germination remains to be identified.

In the present work, we looked at the early events leading to the blockage of germination in durum wheat (*Triticum turgidum* ssp. *durum* Desf. cv. Simeto) seeds exposed to coumarin. To fulfill such an aim, and keeping as a reference scheme the triphasic germination time course (Bewley, 1997), the effects of different coumarin concentrations were evaluated on relevant physiological and biochemical processes associated with seed imbibition and germination.

METHODS AND MATERIALS

Germination Experiments. Seeds of *T. turgidum* ssp. *durum* Desf., cv. Simeto were surface-sterilized for 20 min into 20% (v/v) NaClO solution, and rinsed several times with sterile distilled water. Fifteen seeds were evenly placed into Petri dishes (9 cm diam) on moist filter paper soaked with 5 ml sterilized aqueous coumarin solution adjusted to pH 5.8, whose final concentrations ranged from 0 to 1000 μM . Seeds were incubated in darkness in a growth chamber at 24°C and 70% RH. Seeds showing at least a 2-mm-long extrusion of the radicle after 36 hr of imbibition were considered to have successfully completed their germination. Germination counts were expressed as percentages of the total number of seeds.

In a series of recovery experiments, Simeto seeds were exposed to 0, 100, or 1000 μM coumarin for 1, 3, 6, 12, or 24 hr and then transferred under sterile conditions onto filter paper containing 5 ml sterile distilled water until the end of germination (36 hr). This is referred to as the *ab initio* experiments. In a complementary set of experiments aimed at evaluating the time window that coumarin inhibition is effective, the Simeto seeds were initially placed into distilled water for 1, 3, 6, 12, or 24 hr and then exposed to 0, 100, or 1000 μM coumarin for the time remaining to complete germination (36 hr). This is referred to as the *in itinere* experiments.

Water Content. The amount of water taken up by the seeds was determined as the percent increase in weight of imbibed seed with respect to the initial dry weight of the seed (Aliotta et al., 1994; Baskin et al., 1998). Prior to weighing, seeds were paper-blotted for 10 sec.

Respiration. Oxygen consumption in germinating seeds was measured with a Clark-type electrode (Hansatech Ltd., King's Lynn, UK). For each measurement, two seeds (approx. 150 mg fw each) were placed for approximately 20 min into a cuvette containing 1 ml air-saturated distilled water at $25 \pm 0.5^\circ\text{C}$.

Electrolyte Leakage. For each measurement, 2.0 g seeds (ca. 15 seeds) were bathed in 0, 100, or 1000 μM aerated coumarin solutions (20 ml) and incubated at 25°C for 10 min. The conductivity of the solution was then measured by a MPC227 conductimeter (Mettler-Toledo, Greifensee, Switzerland).

Assays of Metabolites and Enzymes. After 1, 3, 6, 12, 24, or 36 hr during imbibition, seeds were collected, immediately frozen with liquid N_2 , and kept at -80°C until used. Frozen seeds were ground in liquid N_2 , with mortar and pestle. The fine powder (ca. 1 g) was then homogenized with 4 vol. of ice-cold 0.1 M HEPES buffer (pH 7.50), made with 5 mM 2-mercaptoethanol, 10 mM MgCl_2 , 2 mM dithiothreitol, 2 mM Na_2 ethylene diamine tetraacetic acid, 0.1 mM phenylmethylsulfonyl fluoride, 1% (w/v) bovine serum albumin, and 1% (w/v) insoluble polyvinylpyrrolidone. To measure the ascorbate peroxidase (APX) activity, 1 mM ascorbic acid (AsA) was added to the grinding buffer

(Amako et al., 1994). When assaying the glutathione (GSH) pool, 0.1 N hydrochloric acid was used as the extraction medium (Schwanz et al., 1996). The supernatants, obtained after filtering the seed extracts through four layers of cheesecloth and centrifuging at $39,000 \times g$ for 30 min, were used as sources of analytes.

All tested metabolites and enzymes were assayed spectrophotometrically (Lambda 5 double-beam spectrophotometer; Perkin-Elmer, Norwalk, CT, USA), essentially as reported by Sanità di Toppi et al. (2005). Ascorbate peroxidase activity (EC 1.11.1.11) was monitored by following the oxidation of AsA at 290 nm in the presence of H_2O_2 as the cosubstrate. Superoxide dismutase activity (SOD, EC 1.15.1.1) was followed by the superoxide-dependent oxidation of hydroxylamine to nitrite, followed by the colorimetric detection of nitrite at 540 nm; by intercepting O_2^- , SOD is able to inhibit the final colorimetric reaction in a concentration-dependent manner. Catalase activity (CAT, EC 1.11.1.6) was determined by following the consumption of H_2O_2 at 240 nm. Dehydroascorbate reductase activity (DHAR, EC 1.8.5.1) was monitored at 265 nm as it was formed from dehydroascorbic acid (DHAsA). Monodehydroascorbate reductase activity (MDHAR, syn. ascorbate free radical reductase, EC 1.6.5.4) was determined by following the NADH consumed to reduce the monodehydroascorbate radical generated from AsA by the action of ascorbate oxidase. Ascorbate and DHAsA were assayed according to the method Wang et al. (1991), in which the AsA-mediated reduction of Fe^{3+} to Fe^{2+} is followed by the formation of a red chelate among Fe^{2+} and 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline), which absorbs at 534 nm. A standard curve covering the range 0–10 nmol AsA was used. Total and oxidized glutathione (glutathione disulfide, GSSG) were measured by following the 5,5'-dithiobis(2-nitrobenzoic acid)-GS-SG reductase recycling procedure proposed by Griffith (1985); GSSG was determined after removal of GSH from the plant extracts by derivatization with 2-vinylpyridine. Changes in absorbance of the reaction mixtures were measured at 412 nm and 25°C. GSH was determined by subtracting GSSG (as GSH equivalents) from the total glutathione content.

Glucokinase activity (GluK, EC 2.7.1.1) was determined by monitoring the rate of glucose-6-phosphate dehydrogenase-coupled reduction of $NADP^+$ (Espen et al., 1995). Malate dehydrogenase activity (MDH, EC 1.1.1.37) was determined by following the NADH oxidation by α -oxalacetate at 340 nm (Queiroz, 1969). Glucose-6-phosphate dehydrogenase activity (G6PDH, EC 1.1.1.49) was assayed by measuring the reduction of NADP to NADPH in the presence of glucose-6-phosphate (Deutsch, 1983). Pyruvate kinase activity (PyrK, EC 2.7.1.40) was determined by monitoring the rate of NADH oxidation at 340 nm (Pirovano et al., 1996). Both α -amylase (α -AMY, EC 3.2.1.1) and β -amylase (β -AMY, EC 3.2.1.2) activities were determined by monitoring the release of maltose units (assayed against a maltose standard at 546 nm) at 25°C, by using soluble starch as the substrate. α -AMY activity was measured at pH

7.0, and β -AMY at pH 4.8, a value that renders α -AMY inactive (Bergmeyer et al., 1983).

Total soluble protein was estimated according to the method of Bradford (1976) using bovine serum albumin as the standard. All of the above analyses were performed at 4°C.

Nucleic Acid Extraction and Analysis. The procedures used for total RNA extraction, its electrophoretic (25 μ g per sample) transfer to nylon membrane, prehybridization, hybridization and washing of the nylon filters, and Northern blotting were the same as employed by Ciaffi et al. (2000).

cDNA probes were labeled by incorporating digoxigenin-11-dUTP via polymerase chain reaction (PCR), following the method of Ciaffi et al. (1999). As DNA templates, the following sequences were used: *PDICDW-FI-RI* (AJ277379), a 1.695-kbp reverse transcription (RT)-PCR product containing the entire coding sequence of protein disulfide isomerase (*PDI*) gene obtained from ripening caryopses of *T. turgidum* ssp. *durum* cv. Langdon and cloned into pGEM[®]-T (Promega Italia, Linate, Italy; Ciaffi et al., 2000); a partial 1.0-kbp cDNA clone [in pBluescript[®] (SK-)] coding for the luminal binding protein (BiP), isolated from *Triticum aestivum* L. (Grimwade et al. 1996); an RT-PCR product isolated from *T. aestivum* by using a pair of primers designed based on the thioredoxin *h* (*TRX*) gene sequence reported by Gautier et al. (1998).

Experimental Replication and Statistics. Each reported value represents the mean of measurements carried out at least in triplicate and obtained from three independent experiments \pm SD. Two-way ANOVA was performed for germination (*ab initio* and *in itinere* experiments), water content, conductivity, O₂ uptake, and the antioxidant metabolites and enzyme activity parameters, to test the effects of coumarin concentration and time of imbibition. The data were checked for deviations from normality and homogeneity of variances prior to analysis. *A posteriori* comparisons were carried out using the Tukey test to check the significant differences of each parameter among coumarin concentrations within each time of imbibition.

One-way ANOVA and, then, *a posteriori* comparisons by Tukey's test were carried out to check the significant differences of germination parameters (dose-response curve) among the coumarin concentrations. Statistical analysis was conducted using the Systat v.8.0 software package (SPSS Inc., Evanston, IL, USA).

Nucleic acid extraction and analysis were carried out on seeds obtained from three independent experiments.

RESULTS

Seed Germination. Two hundred μ M coumarin was the concentration threshold beyond which coumarin inhibited seed germination (Figure 1a). A

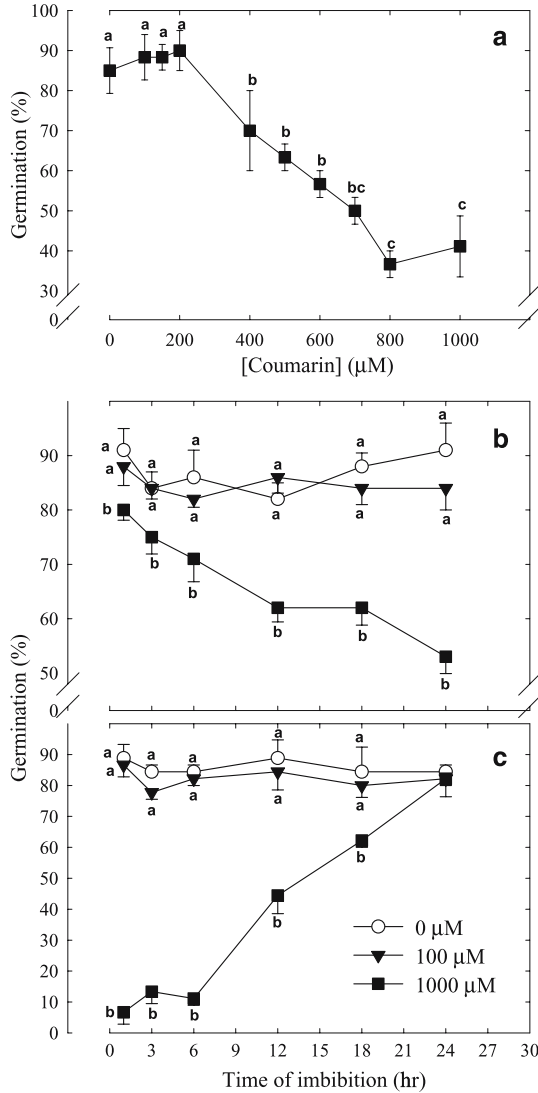


FIG. 1. Effects of coumarin on the germination of durum wheat cv. Simeto seeds. (a) Dose-response curve; (b) the *ab initio* experiment: seeds were exposed to coumarin for the indicated times and transferred into water until completion of germination (36 hr); (c) the *in itinere* experiment: imbibition started in water followed by exposure of the seeds to coumarin for the indicated times. Each value is the mean \pm SD ($N=9$). (a) Different letters indicate significant difference at $P < 0.05$ (Tukey's test). (b, c) Different letters within time of imbibition groups indicate significant difference among treatments ($P < 0.05$, Tukey's test). No letters within time of imbibition groups indicate no significant difference among the treatments ($P > 0.05$, Tukey's test).

maximum of more than 60% inhibition was reached with the highest coumarin concentration, i.e., 1000 μM . No macroscopic change in morphology was observed in coumarin-inhibited seeds (not shown).

Based on Figure 1a, 100 and 1000 μM were adopted in the subsequent experiments as representatives of the no-effect and of the inhibitory coumarin concentration ranges, respectively.

Exposing seeds to different coumarin levels was interrupted at variable periods, allowing them to recover in distilled water (36 hr; referred to as the *ab initio* experiment in [Methods and Materials](#)). A 1-hr exposure to 1000 μM coumarin was sufficient to cause a 20% inhibition of germination. Inhibition increased almost linearly with increasing exposure to the allelochemical, reaching 50% inhibition after 24 hr (Figure 1b).

In a complementary experiment, seeds were first placed into distilled water for variable periods, and then exposed to coumarin until the end of germination (36 hr; the *in itinere* experiment). Exposure to 1000 μM coumarin 1, 3, or 6 hr after the beginning of imbibition, but not to 100 μM at these same times, inhibited germination up to 90% with respect to control seeds (Figure 1c). However, inhibition was significantly decreased when seeds were exposed to 1000 μM coumarin either 12 or 18 hr after the beginning of imbibition, and completely disappeared when the treatment was administered after 24 hr (Figure 1c).

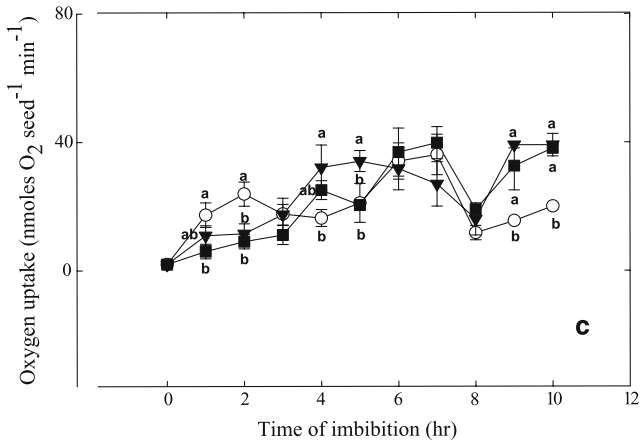
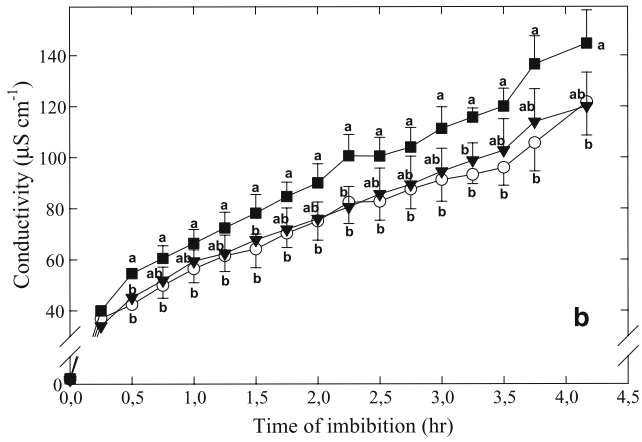
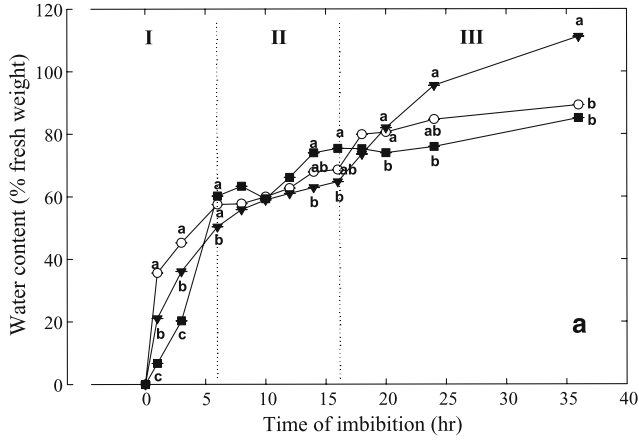
Early Events in Germination. After 1 hr exposure, either 100 or 1000 μM decreased the amount of water taken up by seeds by about 40 or 80%, respectively (Figure 2a). However, seeds recovered to the control level after 3–5 hr. Thereafter, coumarin had no remarkable effect on water (Figure 2a).

A thirty min exposure to 1000 μM coumarin, but not 100 μM , caused a 20% higher solute leakage with respect to the control, and this remained constant until the end of the experiment (Figure 2b).

Respiratory O_2 consumption showed a complex, apparently multiphasic, pattern both in control and in treated seeds (Figure 2c). Coumarin tended to abolish the burst of O_2 consumption during phase I, in a concentration-dependent manner. Both 100 and 1000 μM significantly potentiated the respiratory burst occurring during phase II.

Antioxidant Systems. During germination, AsA was often almost undetectable, although drastic but transient increases were observed after 3 or 12 hr of exposure to either 100 or 1000 μM , respectively (Figure 3). In contrast, the concentration of its oxidized form, DHAsA, was higher and appeared to drastically increase from 24 hr of imbibition onwards, but not in the presence of 1000 μM coumarin.

In the case of GSH, balance among the reduced and oxidized form (GSSG) was almost constantly in favor of the former during germination (Figure 3). However, at the beginning of imbibition, seeds contained almost exclusively GSSG, which increased after 1 hr of water uptake (phase I), regardless of the



presence of coumarin. Control and 1000- μM -treated seeds showed almost identical GSH and GSSG profiles, whereas 100 μM coumarin decreased the GSH level and increased the GSSG level (Figure 3).

In both treated and control seeds, APX activity was detectable only after 24 hr of imbibition (phase III; Figure 3). Enzyme activity dramatically increased thereafter, except in 1000- μM -treated seeds, where only 25% of control activity was present after 36 hr. In contrast, both DHAR and MDHAR activities were detectable, in both treated and control seeds, during the early stages of imbibition (Figure 3). Coumarin (1000 μM) inhibited MDHAR activity after 1 hr of imbibition and then again after 24 hr (about -70% compared to control), a 30% reduction compared with control seeds and being apparent at the end of germination.

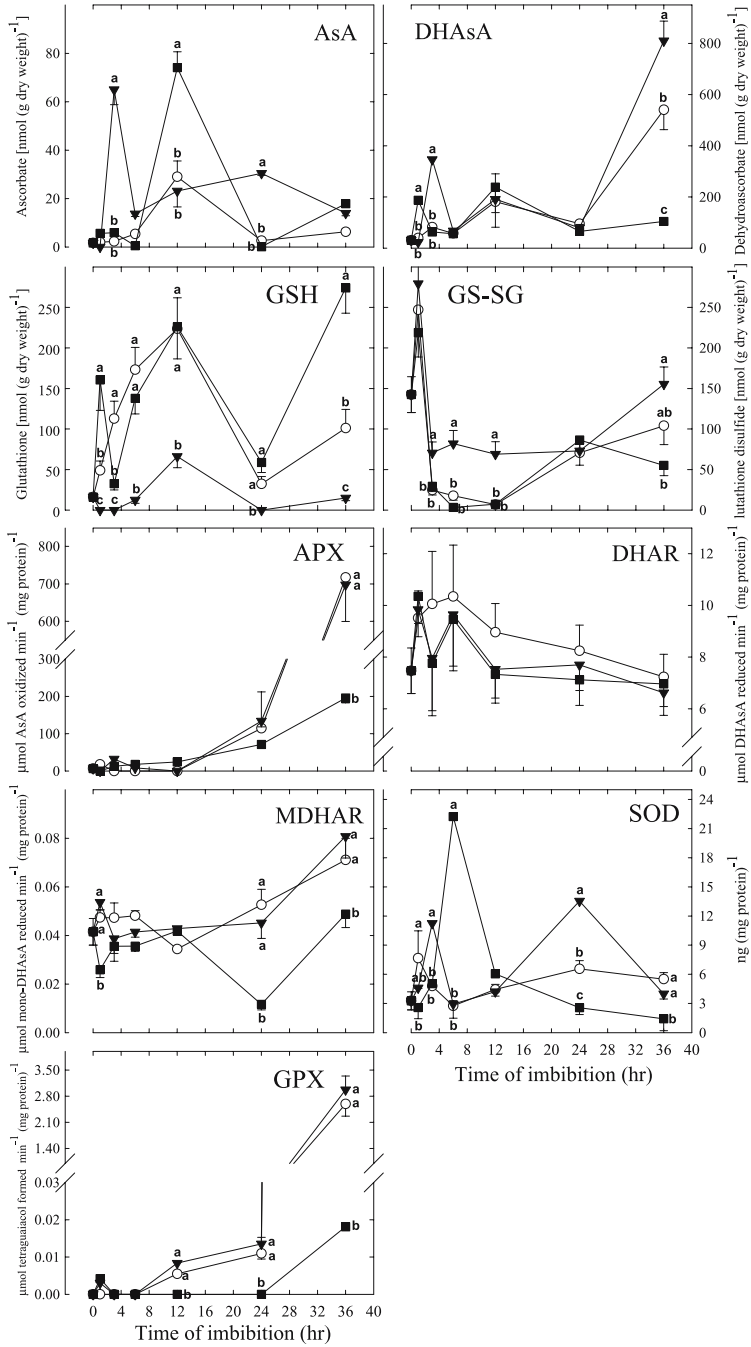
Like APX, GPX activity was also undetectable during the first 12–24 hr of imbibition, with its latency prolonged by the presence of 1000 μM coumarin. Again, like APX, GPX activity increased dramatically during the 12- to 36-hr period up to 400-fold. However, the late burst of activity was almost completely abolished in seeds exposed to 1000 μM coumarin (Figure 3).

Contrary to the peroxidases, SOD activity peaked during the early periods of imbibition (phase I; Figure 3). Coumarin delayed the early upsurge and increased its amplitude, in a concentration-dependent manner. A second peak in SOD activity occurred after 24 hr of imbibition (phase III), but not in the 1000- μM -treated seeds. No CAT activity was detected in germinating seeds.

Marker Enzymes for Metabolic Reactivation. Enzymatic markers for metabolic reactivation, such as α - and β -AMY (starch mobilization), GluK and PyrK (reactivation of glycolysis), G6PDH (oxidative pentose phosphate pathway, OPPP), and MDH (Krebs cycle) were all detectable from the start of germination (Figure 4).

From 12 hr onwards, 1000 μM coumarin caused a 20% decrease in α -AMY activity. This same coumarin level also prevented the gradual increase in GluK activity that occurred in control seeds during the first 12 hr of imbibition, and caused a 20–40% inhibition of enzyme activity through the end of the experimental period. Coumarin at 100 μM also had an inhibitory effect during the early stages of germination, but GluK tended to recover to the control level during phase III. No clear difference among the treatments was observed for

FIG. 2. Water uptake during germination of Simeto seeds in the presence of varying levels of coumarin (a). Solute leakage in germinating Simeto seeds exposed to different levels of coumarin (b). Oxygen consumption in germinating Simeto seeds exposed to different levels of coumarin (c). Symbols for coumarin levels as in Figure 1c. Roman numerals and dotted lines are intended to indicate different phases during seed germination. Each value is the mean \pm SD; $N = 3$. Statistics as in Figure 1b, c.



PyrK and MDH activities. G6PDH was initially depressed by the presence of 1000 μM coumarin, but from 3 hr of imbibition onwards there was no significant difference with respect to the control. Conversely, this same activity was stimulated by the presence of 100 μM coumarin during phase III.

Molecular Chaperones and Protein-Modifying Enzymes. The time courses for *BiP* and *PDI* transcript accumulation were similar (Figure 5). In both cases, a weak Northern hybridization signal appeared after 1 hr of imbibition (phase I) and tended to increase in intensity thereafter. A strong *BiP* and *PDI* hybridization signal appeared after 24 hr of imbibition in the presence of 0 and 100 μM coumarin, but not in 1000- μM -treated seeds. Compared to *BiP* and *PDI*, *TRX* transcripts were abundant from the early stages of imbibition, and did not increase thereafter (Figure 5). In contrast to *BiP* and *PDI*, 1000 μM coumarin enhanced the accumulation of the *TRX* transcripts from 6 hr of imbibition onwards.

DISCUSSION

Our results confirm that coumarin inhibits seed germination (Aliotta et al., 1992, 1994) and that a threshold concentration of about 200 μM is required for inhibition to occur in durum wheat seeds. Notwithstanding, 100 μM coumarin was employed here because it is more representative of the levels of cinnamic acid derivatives commonly found in soils (Macias, 1995).

Coumarin inhibited durum wheat germination in a concentration-dependent manner. Inhibition by coumarin was rapid, apparently irreversible, and cumulative, as it increased along with increasing time of exposure. Our results suggest that coumarin irreversibly blocks or prevents one or more key germination event(s) during phase I or early phase II. Weak or no inhibitory effects were observed when seeds were exposed to the allelochemical during phase III.

Aliotta et al. (1994) reported that inhibition of radish seed germination caused by 5-methoxypsoralen, which belongs to the coumarin family, was associated with an inhibition of water uptake. Such inhibition only became

FIG. 3. Antioxidant metabolites [ascorbate (AsA) and glutathione (GSH)], and their respective oxidized forms [dehydroascorbate (DHAsA) and glutathione disulfide (GS-SG)], and antioxidant enzymes [ascorbate peroxidase (APX), superoxide dismutase (SOD), and guaiacol peroxidase (GPX)] and antioxidant-regenerating enzymes [dehydro- (DHAR) and monodehydroascorbate reductase (MDHAR)], in germinating Simeto seeds exposed to different levels of coumarin. Symbols for coumarin levels as in Figure 1c. Each value is the mean \pm SD ($N = 3$). Statistics as in Figure 1b, c.

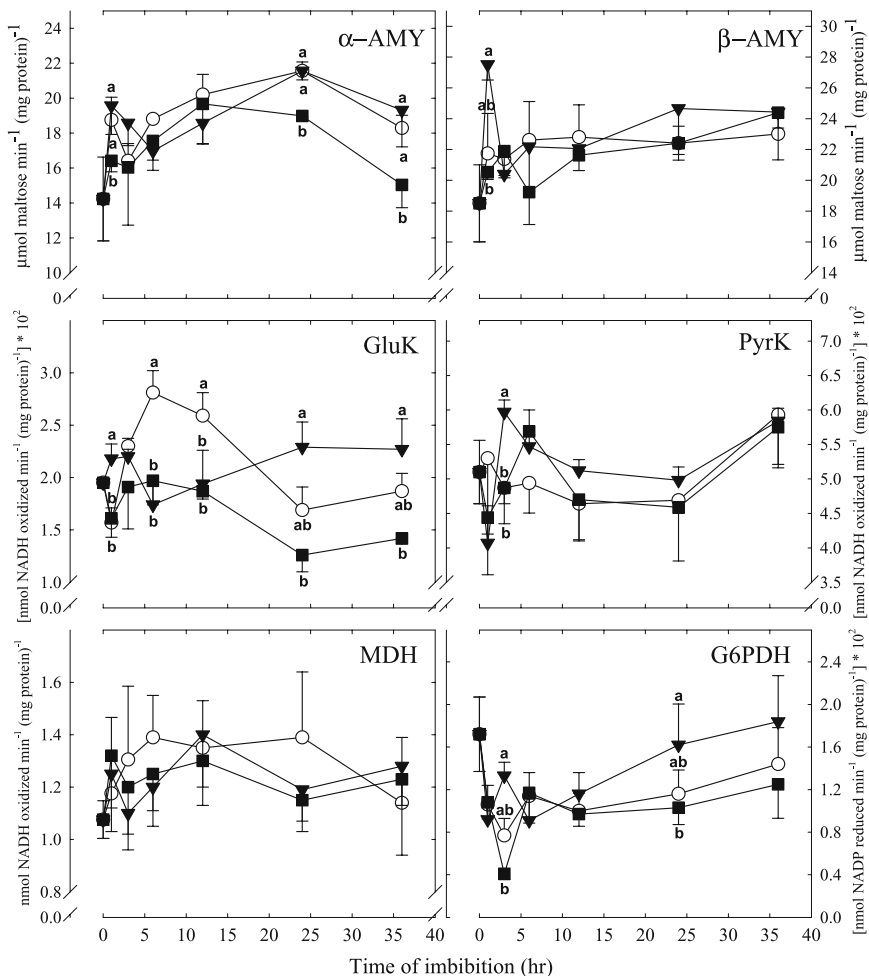


FIG. 4. Marker enzymes for metabolic reactivation during germination of Simeto seeds exposed to different levels of coumarin. α -AMY, α -amylase; β -AMY, β -amylase, respectively. GluK, glucokinase; PyrK, pyruvate kinase; MDH, malate dehydrogenase; G6PDH, glucose 6-P dehydrogenase. Symbols for coumarin levels as in Figure 1c. Each value is the mean \pm SD ($N = 3$). Statistics as in Figure 1b, c.

evident during phase II and was protracted during phase III. In the present work, coumarin reduced water uptake during phase I, but the decrease was recovered during phase II. This suggests that germination arrest occurred during phase I. Interestingly, both 100 and 1000 μM coumarin inhibited water uptake, but only

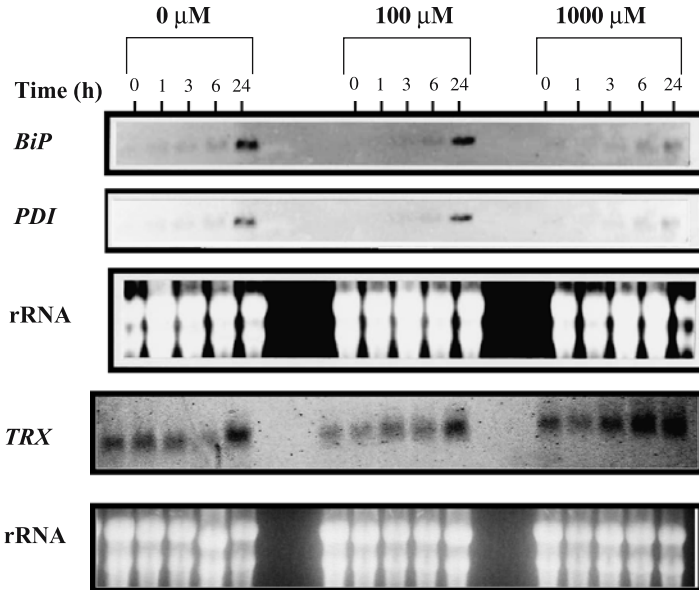


FIG. 5. Typical Northern blot hybridization profile of the luminal binding protein (BiP), protein disulfide isomerase (PDI), and thioredoxin *h* (TRX) mRNA transcripts accumulation during the germination of Simeto seeds exposed to different levels of coumarin. Each lane contained 25 μg total RNA. rRNA, ribosomal RNA.

the latter inhibited seed germination. Only the lesser coumarin concentration allowed reaching that minimal rehydration threshold during phase I that must be achieved for the germination program to be successfully executed.

Water influx into the cells of dry seeds provokes a transient perturbation to membrane structure, which causes an immediate and rapid leakage of solutes into the surrounding medium. After a short time of rehydration, stable configuration is restored and solute leakage stops (Bewley, 1997). Here, as a consequence of early inhibition of water uptake, 1000 μM coumarin could delay, or even prevent, the recovery of a stable membrane configuration. Indeed, phenolic compounds can induce rapid depolarization of plant membranes (Glass and Dunlop, 1974) as well as peroxidation of membrane lipids (Baziramakenga et al., 1995), both of which lead to an increased efflux of solutes.

Interference with membrane functions, and/or delay in taking up an adequate amount of water (Bove et al., 2001), might also explain the early inhibition of O_2 consumption induced by 1000 μM coumarin during phase I. Indeed, Moreland and Novitzky (1987) suggested that coumarins, being able to perturb the membrane systems that sustain electron transport, can inhibit both respiration and photosynthesis.

Water uptake, membrane perturbation, and resumption of respiration may all promote the generation of reactive oxygen species (ROS), including superoxide radical anion, hydroxyl radical, and H_2O_2 , during seed germination (De Gara et al., 1997). However, ROS scavenging may be problematic during germination because, in dry seeds, SOD, which converts superoxide into H_2O_2 , is present with modest activity. Likewise, APX, which removes toxic levels of H_2O_2 , is almost absent during the early stages of germination (De Gara et al., 1997; Figure 3). Therefore, a primary antioxidant strategy during the early stages might be reduction of DHAsA to AsA by using GSH as the electron donor, which is catalyzed by DHAR. Indeed, our results indicate that during the early stages of germination, seeds are substantially devoid of AsA, APX, and CAT activities, but contain DHAsA and show DHAR activity. Coumarin at 1000 μM inhibited DHAR and MDHAR activities, and decreased DHAsA levels, but this occurred only during phase III, i.e., after the arrest of germination had already occurred. Late effects of coumarin were also apparent as far as the glutathione pool and redox ratio is concerned.

Delayed resumption of APX activity could leave the 1000- μM -treated seeds without an adequate ROS scavenging capacity during germination, things being made worse by the absence of a measurable CAT activity. De Gara et al. (1991) reported that a decrease in APX activity may be related to a loss of seed germination capacity in *Dasypyrum villosum* (L.) Borb. Again, however, the coumarin-dependent inhibition of APX reactivation, albeit drastic, became apparent in a late stage of the germination process, so that it probably cannot be regarded as the primary target of coumarin action.

In contrast, the drastic and transient increase in SOD activity induced by 1000 μM coumarin was much closer in time to phase I, during which coumarin-induced arrest of germination was supposed to occur. This transitory stimulation of SOD activity might indirectly indicate a need to face an early burst of superoxide production. However, neither superoxide nor H_2O_2 , the product of SOD catalysis, were measured in this study.

Instead, 1000 μM coumarin prevented the activation of the other peroxidase, GPX, well in advance of phase III. This suggests a rather direct link, on a time basis, with the unknown early event targeted by coumarin. IAA at supraoptimal concentrations may inhibit germination of apple seeds and thus participate in the maintenance of dormancy (Nikolaeva et al., 1987). We might speculate that the 1000- μM -treated seeds, being deficient in GPX, were also lacking in IAA oxidase activity (Foyer et al., 1997). The resulting excess of IAA could then contribute to the blocking of germination. If, instead, a major role of GPX during seed germination is protection from oxidative damage (Stacy et al., 1996), then lack of GPX activation might worsen the consequences of APX inhibition (see above), thus further weakening the overall ROS scavenging capacity of the 1000- μM -treated seeds. According to Bewley

(1997), within a few hours from the beginning of imbibition, glycolysis, OPPP, and the Krebs cycle are activated and seeds achieve their full metabolic status. Since a limited number of marker enzymes was considered here, and coumarin's effects on their *in vivo* activity could not be evaluated, considering the results obtained with 1000 μM coumarin, no obvious failure in the reactivation of the main ATP- and NAD(P)H-producing pathways is apparent.

In germinating cereal seeds, α -AMY activity is rapidly induced through *de novo* synthesis and secretion of the enzyme by the aleurone layer. In contrast, β -AMY is constitutive, deposited during seed development in a zymogenic form, which is converted into the active enzyme during germination (Kruger, 1979). Khan (1969) reported that 340 μM coumarin completely inhibited α -AMY synthesis in barley seeds. Accordingly, here, 1000 μM coumarin inhibited α -AMY activity, but such effect only became evident in an advanced stage of germination.

One function of the endoplasmic reticulum (ER) is to synthesize a large group of vacuolar or secreted proteins, such as proteases, hydrolases, and amylases. Among the intrinsic components of the ER, central roles in the correct folding, and oligomerization, and formation of disulfide bridges of nascent secretory proteins are assigned, respectively, to chaperonine-like enzymes, such as BiP, and to protein-modifying enzymes, such as PDI. The former is likely to bind transiently to all nascent protein chains and to associate permanently to malfolded proteins; the latter is a member of the thioredoxin superfamily, responsible for the introduction and isomerization of disulfide bonds that are often present in secreted- and cell surface proteins (Ciaffi et al., 2000). Coumarin (1000 μM) had opposite effects on the transcript abundances of ER-resident- (BiP and PDI) and of cytosolic molecular chaperons (TRX h in the present study). Livesley et al. (1992) reported that the germination rate of bread wheat (*T. aestivum* L.) seed populations was correlated with the PDI activity of microsome-enriched fractions extracted from the aleurone layer. Our results indirectly support these findings and suggest that one coumarin target could be the transcriptional activation of genes involved in the assemblage and stabilization of secretory proteins, which could decrease α -AMY synthesis and secretion (Figure 4), eventually affecting storage mobilization and metabolic reactivation during seed germination. On the other hand, early increase in the TRX transcript abundance suggests that exposure to 1000 μM coumarin causes oxidative perturbation in the cytosol, requiring the activation of compartment-specific mechanisms of redox homeostasis.

In summary, beyond a concentration threshold of about 200 μM , coumarin was able to rapidly and significantly inhibit the germination of durum wheat seed during the early stages of imbibition. This inhibitory effect produced a series of changes that were either transitory (e.g., the inhibition of seed water uptake), too late to be directly traceable to the early arrest during the ger-

mination process, or whose extent was much lower than proportional to the severity of the inhibition observed. As a result, we cannot precisely identify the primary structure or function targeted by coumarin. Neither can we assess whether one or more critical events were affected.

We are prompted to speculate that, in Simeto seeds exposed to 1000 μM coumarin, a less-than-sufficient rehydration in the very early stage of imbibition may prevent or delay the attainment of a stable configuration of membrane systems. This could lead to a prolonged loss of osmotically active substances and substrates, and delay resumption of respiration. A reduced utilization of O_2 as the terminal electron acceptor may lead to an increase in mitochondrial $p\text{O}_2$, and result in an electron overflow. Both of these conditions are conceivably conducive to increased ROS generation. This may explain the observed drastic increase in SOD activity during phases I–II, with compensatory purposes, which could in turn result in a burst of H_2O_2 . H_2O_2 overproduction could escape the control operated by the endogenous scavenging systems, since both GPX, during phase II, and APX, later on, failed to reactivate in the 1000- μM -treated seeds. This may eventually cause oxidative stress, which explain the enhanced transcription, already occurring during phase II, of genes involved in redox homeostasis, such as *TRX*. Since redox regulation is of critical importance for most, if not all, processes in the aerobic cell, including signal perception and transduction, cell-to-cell communication, and the cell cycle itself (Foyer et al., 1997), an altered redox balance caused by exposure to coumarin may ultimately hamper, or even block, the execution of the germination program.

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INHIBITION OF CYP6B1-MEDIATED DETOXIFICATION OF XANTHOTOXIN BY PLANT ALLELOCHEMICALS IN THE BLACK SWALLOWTAIL (*Papilio polyxenes*)

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Abstract—The structural and biosynthetic diversity of allelochemicals in plants is thought to arise from selection for additive toxicity as a consequence of toxin mixture or for enhanced toxicity as a result of synergism. In order to understand how insects cope with this type of plant defense, we tested the effects of some allelochemicals in host plants of the black swallowtail *Papilio polyxenes* on the xanthotoxin-metabolic activity of CYP6B1, the principal enzyme responsible for the detoxification of furanocoumarins in this caterpillar. Additionally, the effects of some synthetic compounds not normally encountered by *P. polyxenes* on CYP6B1 were tested. These studies demonstrate that the integrity of furanocoumarin structure is important for competitive binding to the active site of CYP6B1, even though the carbonyl group on the pyranone ring apparently does not affect its inhibitory capacity, as in the case of furanochromones. Angular furanocoumarins are generally less phototoxic to many organisms than linear furanocoumarins due to their reduced capacity for cross-linking DNA strands, yet they are more toxic than linear furanocoumarins to black swallowtail larvae. This enhanced toxicity *in vivo* may be due to the ability of angular furanocoumarins to bind to the active site of CYP6B1 without being rapidly metabolized. This binding reduces the availability of CYP6B1 to metabolize other linear furanocoumarins. The structure-activity relationships for methylenedioxyphenyl compounds, flavonoids, imidazole, and imidazole derivatives are also discussed in light of their capacity to inhibit the xanthotoxin-metabolic activity of CYP6B1.

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Key Words—Cytochrome P450 monooxygenases (P450s), enzyme inhibition, furanocoumarins, furanochromones, methylenedioxyphenyl compounds, flavonoids, plant-insect interactions.

INTRODUCTION

Virtually all plant species produce a diversity of secondary plant compounds or allelochemicals for defense against herbivores. Among the compounds that are toxic to a broad range of organisms are furanocoumarins, which are found in plants from several families including Apiaceae and Rutaceae (Berenbaum, 1981, 1991). These compounds are toxic owing to their ability to interfere with the processes of DNA replication and transcription by forming covalent cross-links with DNA helices in the presence of long-wavelength UV light and/or to inactivate proteins by irreversible binding (Berenbaum, 1991). Despite this toxicity, some specialist insects, e.g., the black swallowtail (*Papilio polyxenes*) and the parsnip webworm (*Depressaria pastinacella*), are able to feed exclusively on host plants containing furanocoumarins. They rapidly and efficiently detoxify furanocoumarins encountered in their diet via cytochrome-P450-mediated detoxification systems (Ivie et al., 1987; Nitao, 1989; Schuler, 1996; Berenbaum, 2002; Nitao et al., 2003). Metabolic transformation of the furan ring is mediated by P450s in both mammals (Koenigs and Trager, 1998a) and specialist insects (Ivie et al., 1986, 1987; Baudry et al., 2003; Nitao et al., 2003). In mammals, the epoxidation and subsequent opening of the furan ring lead to irreversible inactivation of some P450s due to the formation of stable P450 metabolite complexes through covalent bonding, a process called suicide inactivation or mechanism-based inhibition (Koenigs and Trager, 1998a). The epoxidation and subsequent opening of the furan ring lead to the detoxification and excretion of furanocoumarin metabolites in both the black swallowtail (Bull et al., 1986) and the parsnip webworm (Nitao et al., 2003). Cloning and expression of P450s from the black swallowtail have identified CYP6B1 as the principal enzyme responsible for the detoxification of furanocoumarins (Cohen et al., 1992; Ma et al., 1994; Chen et al., 2002; Baudry et al., 2003; Wen et al., 2003). CYP6B3 was identified in this butterfly species as a related enzyme with an overall lower capacity for furanocoumarin metabolism (Hung et al., 1995a; Wen et al., unpublished).

In addition to furanocoumarins, many other allelochemicals occur in the host plants of *P. polyxenes*. Among these are methylenedioxyphenyl (MDP) compounds including myristicin and safrole, simple coumarins, furanochromones including visnagin and khellin, and flavonoid compounds including flavone (Berenbaum, 1985, 1990; Berenbaum and Neal, 1987; <http://www.arsgrin.gov/duke/index.html>). Some of these chemicals are known to modulate

P450 activities by acting either as P450 inducers or inhibitors (Cohen et al., 1989; Prapaipong et al., 1994; Hung et al., 1995a,b; Ono et al., 1996; Scott, 1996; Koenigs et al., 1997; Koenigs and Trager 1998a,b; Guo et al., 2000; Scott et al., 2000; Ho et al., 2001; Petersen et al., 2001; Tantcheva-Poor et al., 2001; Zhang et al., 2001; Baudry et al., 2003). In the same way that plant allelochemicals consumed in the human diet alter the bioavailability and the efficacy of certain drugs by modulating drug-metabolizing P450 activities (McKinnon and Evans, 2000; Zhou et al., 2004), modulation of toxin-metabolizing P450s in herbivorous insects by chemicals coexisting with host plant toxin(s) have potential for affecting their ability to detoxify plant allelochemicals and, consequently, to utilize certain plants. Some allelochemicals in complex mixtures may not be toxic themselves, but they may enhance the toxicity of co-occurring toxicants by inhibiting key enzymes (e.g., P450s) that detoxify them. While certain toxic chemicals can significantly alter host plant utilization patterns by particular groups of insects, suites of allelochemicals via additive toxicity or synergism can provide more effective defense against a range of herbivorous insects (Berenbaum, 1985; Berenbaum and Neal, 1987).

In *P. polyxenes*, Neal and Berenbaum (1989) demonstrated that both myristicin and safrole inhibit ρ -nitrophenol and ρ -nitroanisole O-demethylation activities in *P. polyxenes* to an extent that is much lower than their ability to inhibit these same activities in the caterpillar *Helicoverpa zea*, a polyphagous species that rarely utilizes furanocoumarin-containing host plants. Zumwalt and Neal (1993) further demonstrated that xanthotoxin inhibits ρ NA O-demethylation activity in *P. polyxenes* to a lower extent than in *Manduca sexta*, a sphingid specialist on Solanaceae that never encounters furanocoumarins. The reverse is true, however, for xanthotoxin inhibition of aldrin epoxidation activity in these two species. Additionally, xanthotoxin inhibition of aldrin epoxidation activity in both species and ρ NA O-demethylation activity in *M. sexta* is irreversible, whereas its inhibition of ρ NA O-demethylation activity in *P. polyxenes* is reversible. Given the complexities of P450 systems due to isoform multiplicity and often broad yet overlapping substrate specificity, any given midgut P450 activity of *P. polyxenes* against synthetic substrates may represent the combined activities of multiple individual P450s. The inhibitory profiles for such activities using synthetic substrates may not be the same as the inhibitory profiles for P450 activities that have evolved in a particular ecological and phytochemical context. Examination of inhibitory profiles for individual toxin-metabolizing P450s by allelochemicals that coexist with the major furanocoumarin toxins is more relevant for understanding the complexity of plant-insect interactions.

The identification of CYP6B1 as the principal furanocoumarin-metabolizing enzyme in *P. polyxenes*, and the successful establishment of a heterologous

CYP6B1-house fly P450 reductase coexpression system have allowed us to directly evaluate not only the binding of host plant-specific allelochemicals and synthetic compounds with CYP6B1 but also the effects of these chemicals on CYP6B1 metabolism of xanthotoxin, the major toxin in the host plants of *P. polyxenes* (Baudry et al., 2003; Wen et al., 2003). These studies provide insight into the mechanisms underlying the evolution of phytochemical diversity in plants by demonstrating that compounds with structures different from that of the furanocoumarin nucleus can significantly block CYP6B1 metabolism of xanthotoxin.

METHODS AND MATERIALS

Chemicals. Xanthotoxin (8-methoxypsoralen), bergapten (5-methoxypsoralen), psoralen, and angelicin were purchased from Indofine Chemical Co. (Belle Mead, NJ, USA). Coumarin, myristicin, cimetidine, imidazole, ketoconazole, β -NADPH, heat-inactivated fetal bovine serum (FBS), and hemin were obtained from Sigma (St. Louis, MO, USA). Visnagin, khellin, flavone, flavanone, α -naphthoflavone, piperonyl butoxide (PBO), safrole, isosafrole, and 2,3-benzofuran were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Molecular biology reagents, Sf9 insect cells, SF-900 serum-free medium, pFastBac1 expression vector, and DH₁₀BAC competent cells were purchased from GibcoBRL/Life Technology (Grand Island, NY, USA). Solvents for HPLC were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Expression of CYP6B1 Protein. CYP6B1 was coexpressed with house fly cytochrome P450 reductase at a multiplicity of infection (MOI, pfu/cell) ratio of 2:0.05 or 2:2 in a baculovirus-Sf9 cell system as described in Wen et al. (2003) and Pan et al. (2004). Briefly, Sf9 cells infected with CYP6B1 and house fly P450 reductase virus were harvested 72 hr post infection by centrifugation at $3000 \times g$ for 10 min, washed once with one-half cell culture volume of 100 mM cold sodium phosphate buffer (pH 7.8) and once with one-tenth cell culture volume of cold cell lysate buffer [100 mM sodium phosphate (pH 7.8), 1.1 mM EDTA, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μ g/ml (w/v) leupeptin, 20% glycerol]. The cells were resuspended in one-tenth cell culture volume of cold cell lysate buffer, sonicated twice for 30 sec with an interval of 1 min in 5-ml batches on ice, vortexed for 15 sec, and centrifuged at $3000 \times g$ for 10 min. These low-speed cell lysates cleared of cell debris were analyzed directly or frozen in liquid nitrogen and stored at -80°C . P450 content was determined as described previously (Wen et al., 2003).

Assays for CYP6B1 Inactivation by Xanthotoxin. The effects of xanthotoxin on the metabolic activity of CYP6B1 against xanthotoxin were assayed in two-phase reactions using CYP6B1 coexpressed with house fly P450 reductase

at an MOI ratio of 2:2. In the first phase, three sets (A, B, C) of 500- μ l reactions containing 0.1 μ M CYP6B1 in cell lysate buffer were set up in duplicate: Set A reactions, which served as control, contained 3 μ l methanol; set B reactions, which examined the effects of NADP⁺ on CYP6B1 activity, contained 0.3 mM NADP⁺ and 3 μ l methanol; and set C reactions contained 0.3 mM β -NADPH and varying concentrations of xanthotoxin (0.1, 1, 10, and 25 μ M) delivered in 3 μ l methanol. The first-phase reactions were incubated for 30 min at 30°C in a shaking water bath starting with the addition of NADPH to set C reactions. Under these conditions, xanthotoxin in all of the set C reactions disappeared at the end of the first-phase reaction as analyzed by HPLC. At the end of this reaction, 100 μ l of a solution containing xanthotoxin (3 μ l 20 mM stock in methanol) and NADPH (60 μ l 3 mM stock) in cell lysate buffer were added to all reactions (sets A, B, and C), bringing the total volume of all second-phase reactions to 600 μ l, with a final xanthotoxin concentration of 100 μ M and NADPH concentration of 0.3 mM or higher (if NADPH was not completely consumed in set C reactions in the first phase). The second-phase reactions were incubated at 30°C for 20 min and terminated by adding 150 μ l 2 N HCl. Psoralen, which served as an internal standard for extraction efficiency, was added to all reactions at the same xanthotoxin concentration. Unmetabolized xanthotoxin was extracted with 2 ml ethyl acetate, and 10 μ l of the extract were directly analyzed on a normal-phase HPLC system using an isocratic solvent containing 80% cyclohexane/18% ethyl ether/2% 1-butanol. Experiments run in duplicate were independently replicated at least three times. CYP6B1 activity in the second-phase reactions was expressed as xanthotoxin turnover. Xanthotoxin turnover by CYP6B1 in set A reactions that were devoid of both xanthotoxin and NADP⁺ in the first-phase reactions was set as 100% CYP6B1 activity. Comparison of CYP6B1 activity in sets B (containing NADP⁺ in the first phase) and C (containing both NADPH and xanthotoxin in the first phase) reactions with that of set A (control) allowed the evaluation of the effects of NADP⁺ and xanthotoxin on CYP6B1 metabolism of xanthotoxin.

Assays for CYP6B1 Inhibition by Other Allelochemical and Xenobiotics. Chemicals were tested for their effect on xanthotoxin metabolism by CYP6B1 using a series of concentrations ranging from 0 to 3 mM, which varied depending on the compounds tested, in 500 μ l reactions. For each, chemicals (imidazole delivered in 15 μ l cell lysate buffer and all other chemicals in 15 μ l methanol) were preincubated with cell lysates containing 0.05 nmol CYP6B1 coexpressed with housefly P450 reductase (MOI ratio of 2:0.05) in a volume of 350 μ l for 5 min at 30°C in a shaking water bath. At the end of this time period, 100 μ l of a xanthotoxin solution containing 2.5 μ l of 5 mM xanthotoxin stock (final concentration 25 μ M) in methanol and 97.5 μ l cell lysate buffer were added to each incubation, and the reaction was incubated for an additional 20 min at 30°C in a shaking water bath after addition of 50 μ l 3 mM NADPH (final

concentration of 0.3 mM) to initiate the reactions. Following this incubation, the reaction was stopped by adding 125 μ l 2 N HCl, and psoralen at the same concentration as xanthotoxin was added as an internal standard. Unmetabolized xanthotoxin was extracted with 500 μ l ethyl acetate, and 10 μ l of this extract were directly analyzed on a normal-phase HPLC system as described above. Control reactions were either stopped at time zero or contained no NADPH. Xanthotoxin metabolic activity of CYP6B1 incubated with varied concentrations of each test chemical was expressed as percentage of that obtained for CYP6B1 incubated without inhibitors. IC₅₀ values (the concentration of a test chemical that inhibits 50% of CYP6B1 metabolic activity) were obtained using the nonlinear regression program of GraphPad software (San Diego, CA, USA).

Spectral Binding Analysis. Spectral binding analyses were performed using split cuvette and double beam mode on a CARY 100 spectrophotometer as described by Wen et al. (2003). Briefly, 1 ml cell lysate buffer was added to half of each reference and sample cuvette, and 1 ml of cell lysate containing 50 nM CYP6B1 coexpressed with house fly P450 reductase (MOI ratio of 2:0.05) was added to the other half of each reference and sample cuvette. Following a baseline correction scan from 350 to 500 nm, test compounds with stock concentrations ranging from 5 to 200 mM were added sequentially in small volumes (0.2–2 μ l) to the cell lysate buffer in the reference cuvette and to the P450-containing cell lysate in the sample cuvette. Spectra were recorded 30 sec after each addition. Given the fact that some CYP6B1 substrates elicit strong, while others elicit much weaker binding spectra as exemplified in the type I binding of angelicin to CYP6B1 (Wen et al., 2003), we used the minimum concentration that elicits a type I or type II binding spectrum as a parameter to measure each compound's relative ability to bind to the heme in the CYP6B1 catalytic site.

RESULTS

Xanthotoxin is a well-known mechanism-based inhibitor for many P450s. To determine if xanthotoxin similarly inhibits the CYP6B1 protein, we set up a two-phase reaction system assaying CYP6B1 activity after preincubating it in the first phase with different concentrations of xanthotoxin in the presence of NADPH. If xanthotoxin is a mechanism-based inhibitor of CYP6B1, xanthotoxin metabolism in the second phase should be dependent on the concentration of xanthotoxin in the first-phase preincubated reaction containing NADPH. Results of these assays (Figure 1) indicate that the remaining CYP6B1 activities toward xanthotoxin in the second phase were nearly the same for reactions with

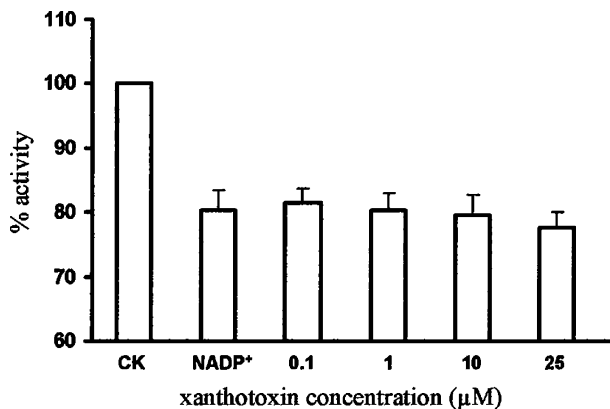


FIG. 1. Effects of xanthotoxin preincubation in the presence of NADPH on CYP6B1 activity toward xanthotoxin. The metabolic activity of CYP6B1 against xanthotoxin was analyzed in a two-phase reaction. Following the first-phase preincubation of 50 pmol CYP6B1 with various concentrations of xanthotoxin in the presence of NADPH (bars designated 0.1, 1, 10, 25), metabolism of xanthotoxin by CYP6B1 was compared with that of CYP6B1 preincubated devoid of xanthotoxin and NADPH in the first phase (bar designated CK) and that of CYP6B1 preincubated in the presence of only NADP⁺ in the first phase (bar designated NADP⁺). Bars show mean % activity \pm SE ($N = 3$).

varying concentrations of xanthotoxin (0.1–25 μM) preincubated in the presence of NADPH in the first phase and for reactions preincubated in the presence of NADP⁺ but devoid of xanthotoxin in the first phase. The decrease in the activities of CYP6B1 preincubated with different concentrations of xanthotoxin in the presence of NADPH in the first-phase reactions is apparently due to the feedback inhibition by NADP⁺, the product of NADPH, but not to the suicide inactivation of CYP6B1 by xanthotoxin.

To define the extent to which CYP6B1 metabolism of furanocoumarins is affected by allelochemicals co-occurring with furanocoumarins in the host plants of *P. polyxenes* and by synthetic chemicals, each chemical was tested at a series of concentrations (0 to 3 mM depending on the compound under analysis) for its effect on CYP6B1 metabolism of xanthotoxin. In these assays (Figure 2), CYP6B1 activity toward xanthotoxin in the presence of a test chemical, expressed as percentage of CYP6B1 activity toward xanthotoxin in the absence of inhibitors, is plotted as a function of the log concentration of that compound. IC₅₀ values, the concentration of a test chemical at which 50% of CYP6B1 activity is inhibited, were obtained using nonlinear regression (curve fitting) of GraphPad Prism software.

It is evident from this analysis that furanocoumarins inhibit xanthotoxin metabolism by CYP6B1, with linear furanocoumarins being slightly more

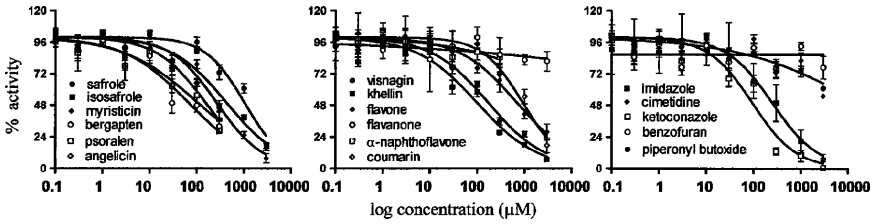


FIG. 2. Xanthotoxin metabolic activity of CYP6B1 in the presence of plant allelochemicals and synthetic compounds compared to that of CYP6B1 in the absence of test chemicals (defined as 100% activity). IC₅₀ (µM), the concentration of a test chemical that inhibits 50% of the CYP6B1 metabolic activity, was obtained using the nonlinear regression program of GraphPad software. Mean % activity ± SE are given. N = 3 for each component tested.

inhibitory than angular furanocoumarins. The order of IC₅₀ values in this study was 66.8, 105, and 154 µM for bergapten, psoralen (linear), and angelicin (angular), respectively (Figure 3). All of these furanocoumarins elicited typical type I binding spectra with an absorbance trough at 420 nm and a peak at 390 nm. Type I binding reflects the ability of substrates/inhibitors to displace water as the sixth ligand to the P450 heme iron (Jefcoate, 1978). The minimum concen-

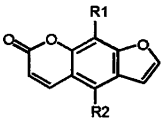
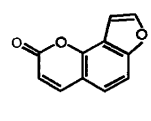
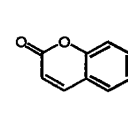
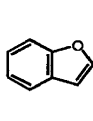
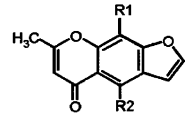
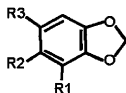
		furanocoumarins		Furanocoumarin analogs							
											
		linear		angular (angelicin)		coumarin		benzofuran		furanochromones	
chemicals	structure	R1 R2		IC50 (95% CI) (µM)	spectral binding						
		type	min con (µM)								
furanocoumarins		xanthotoxin		OCH ₃	H		I	1.0-2.5			
linear	bergapten	H	OCH ₃	66.8 (37.1 - 120)	I	1.0-2.5					
	psoralen	H	H	105 (41.4 - 267)	I	5-10					
angular	angelicin			154 (110 - 216)	I	5-10					
furanocoumarin analogs		coumarin				925 (626-1361)	I	100			
	benzofuran					>3000	ND				
furanochromones	khellin	OCH ₃	OCH ₃	100 (70-142)	ND						
	visnagin	H	OCH ₃	172 (129-231)	ND						

FIG. 3. Effects of furanocoumarins and analogs on xanthotoxin-metabolic activity of CYP6B1. IC₅₀ was expressed in micromoles per liter with the 95% confidence interval shown in parentheses. Min con, minimum concentration to elicit type I binding spectrum; ND, no type I binding detected. N = 3.

methylenedioxyphenyl compounds

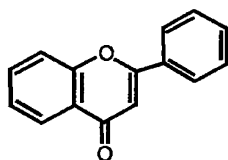


chemicals	structure			IC ₅₀ (95% CI) (μ M)	spectral binding	
	R1	R2	R3		type	min con (μ M)
safrole	H	CH ₂ CH=CH ₂	H	1030 (732-1449)	ND	
isosafrole	H	CH=CHCH ₃	H	428 (290-632)	ND	
myristicin	OCH ₃	H	CH ₂ CH=CH ₂	265 (188-374)	I	40 - 100
piperonyl butoxide	H	CH ₂ CH ₂ CH ₃	CH ₂ O(CH ₂ CH ₂ O) ₂ (CH ₂) ₃ CH ₃	>3000	ND	

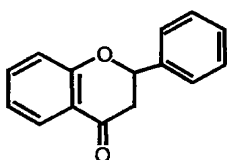
FIG. 4. Effects of methylenedioxyphenyl (MDP) compounds on xanthotoxin-metabolic activity of CYP6B1. IC₅₀ was expressed in micromoles per liter with the 95% confidence interval in parentheses. Min con, minimum concentration to elicit type I binding spectrum; ND, no type I binding detected. $N = 3$.

trations needed for detection of a type I spectrum are 1.0–2.5 μ M for bergapten and 5–10 μ M for psoralen and angelicin. In contrast, coumarin, which lacks a furan ring, had an IC₅₀ value of 925 μ M and a minimum concentration of 100 μ M to elicit a type I binding spectrum. Benzofuran, which lacks a pyran ring, had an IC₅₀ value of >3000 μ M and did not elicit a type I binding spectrum. Khellin and visnagin, furanochromones that differ from linear furanocoumarins

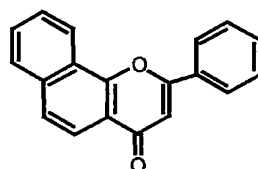
flavonoids



flavone



flavanone

 α -naphthoflavone

chemicals	IC ₅₀ (95% CI) (μ M)	spectral binding	
		type	min con (μ M)
flavone	698 (402-1213)	I	20-40
flavanone	>3000	ND	
α -naphthoflavone	>3000	ND	

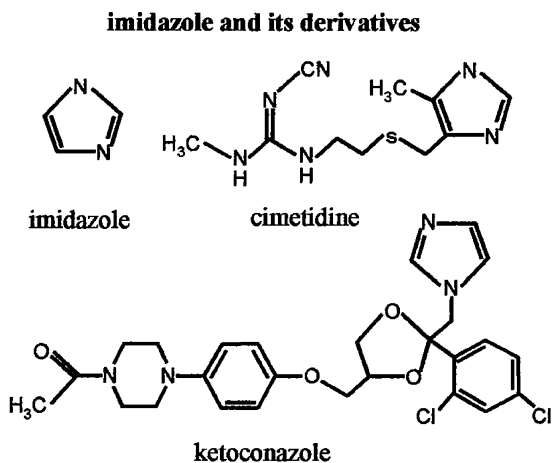
FIG. 5. Effects of flavonoids on xanthotoxin-metabolic activity of CYP6B1. IC₅₀ was expressed in micromoles per liter with the 95% confidence interval in parentheses. Min con, minimum concentration to elicit type I binding spectrum; ND, no type I binding detected. $N = 3$.

mainly in the position of the carbonyl group on the pyran ring, had IC_{50} values of 100 and 172 μM , respectively, and did not elicit any type I binding spectra.

Among the MDP compounds tested, the potency of compounds inhibiting CYP6B1 metabolism of xanthotoxin varied greatly, with IC_{50} values ranging from 265 μM for myristicin to >3000 μM for PBO (Figure 4). Isosafrole, with an IC_{50} value of 428 μM , was twofold more potent than its structural isomer safrole, which had an IC_{50} value of 1030 μM . Among these compounds, only myristicin, the most potent CYP6B1 inhibitor, elicited a typical type I binding spectrum at concentrations higher than 40–100 μM .

Among the flavonoid compounds, flavone did not strongly inhibit CYP6B1 metabolism of xanthotoxin (IC_{50} value of 698 μM) although it elicited a type I binding spectrum at concentrations higher than 20–40 μM (Figure 5). Both flavanone and α -naphthoflavone had IC_{50} values >3000 μM , and neither compound elicited any discernible change in spin state of the heme.

Among the imidazole derivatives, ketoconazole and imidazole had IC_{50} values of 95 and 251 μM , respectively (Figure 6). At very low concentrations



chemicals	IC_{50} (95% CI) (μM)	spectral binding	
		type	min con (μM)
imidazole	251 (115-548)	II	1
cimetidine	>3000	II	200 – 400
ketoconazole	95 (62-145)	II	2

FIG. 6. Effects of imidazole and its derivatives on xanthotoxin-metabolic activity of CYP6B1. IC_{50} was expressed in micromoles per liter with the 95% confidence interval in parentheses. Min con, minimum concentration to elicit type II binding spectrum. $N = 3$.

(1–2 μM), both compounds elicited typical type II binding spectra with an absorbance trough at 414 nm and a peak at 434 nm, respectively, typical of N-containing compounds coordinating with the heme. Although cimetidine was capable of eliciting a typical type II binding spectrum at high concentrations (200–400 μM), its IC_{50} value was >3000 μM (Figure 6).

DISCUSSION

Evolution of efficient detoxification of plant toxins by P450-mediated reactions is one of the most important mechanisms responsible for the adaptation of specialist insects to host plants containing toxic allelochemicals (Berenbaum, 1999). Exclusive feeding by *P. polyxenes* larvae on host plants containing furanocoumarins is attributable largely to the rapid detoxification of these toxins by P450s, specifically CYP6B1, a protein that has been extensively characterized (Ma et al., 1994; Berenbaum, 1999; Chen et al., 2002; Baudry et al., 2003; Wen et al., 2003, 2005; Pan et al., 2004). Here, we provide evidence that xanthotoxin does not suicide-inactivate CYP6B1. In combination with the facts that furanocoumarins are capable of inducing CYP6B1 expression (Prapaipong et al., 1994; Hung et al., 1995a,b; Petersen et al., 2001) and are efficiently detoxified by CYP6B1 (Ma et al., 1994; Chen et al., 2002; Baudry et al., 2003; Wen et al., 2003, 2005; Pan et al., 2004), our results support the identification of CYP6B1 as the principal enzyme responsible for the detoxification of furanocoumarins in *P. polyxenes*.

Our data indicate that both linear (bergapten, psoralen) and angular (angelicin) furanocoumarins inhibit xanthotoxin-metabolic activity of CYP6B1. The rank order of the IC_{50} values for the tested furanocoumarins (66.8, 105, and 154 μM for bergapten, psoralen, and angelicin, respectively) is the same as the rank order of the minimum concentrations needed to elicit type I binding spectra (1.0–2.5, 5–10, and 5–10 μM for bergapten, psoralen, and angelicin) that define the ability of these chemicals to displace water coordinated to the heme iron in the CYP6B1 catalytic site (Jefcoate, 1978). These results, together with the fact that these furanocoumarins are metabolized by CYP6B1 in an order inversely related to their ability to inhibit xanthotoxin metabolism (19.9, 16.9, and 4.5 $\text{nmol min}^{-1} \text{nmol}^{-1}$ P450 for bergapten, psoralen, and angelicin, respectively; Wen et al., 2005), suggest that the inhibition of xanthotoxin metabolism is most likely due to the competition of these chemicals with xanthotoxin for the CYP6B1 active site.

Angular furanocoumarins are generally less phototoxic to many organisms than linear furanocoumarins due to their ineffectiveness at cross-linking DNA strands in the presence of UV light (Berenbaum, 1991). That they are more toxic *in vivo* than linear furanocoumarins to black swallowtail larvae (Berenbaum and

Feeny, 1981) may be a reflection more of their capacity for analogue synergism than of their ability to intercalate into DNA. Black swallowtail larvae metabolize angelicin (an angular furanocoumarin) at rates threefold lower than rates at which the linear furanocoumarin psoralen is metabolized. This ratio is similar to the overall ratio at which these compounds are metabolized *in vitro* by CYP6B1 in the presence or absence of CYP6B3 (3.3-fold by CYP6B1 and CYP6B3 combined, 3.7-fold by CYP6B1 alone; Wen et al., unpublished). The fact that the IC_{50} ratios for these compounds indicate that psoralen is 1.5-fold more competitive for the CYP6B1 active site than angelicin suggests that, in the absence of rapid metabolism, binding of the angular furanocoumarin angelicin in the CYP6B1 active site limits the amount of enzyme available for metabolism of co-occurring linear furanocoumarins psoralen and xanthotoxin.

The competitive inhibition of CYP6B1 by furanocoumarins contrasts with furanocoumarins acting as mechanism-based inhibitors for several well-known mammalian P450 activities [e.g., coumarin 7-hydroxylase of human CYP2A6 (Koenigs and Trager, 1998a)] and insect P450 activities [e.g., ρ NA O-demethylase of *M. sexta*, aldrin epoxidase of *M. sexta* and *P. polyxenes* (Zumwalt and Neal, 1993; Neal and Wu, 1994)]. While it is not clear whether CYP6B1 contributes to the ρ NA O-demethylation activity that can be reversibly inhibited by xanthotoxin (Zumwalt and Neal, 1993), the mechanistic inhibition of *P. polyxenes* aldrin epoxidation activity by xanthotoxin suggests that at least one P450 isoform other than CYP6B1 is responsible for the aldrin epoxidation activity in this species.

The integrity of the coumarin nucleus and furan ring present in furanocoumarins is apparently important for their biochemical properties. Compared with furanocoumarins, the inhibition potency is dramatically lower for coumarin ($IC_{50} = 925 \mu\text{M}$), which lacks the furan ring, and absent for benzofuran ($IC_{50} > 3000 \mu\text{M}$), which lacks the coumarin core structure (this work; Baudry et al., 2003). Metabolic analysis has demonstrated that coumarin is only marginally metabolized by CYP6B1 ($0.08 \text{ pmol min}^{-1} \text{ pmol}^{-1}$ P450; Wen et al., 2005). Moreover, molecular modeling of CYP6B1-coumarin interactions suggests that this compound inhibits effectively by binding in a distal region of the catalytic site and preventing xanthotoxin from being metabolized (Baudry et al., 2003).

Furanochromones, whose structures are comparable to furanocoumarins except for the position of the carbonyl group on their pyran ring, inhibit xanthotoxin metabolism by CYP6B1 with potencies similar to psoralen and angelicin ($IC_{50} = 100 \mu\text{M}$ for khellin and $172 \mu\text{M}$ for visnagin), but they fail to elicit type I binding spectra. Given that furanochromones are poorly metabolized by CYP6B1 ($0.14 \text{ pmol min}^{-1} \text{ pmol}^{-1}$ P450 for khellin; $0.36 \text{ pmol min}^{-1} \text{ pmol}^{-1}$ P450 for visnagin; Wen et al., 2005), it is likely that furanochromones also inhibit CYP6B1 by active site competition.

The co-occurrence of MDP compounds, which are well-known P450 inhibitors, with toxic plant allelochemicals has been hypothesized to enhance plant defense against insect herbivores (Berenbaum, 1985; Berenbaum and Neal, 1985, 1987; Neal, 1989). However, inhibitory capacity of MDP compounds varies substantially depending on their functional groups, the length of side chains, and even the position of the double bond in the side chain attached to the benzene ring. Among the compounds tested, myristicin, which contains a methoxy group and a short side chain at the R3 position, was the most potent CYP6B1 inhibitor ($IC_{50} = 265 \mu\text{M}$) and the only MDP compound capable of eliciting a typical type I binding spectrum. Compounds such as safrole, which lacks this methoxy group and has the same short side chain at an alternate R2 position, are significantly less potent CYP6B1 inhibitors ($IC_{50} = 1030 \mu\text{M}$). Simply switching the double bond within this side chain alters the IC_{50} value more than twofold, from $1030 \mu\text{M}$ for safrole to $428 \mu\text{M}$ for isosafrole. Piperonyl butoxide (PBO), widely used as insecticide synergist and tool to demonstrate the involvement of P450s in insecticide metabolism, failed to inhibit CYP6B1, even though the original demonstration of P450s in furanocoumarin detoxification in black swallowtails utilized PBO for the general inactivation of P450s (Bull et al., 1986). Based on our results, it is likely that CYP6B1 isoforms other than CYP6B1 and CYP6B3 that are sensitive to PBO inhibition also contribute to the efficient detoxification of furanocoumarins in this species.

Flavonoids are generally poor inhibitors for CYP6B1, with flavone being most potent, having an IC_{50} of $698 \mu\text{M}$. Flavanone, lacking a double bond in the pyran ring, and α -naphthoflavone failed to inhibit CYP6B1 activity. In view of the fact that they are poorly metabolized by CYP6B1 ($0.56 \text{ pmol min}^{-1} \text{ pmol}^{-1}$ P450 for flavone and α -NF; Wen et al., 2005), and that only high concentrations of flavone ($20\text{--}40 \mu\text{M}$) elicit type I binding spectra, our results suggest that flavonoids, due to their size, may not fit well into the catalytic site of CYP6B1. Imidazole and its derivatives inhibit P450 by binding to the heme iron. Although all three compounds elicit a typical type II binding spectrum for CYP6B1, ketoconazole was the most potent inhibitor for CYP6B1 among imidazole and its derivatives.

One major strategy allowing specialist insects to feed exclusively on toxin-containing host plants is the evolution of P450-mediated detoxification of plant toxins. As a coevolutionary response to this process, plants accumulate a range of secondary compounds that act collectively against these herbivores (Berenbaum, 1985; Berenbaum and Neal, 1987). This study contributes toward a mechanistic explanation for how naturally occurring synergists can interfere with insect metabolism of (and hence resistance to) plant defense compounds, and in doing so sheds light on the functional significance of the phytochemical diversity that characterizes angiosperm plants in general. In view of the ubiquity

of P450-mediated insect resistance to host plant allelochemicals in plant-insect interactions, it is likely that the type of inhibition we have documented here is a widespread and evolutionarily important phenomenon.

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CYTOCHROME P450-MEDIATED METABOLISM OF XANTHOTOXIN BY *Papilio multicaudatus*

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Abstract—Within the genus *Papilio*, the *P. glaucus* group contains the most polyphagous *Papilio* species within the Papilionidae. The majority of *Papilio* species are associated with hostplants in the families Rutaceae and Apiaceae, and characterizing most are secondary metabolites called furanocoumarins. Recent phylogenetic studies suggest that furanocoumarin metabolism is an ancestral trait, with the *glaucus* group derived from ancestors associated with furanocoumarin-containing Rutaceae. In this study, we examined this relationship by conducting a gravimetric analysis of growth that used various concentrations of the furanocoumarin xanthotoxin. *Papilio multicaudatus*, the putative ancestor of the *glaucus* group, includes at least one furanocoumarin-containing rutaceous species among its hostplants; this species can consume leaf tissue containing up to 0.3% xanthotoxin with no detectable effect on relative growth rate, relative consumption rate, or efficiency of conversion of ingested food. As is the case for other *Papilio* species, xanthotoxin metabolism is mediated by cytochrome P450 monooxygenases (P450s). Ingestion of xanthotoxin by ultimate instar *P. multicaudatus* increases activity up to 30-fold in a dose-dependent fashion. Midguts of induced larvae can also effectively metabolize six other furanocoumarins, including both linear (bergapten, isopimpinellin, imperatorin) and angular (angelicin, sphondin) forms. A metabolite of xanthotoxin in the frass from xanthotoxin-treated larvae, identified as 6-(7-hydroxy-8-methoxycoumaryl)-acetic acid by MS–MS and NMR analyses, is identical to one from the frass of *P. polyxenes*. The occurrence of this metabolite in two swallowtails and the presence of a second metabolite of xanthotoxin, 6-(7-hydroxy-8-methoxycoumaryl)-hydroxyethanol in the frass of both *P. polyxenes* and *Depressaria pastinacella* are consistent with the suggestion that lepidopterans share as the first step of xanthotoxin metabolism the P450-mediated epoxidation of the furan ring 2'–3' double bond.

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Key Words—detoxification, furanocoumarin, induction, Lepidoptera, metabolite, Rutaceae, swallowtail.

INTRODUCTION

More than 40 years ago, Ehrlich and Raven (1964) demonstrated that phylogenetic patterns of hostplant use within the Lepidoptera can provide insights into the evolution of specialization and adaptation to plant chemical defense. In coevolutionary interactions, key innovations in behavior or physiology are thought to underlie host use patterns and facilitate hostplant switches; molecular methods now allow the identification and characterization of genes encoding enzymes that play key roles in this reciprocal adaptive process (Berenbaum et al., 1996). The swallowtail butterflies of the family Papilionidae, a cosmopolitan group of 500+ species, has been the focus of coevolutionary studies for many years (Feeny, 1992). Early estimates of phylogenetic relationships within the family were based on morphological characteristics, larval food plant associations, and geographic distributions. Munroe (1961) split *Papilio* into five sections, which corresponded to differences in utilization of the principal food plants in the families Annonaceae, Lauraceae, Rutaceae, and Apiaceae (Berenbaum, 1995). Plants in the Apiaceae and Rutaceae contain furanocoumarins as defense chemicals (Berenbaum, 1983). *Papilio* sections II and IV generally utilize species in the family Apiaceae and Rutaceae as hostplants (Scriber et al., 1991; Scriber, 1995), whereas section III species, which tend to have more polyphagous diets, only occasionally use these furanocoumarin-containing hostplants (Cohen et al., 1992). There are no known associations between species in sections I and V and furanocoumarin-containing plants.

Prior to molecular analysis, sections III and V were believed to be basal within the genus *Papilio* based largely on male genitalia, morphology, and larval hostplant preferences. Analyses have utilized allozyme variations (Hagen and Scriber, 1991), mitochondrial RNA (Sperling, 1993; Sperling and Harrison, 1994; Caterino and Sperling, 1999; Reed and Sperling, 1999), and DNA sequencing (Aubert et al., 1999; Vane-Wright et al., 1999; Yagi et al., 1999) to elucidate relationships within the genus. Zakharov et al. (2004), conducting a meta-analysis to generate an accurate phylogeny, split the genus into two distinct lineages, one including the subgenera *Princeps* and *Papilio* (*sensu stricto*) (section II) and the other including the subgenera *Heraclides*, *Pterourus*, and *Chilasa* (sections IV, III, and V, and I, respectively). Although *Pterourus* is paraphyletic, the *glaucus* group is considered the most advanced of these clades. According to this analysis, *P. multicaudatus* is the basal species within section III, the *glaucus* group in the subgenus *Pterourus*.

In both polyphagous section III species and oligophagous section II species, the ability to utilize furanocoumarin-containing hostplants in Apiaceae and

Rutaceae is associated with metabolism by cytochrome P450 monooxygenases (P450s), which are detoxificative enzymes that catalyze the NADPH-associated reductive cleavage of oxygen to produce a functionalized product and water. The genes encoding these enzymes (<http://drnelson.utmem.edu/CytochromeP450.html>) constitute one of the largest superfamilies known. The furanocoumarin-metabolic activity of CYP6B proteins in *Papilio* species is associated with the probability of encountering hostplant furanocoumarins. Catalytic activity was compared in two closely related CYP6B4 and CYP6B17 groups in the polyphagous congeners *Papilio glaucus* and *Papilio canadensis* (Li et al., 2003). Generally, P450s from *P. glaucus*, which feeds occasionally on furanocoumarin-containing hostplants, display higher activities against furanocoumarins than those from *P. canadensis*, which normally does not encounter furanocoumarins. These P450s in turn catalyze a larger range of furanocoumarins with lower activity than CYP6B1, a P450 from *Papilio polyxenes*, which feeds exclusively on furanocoumarin-containing apiaceous hostplants. Reconstruction of the ancestral CYP6B sequences using maximum likelihood predictions and comparisons of the sequence and geometry of their active sites to those of contemporary CYP6B proteins indicate that hostplant diversity is inversely related to substrate specificity. These predictions suggest that, in the lineage leading to *Papilio* P450s, the ancestral highly versatile CYP6B protein presumed to exist in a polyphagous ancestor evolved through time into a more efficient and specialized CYP6B1-like protein in *Papilio* species with continual exposure to furanocoumarins.

P. multicaudatus specializes on only three genera of plants within three families: Rutaceae, Rosaceae, and Oleaceae. One host genus, *Ptelea*, is known to contain furanocoumarins (Murray et al., 1982). The purpose of our study was to examine furanocoumarin disposition in this species, putatively basal to the polyphagous species in section III, in order to determine whether (1) tolerance is intermediate between that of section II furanocoumarin specialists and section III generalists, (2) inducibility of furanocoumarin metabolism is conserved between sections, and (3) metabolites generated by P450-mediated metabolism, reflective of the detoxificative transformation, are similar across sections. Interpreting detoxificative metabolism of hostplant phytochemicals in a phylogenetic context can allow for reconstruction of host shifts and diversification.

METHODS AND MATERIALS

Insects. Adult *P. multicaudatus* were captured at Kitt Peak in Pima County, Arizona, at 5000 ft elevation on 11 August 2004 and subsequently transported to Champaign County, Illinois. The captive butterflies were allowed to mate within a 1 × 1 × 1-m screen mesh cage. Juvenile *Ptelea trifoliata* and *Prunus serotina* trees were placed within the cage for oviposition. The eggs were allowed to

hatch within the cage. Second instar larvae were removed from the cage and placed in 15l rearing tubs. Each rearing tub was constructed of durable, opaque plastic lined with moist paper towels. Air vents were cut into the lids. Fresh *P. serotina* leaves were exchanged on a daily basis. No rearing tub contained more than 30 larvae at any time. The caterpillars were maintained at 25°C and 16:8 light/dark photoperiod for the duration of the experiment.

Reagents for Detoxification Studies. Xanthotoxin used in gravimetric analyses of performance was obtained from Sigma (St. Louis, MO, USA). Other furanocoumarins, used as substrates in metabolism assays, including angelicin and isopimpinellin from Indofine Chemical Co. (Belle Mead, NJ, USA), bergapten and psoralen from Sigma (St. Louis, MO, USA), and imperatorin from Feinbiochemical (Heidelberg, Germany); sphondin was a gift from Ralph Mumma. NADPH was obtained from Sigma, and HPLC solvents from Fisher Scientific L.L.C. (Pittsburgh, PA, USA).

Gravimetric Estimates of Xanthotoxin Tolerance. Using ultimate instar *P. multicaudatus* larvae, we conducted a gravimetric estimate of performance with varying concentrations of xanthotoxin, a linear furanocoumarin, topically applied to the hostplant. Fifth instar larvae were collected from the rearing tubs within 2 hr of the final larval molt. Each caterpillar was weighed and placed in a 9-cm Petri dish with moistened filter paper. Approximately 1 g of *P. serotina* leaf material, which lacks furanocoumarins, was weighed and topically treated with either acetone or an acetone solution of xanthotoxin. Xanthotoxin solutions of different concentrations were prepared, so that equal volumes could be applied to obtain diets containing xanthotoxin at 0.1, 0.2, 0.3, and 0.4% fresh weight. These concentrations were based on previous studies of *P. polyxenes* and its host plant, flat-leaf parsley, *Petroselinum sativum* (Cohen et al., 1989). One hundred larvae were distributed among the five treatments.

Larvae were allowed to feed for 24 hr and then placed in a -20°C freezer for 30 min. Subsequently, larvae, remaining leaf material, and frass were placed in a 60°C drying oven for 24 hr to obtain dry weight. All fresh and dry weights were recorded, and fresh weight was converted to dry weight by using a conversion factor based upon five caterpillars that were frozen and dried within 2 hr of the final molt and five dried *P. serotina* leaves.

Standard gravimetric parameters of performance were calculated, including relative growth rate (RGR), the efficiency of conversion of ingested food to body substance (ECI), and relative consumption rate (RCR) or weight gain (Waldbauer, 1968). Data were analyzed by using analysis of variance (ANOVA), Dunnett's test, and analysis of covariance (ANCOVA) (Raubenheimer and Simpson, 1992). Regression analysis was conducted to determine the effect of xanthotoxin on relative growth rate.

Inducibility of Xanthotoxin Metabolism. Newly molted fifth instars were individually placed on 1 g fresh weight foliage of *P. serotina*, topically treated

with varying amounts of xanthotoxin dissolved in 250 μ l acetone (0, 1, 2, and 4 mg). Four replicate larvae were used for each concentration. After 48 hr, midguts were dissected on ice, and each midgut was homogenized in 600 μ l of 0.1 M ice-cold sodium phosphate buffer (pH 7.8) containing 20% glycerol, 1.1 mM EDTA, 0.5 mM PMSF, and 5 μ g/ml (w/v) leupeptin. The homogenates were centrifuged at 5000 rpm for 5 min at 4 °C. Metabolism reactions were set up with 30 μ l of the supernatant. Each reaction mixture contained 0 (control) or 50 μ l of NADPH (10 mg/ml in 0.1 M phosphate buffer, pH 7.8), 2 μ l of xanthotoxin (10 mM in methanol), and 468 μ l (in control) or 418 μ l of 0.1 M phosphate buffer, pH 7.8. The reactions were incubated for 30 min in a 30°C shaking water bath followed by incubation for 5 min at 70°C to inactivate the P450s. The reaction mixture was extracted with 500 μ l of ethyl acetate after addition of 5 μ l of 1 mM psoralen as an internal standard and centrifuged at 14,000 rpm for 5 min using a benchtop centrifuge at room temperature. Ten microliters of the ethyl acetate phase were removed and analyzed by normal-phase HPLC (Econosphere 5- μ m silica column, 150 \times 4.6 mm) with a solvent system containing 55% cyclohexane, 42% isopropyl ether, and 3% amyl alcohol to determine the amount of unmetabolized xanthotoxin remaining.

Metabolism of Furanocoumarins by Homogenates of Xanthotoxin-Induced Larval Midguts. To determine the substrate-specificity of P450-mediated furanocoumarin metabolism in *P. multicaudatus*, metabolism reactions were carried out as described except that homogenate supernatants were prepared from midguts of larvae induced with 4 mg xanthotoxin per gram wild cherry leaves and six furanocoumarin substrates, including the angular furanocoumarins angelicin and sphondin, and the linear furanocoumarins bergapten, imperatorin, isopimpinellin, and psoralen. They were analyzed, with 5 μ l of 100 nM xanthotoxin added to the reaction as an internal standard before ethyl acetate extraction.

Isolation and Structural Elucidation of Xanthotoxin Metabolite from Frass. *P. multicaudatus* larvae starved for 3 hr were presented with foliage of wild cherry treated with 0 mg (control) or 4 mg xanthotoxin/g fresh weight (fw) foliage (treatment). After 24 hr of feeding, frass was collected. To detect the xanthotoxin metabolite in frass and determine a suitable metabolite extraction method, frass from caterpillars on each diet was extracted with water and methanol, and the extracts were analyzed by reverse-phase HPLC (4- μ m C-18 column, Waters Novapak, 4.7 \times 150 mm). To purify the metabolite, gradient elution (solvent A, 5% glacial acetic acid in water; solvent B, 100% acetonitrile) was performed at a flow rate of 1 ml/min with gradient conditions ranging from 100% A/0% B to 90% A/10% B over 5 min, to 60% A/40% B over 30 min; after 5 min at 60% A/40% B, a gradient was run to 100% A/0% B. The spectra for water and methanol extracts of treatment frass were compared with those of the corresponding control frass at wavelengths

between 210 and 400 nm (996 Diode array detector, Waters, Milford, MA, USA); peaks unique to xanthotoxin-fed larvae were considered to represent metabolites.

To obtain sufficient amounts of metabolites to characterize, frass was again collected as described from fifth instars on control or treated (4 mg xanthotoxin/gfw) foliage. The frass collected was extracted with methanol. After drying the extract with N₂, the residue was redissolved in the same original volume of HCl-acidified distilled water (pH 2). The water phase was then extracted with the same volume of ethyl acetate after extraction with the same volume of chloroform. The water phase was collected, frozen in liquid nitrogen, and lyophilized. Finally, the residue was resuspended in 0.5 ml methanol for separation by reverse-phase HPLC (as described earlier, except that UV absorbance was set at 325 nm).

Mass spectrometric analysis of purified metabolite was performed at the Mass Spectrometry Service Facility at the University of Illinois at Urbana-Champaign; the instrument used was a Finnigan LCQ Deca XP MAX ion trap mass spectrometer (Thermo Electron Corporation). For MS-MS analysis, a collision energy of 25 was used for the metabolite of MW 250. The ¹H NMR spectrum was obtained with a Bruker (Billerica, MA, USA) Avance 400 NMR spectrometer equipped with a 5-mm inverse broadband Z-gradient probe (¹H, 400 MHz). The NMR spectrum was recorded in methanol-*d*₄, which served as the internal reference (¹H NMR, 3.30 ppm). Data were analyzed using the Advanced Chemistry Development, Inc., SpecManager 1D Processor and the HNMR Predictor software suite (Toronto, Ontario, Canada).

RESULTS

Gravimetric Estimates of Xanthotoxin Tolerance. There were no statistically significant differences among treatments in consumption rate or efficiency of conversion (Table 1). The only significant effect of treatment evidenced in calculated gravimetric ratios evaluated by ANOVA was for RGR ($F = 3.004$, df 4, 97, $P = 0.022$) (Table 1). Growth rates in diet containing 0.2% xanthotoxin were lower than growth rates of caterpillars placed on control diet ($P = 0.031$), and RGR for caterpillars exposed to 0.4% xanthotoxin were marginally lower than those on control ($P = 0.053$).

ANCOVAs were performed to compare consumption and weight gain on the five diets. In both analyses, the assumption of homogeneity of slopes was not violated ($P > 0.12$), but there were no significant differences in consumption or growth among treatments. A marginally significant negative regression of

TABLE 1. GRAVIMETRIC ESTIMATES OF PERFORMANCE RATIOS

Percent xanthotoxin/fresh weight leaf	N	RGR ± SD	ECI ± SD	RCR ± SD
Control	21	0.480 ± 0.191	32.333 ± 12.082	1.527 ± 0.641
0.1	18	0.428 ± 0.190	25.510 ± 31.605	1.260 ± 0.561
0.2	21	0.340 ± 0.168 ^a	26.219 ± 23.685	1.359 ± 0.523
0.3	19	0.468 ± 0.155	37.125 ± 12.576	1.341 ± 0.467
0.4	21	0.351 ± 0.142 ^a	24.825 ± 17.404	1.216 ± 0.560
Average	20	0.411 ± 0.177	29.086 ± 20.868	1.343 ± 0.555

Calculated gravimetric estimated ratios per treatment of xanthotoxin. The only significant treatment effect was for RGR ANOVA ($F = 3.004$, $df 4, 97$, $P = 0.022$). RGR = relative growth rate ($\text{g g}^{-1} \text{d}^{-1}$ dry weight); ECI = the efficiency of conversion of ingested food to body substance (%); RCR = relative consumption rate ($\text{g g}^{-1} \text{d}^{-1}$ dry weight).

^aTreatments that were significantly different from control (Dunnett’s test, $P < 0.05$).

relative growth rate on xanthotoxin concentration (Figure 1) suggests a mild negative effect of ingesting increasing amounts of xanthotoxin ($P = 0.061$, $r^2 = 0.04$).

Inducibility of Xanthotoxin Metabolism. Cytochrome P450-mediated xanthotoxin metabolism in the midgut of *P. multicaudatus* was induced in a dose-dependent fashion, increasing 13-, 16-, and 23-fold when ultimate instars were fed foliage treated with xanthotoxin at 1, 2, and 4 mg/g fw, respectively; constitutive levels of metabolism, in the absence of furanocoumarins, were very low (Figure 2). Larvae with midgut metabolism induced by ingestion of hostplant treated with xanthotoxin (4 mg/g fw) were capable of metabolizing six

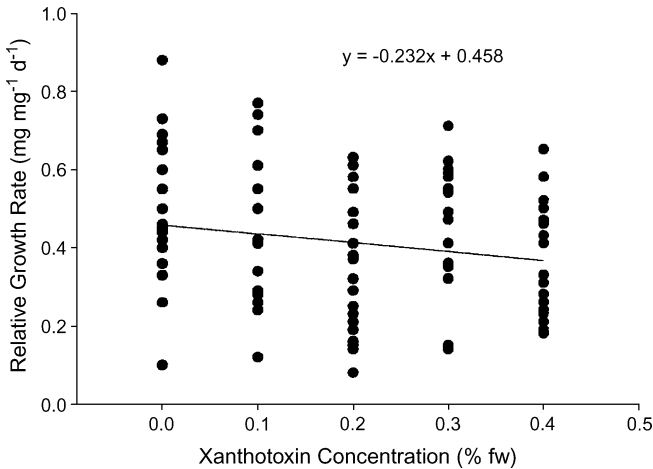


FIG. 1. Regression plot of relative growth rate as a function of xanthotoxin concentration.

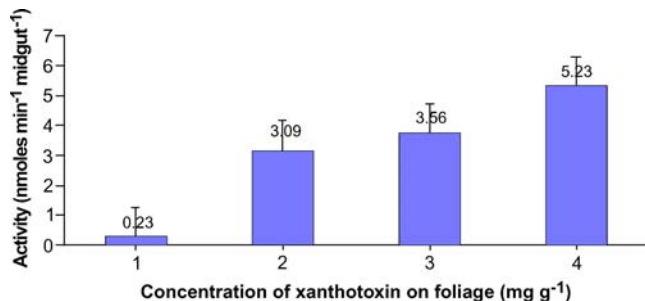


FIG. 2. Dose-dependent inducibility of xanthotoxin metabolism in *P. multicaudatus* midgut microsomes.

furanocoumarins other than xanthotoxin, including not only linear furanocoumarins (bergapten, imperatorin, isopimpinellin, psoralen) but also angular furanocoumarins (angelicin, sphondin). Metabolism rates were 4.38, 12.95, 6.28, 12.62, 4.97, and 10.89 nmol/min per midgut for angelicin, bergapten, imperatorin, isopimpinellin, psoralen, and sphondin, respectively (Figure 3).

Isolation and Structural Elucidation of Metabolite. Comparison of UV spectra of aqueous and methanolic extracts of frass from caterpillars on control and xanthotoxin diets revealed only a single metabolite, which absorbed strongly at 325 nm. This was isolated and purified by reverse-phase HPLC after chloroform and ethyl acetate extraction (Figure 4) and found to have a molecular weight of 250 (Figure 5). Collection of the metabolite fraction from

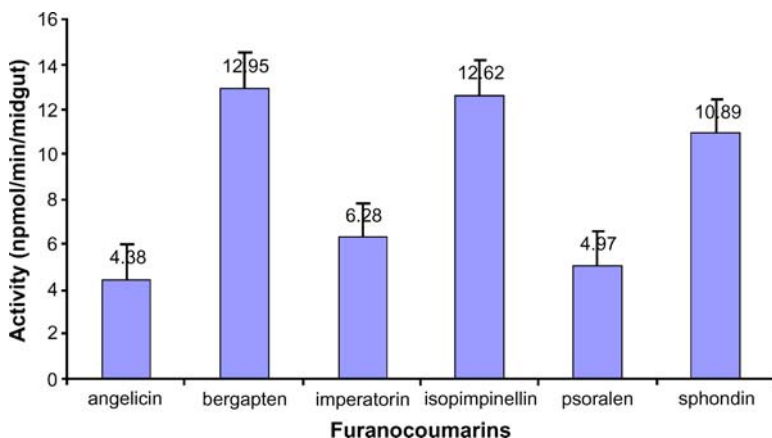


FIG. 3. Metabolism of six furanocoumarins by the midgut of *P. multicaudatus* larvae fed with 4 mg xanthotoxin per gram leaf of *P. serotina* (wild cherry).

reverse-phase HPLC and subsequent drying by speed vacuum method yielded sufficient crystalline material to perform structure-elucidation analyses.

Identification of the isolated metabolite was accomplished by both MS–MS and NMR spectroscopy. The MS–MS spectrum of the metabolite shows that the coumarin ring is intact, but the furan ring is no longer closed (Figure 5). Proton NMR shows a singlet of three protons at $\delta = 3.98$ ppm corresponding to the 8-methoxy group. The protons at C-3 and C-4 give rise to two doublets at $\delta = 6.22$ and 7.85 ppm, respectively, whereas the isolated proton at C-5 shows a singlet at $\delta = 7.20$ ppm. A singlet of two aliphatic protons at $\delta = 3.67$ indicates the adjacent presence of a carboxylic acid resulting from metabolic cleavage of the furan ring (Figure 6). Thus, both MS–MS and NMR spectra suggest that the metabolite in frass is HCA, 6-(7-hydroxy-8-methoxycoumaryl)-acetic acid (Figures 5 and 6).

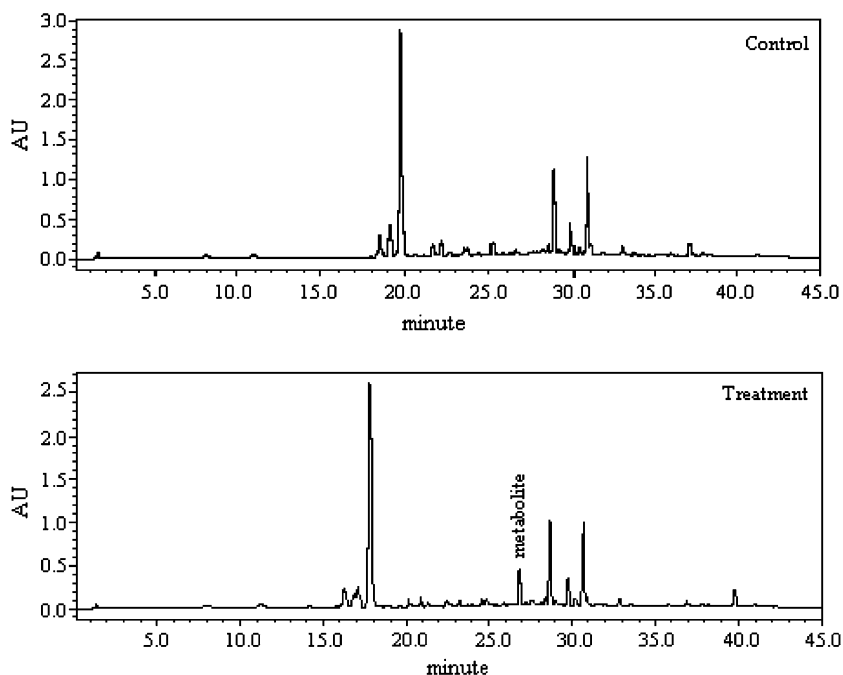


FIG. 4. Isolation of metabolite in frass on reverse-phase HPLC column (C-18 column, Waters Novapak, 4.7×150 mm) after chloroform and ethyl acetate extraction. (A) Depicts the extract of frass from *P. multicaudatus* larvae fed leaves treated with acetone (control); (B) depicts the extract of frass from *P. multicaudatus* larvae fed leaves treated with xanthotoxin dissolved in acetone (treatment).

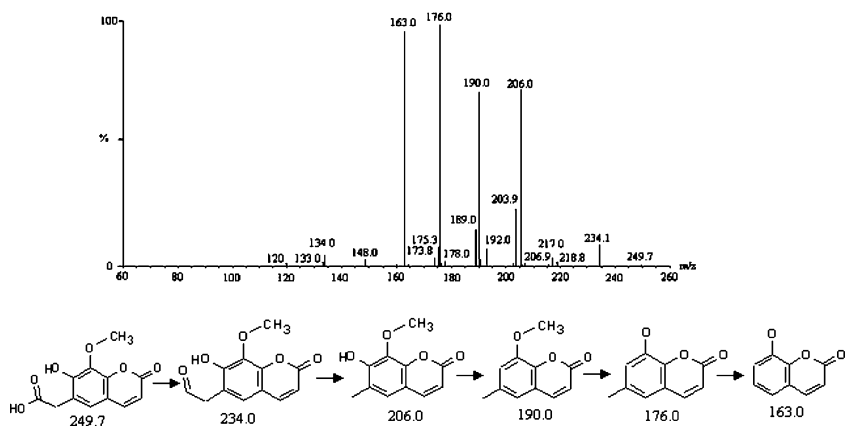


FIG. 5. MS-MS spectrum and mass fragments of xanthotoxin metabolite.

DISCUSSION

Physiological and biochemical analyses confirm that *P. multicaudatus*, the putative ancestor to the *glaucus* group swallowtails, maintains the ancestral capacity to tolerate furanocoumarins, in contrast with the more derived species, *P. canadensis* and *P. glaucus*, which have a reduced ability to tolerate these compounds (Li et al., 2002) (Table 1). This conclusion is supported by larval hostplant associations; most section III *Papilio* species utilize Lauraceae as primary larval hostplants and Magnoliaceae as secondary larval hostplants.

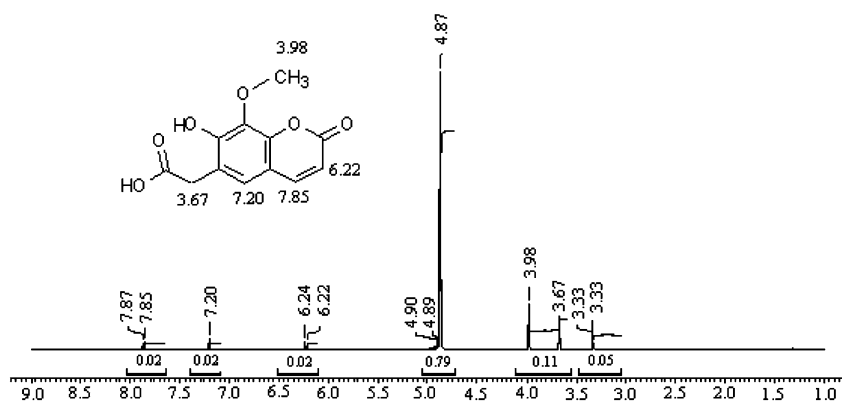


FIG. 6. 400 MHz NMR spectrum of metabolite dissolved in methanol- d_4 . Peaks 4.87 and 3.33 represent H_2O and methanol- d_4 peaks, respectively.

P. multicaudatus and *P. glaucus* are the only two section III species known to use Rutaceae as larval hostplants (Aubert et al., 1999).

As is the case for both Section II (Cohen et al., 1989) and Section III swallowtails (Li et al., 2003), xanthotoxin is a strong inducer of its own P450-mediated metabolism. When larvae were induced with 0.4% fresh weight xanthotoxin, the rate of xanthotoxin metabolism increased to over 5.23 nmol/min per midgut, more than 20 times the rate of its constitutive metabolism (0.23 nmol/min per midgut). Compared with the Section II black swallowtail, *P. polyxenes*, in which induction increased xanthotoxin metabolism 3- to 8-fold over constitutive levels at dietary concentrations of 0.1 to 0.5%, and the section III tiger swallowtail, *P. glaucus*, in which induction increased 30-fold over constitutive levels at dietary concentrations of 0.2% (Figure 2, Li et al. 2001), induced metabolism and maximum tolerance increased induction approximately 20-fold in *P. multicaudatus* at dietary concentrations of 0.2% fresh weight xanthotoxin, levels that are intermediate, as predicted. This intermediate inducibility and tolerance are consistent with the frequency with which furanocoumarins are encountered by these species. *P. polyxenes*, all of whose hosts contain furanocoumarins, maintains high constitutive levels of furanocoumarin metabolism that are only slightly inducible before maximum capacity is reached; *P. multicaudatus*, one-third of whose known hosts contain furanocoumarins, maintains intermediate constitutive levels of furanocoumarin metabolism that are substantially inducible; and *P. glaucus* and *P. canadensis*, which rarely and never encounter furanocoumarins in their respective diets, maintain vanishingly low levels of constitutive xanthotoxin metabolism that are highly inducible if furanocoumarins are encountered (Li et al., 2003).

Like the other more generalized section III swallowtails, *P. multicaudatus* larvae can effectively metabolize not only xanthotoxin, but six other furanocoumarins, including linear and angular forms. In the section III swallowtails, this ability to metabolize a diverse array of furanocoumarins is attributable in part to a greater number of furanocoumarin-inducible P450 genes, each of which has broad substrate specificity (Li et al., 2003). *CYP6B4*, *CYP6B17*, and *CYB6B2*, which are all furanocoumarin-inducible in *P. glaucus*, and *CYP6B26*, which is furanocoumarin-inducible in *P. canadensis* (Li et al., 2001), have overlapping substrate specificities, and all can metabolize both linear and angular furanocoumarins (Li et al., 2003). In contrast, in the furanocoumarin specialist *P. polyxenes*, the enzyme CYP6B1 (Cohen et al., 1992) is furanocoumarin-inducible, but it is highly specialized and can metabolize angular furanocoumarins only to a limited extent and cannot metabolize imperatorin, a linear furanocoumarin. Whether *P. multicaudatus* owes its tolerance to furanocoumarins to multiple furanocoumarin-inducible P450s with broad substrate specificities, like the other *glaucus* group swallowtails, remains to be determined.

Some insight into the nature of the metabolic transformations that contribute to *P. multicaudatus* furanocoumarin tolerance can be gained by an examination of the structure of the metabolites produced. Only a single metabolite was isolated and identified from frass. The metabolite, HCA (Figures 5, 6, and 7), likely arises as a result of cleavage of the furan ring at the 2'-3' position, because the electron density at that position is higher than at the 3-4 position, and oxidative pathways usually prefer electron-rich π bonds (Schmid et al., 1980). This metabolite has also been found in frass of *P. polyxenes* as well as in the noctuid generalist *Spodoptera frugiperda* after xanthotoxin consumption. Both of these species produce a second metabolite, 6-(7-hydroxy-8-methoxycoumeryl)-hydroxyacetic acid (HCHA) (Ivie et al., 1983); HCHA constitutes the sole xanthotoxin metabolite in frass of the oecophorid caterpillar *Depressaria pastinacella*, which feeds exclusively on furanocoumarin-containing species in the apiaceous genera *Pastinaca* and *Heracleum* (Nitao et al., 2003). Collectively, these findings suggest that the furan ring is an important site of metabolic alteration of xanthotoxin in Lepidoptera. In this context, we suggest that P450-mediated epoxidation at the 2'-3' position on the furan ring is the first step of xanthotoxin metabolism in *P. multicaudatus* (Figure 7), as is postulated to occur in *P. polyxenes*, *S. frugiperda* (Ivie et al., 1983), and *D. pastinacella* (Ivie et al., 1983; Nitao et al., 2003).

Formation of an epoxide as part of a metabolic transformation presents a toxicological challenge to organisms. In general, epoxides are highly reactive, so the epoxide metabolites of furanocoumarins, if they accumulate in any quantity, may seriously interfere with normal physiological function. In mammals, this problem is resolved by use of rapid conjugation of xanthotoxin with glucuronic acid, glutathione, and sulfate to increase their stability and hydrophilicity (Hayes and Pulford, 1995), for ultimate transport and excretion via ATP-binding cassette (ABC) transporter genes (Dean and Annilo, 2005). To date, no conjugates of xanthotoxin or any other furanocoumarins have been found in insects. The widespread occurrence of metabolite products consistent with epoxidation in Lepidoptera that encounter furanocoumarins suggests that

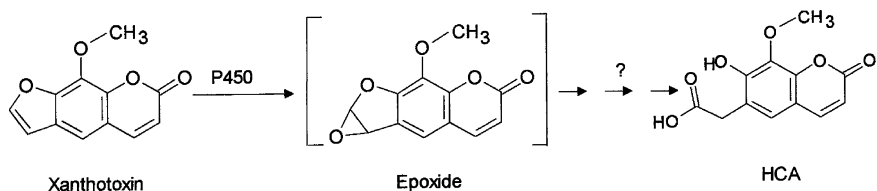


FIG. 7. Proposed pathway of xanthotoxin metabolism in *P. multicaudatus* larvae. The presumptive epoxide is in brackets.

conjugation may play a more important role in detoxification and hostplant specialization than has hitherto been realized.

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NEWLY HATCHED NEONATE LARVAE CAN GLYCOSYLATE: THE FATE OF *Betula pubescens* BUD FLAVONOIDS IN FIRST INSTAR *Epirrita autumnata*

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Abstract—*Betula pubescens* bud flavonoid aglycones reportedly have negative effects on the performance of first instar *Epirrita autumnata* and, thus, may defend birch leaves from larval defoliation. We hypothesized that the detrimental effects of these lipophilic flavonoids on larvae are due to their high levels in birch buds and/or the inability of naïve neonates to glycosylate them, which we have shown to occur in fifth instars. To test the latter hypothesis, we investigated the biochemical transformation of bud flavonoids in first instar *E. autumnata*. We found that newly hatched larvae have the ability to glycosylate birch bud/leaf flavonoid aglycones into corresponding glycosides. Moreover, we suggest that glycosylation may depend upon the chemical character of the aglycone and is an important factor in the performance of first instars.

Key Words—*Betula pubescens*, *Epirrita autumnata*, flavonoid aglycones, first instar.

INTRODUCTION

The highest mortality of lepidopteran larvae usually occurs during their first instar. The causes are not always clear, and may be the sum of various factors that

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affect larval survivorship. The first days are critical in larval life; during these neonates need to establish a feeding site and cope with different plant characters, microclimatic conditions, predators, parasitoids, and pathogens (Zalucki et al., 2002). The first stages of larval development are critical from the point of view of the plant as well. If conditions on the host plant are not favorable for larval development, this could dramatically reduce future biomass losses of the plant (Karban and Baldwin, 1997). It has been proposed that the most effective strategy for plant defense against herbivores is the synthesis of defensive compounds in young leaves rather than mature ones (Laitinen et al., 2002). Interaction between the timing of herbivory and the seasonally changing leaf chemistry was first suggested by Paul Feeny in his seminal paper (Feeny, 1970).

Even though the first instar may well be the most important—or at least the most vulnerable—stage in the life of herbivore larvae, there have been few studies conducted with neonates. This is presumably due to methodological problems, e.g., the small size of first instars (Zalucki et al., 2002). In earlier studies concerning the mechanisms of birch resistance and interactions between herbivores and birch foliar chemistry (e.g., Kause et al., 1999; Ossipov et al., 2001; Haukioja et al., 2002; Henriksson et al., 2003), the larvae have been in their later (third, fourth, or fifth) instars. A study with first instar *E. autumnata* was reported in Lahtinen et al. (2004), in which the biological activities of white birch (*Betula pubescens*) leaf surface exudates were investigated. We showed that tree-specific mortality of first instars correlated strongly with tree-specific contents of surface flavonoid aglycones in emerging leaves. Correlations between the contents of individual aglycones and larval performance [relative growth rate (RGR) and instar duration] also were found. In the same study, it was observed that flavonoid aglycones did not have harmful effects on fifth instar *E. autumnata* that feed on fully expanded leaves. This may be due to the lower contents of flavonoid aglycones in mature leaves (Valkama et al., 2004). In addition, we have previously shown that fifth instar *E. autumnata* can at least partially detoxify birch leaf flavonoid aglycones *via* glycosylation (Salminen et al., 2004), which may explain how last instars cope with lipophilic aglycones.

On the basis of these earlier findings, we suggested that the explanation for the strong negative correlations between the contents of certain birch leaf surface flavonoid aglycones and performance of first instar *E. autumnata* could be the high contents of these aglycones on buds. We also hypothesized that first instars may not have the ability to glycosylate flavonoid aglycones. To investigate the latter hypothesis, the biochemical transformation of birch bud flavonoids in first instar *E. autumnata* was studied by comparing the composition of flavonoid compounds (aglycones and glycosides) in larval frass to that in their leaf diet. This is the first time that individual compounds have been analyzed in the frass of first instar *E. autumnata*.

METHODS AND MATERIALS

Study Organisms. *Epirrita autumnata* (Borkhausen) is a univoltine geometrid moth commonly found on birch species in northern Europe. It is a destructive pest especially in northern and high-altitude populations of mountain birch [*B. pubescens* ssp. *czerepanovii* (Orlova) Hämet–Ahti], where its population densities fluctuate at intervals of approximately 9 to 10 yr, causing severe local outbreaks (Tenow, 1972; Haukioja et al., 1988; Ruohomäki et al., 2000). *E. autumnata* is best classified as a polyphagous species; it is able to attain comparable performance on leaves of host trees from several genera (Neuvonen et al., 1987) and can be found feeding on other hosts besides *Betula* in the field. Larvae of the autumnal moth hatch in the spring simultaneously with bud break and feed on young growing birch foliage.

Bioassay Design. About 200 first instar *E. autumnata* were reared in two large (12 l) pails on April 23–28, 2004. One branch of *B. pubescens*, with buds at the appropriate early phenological stage, was placed into each pail in a jar filled with water (100 ml) with added sucrose (1 g/vase) and sealed with parafilm. In practice, the appropriate phenological stage means opening buds with the tips of the emerging leaves visible. Larval frass was collected every 24 hr on filter-paper discs, which were placed at the bottom of each pail. Filter papers were the size of the bottom of the pail. Since despite the relatively high number of larvae, the amount of frass produced was low so the frass from 2 d at a time (1st + 2nd, 3rd + 4th, and 5th + 6th) was pooled for HPLC samples. Bud samples (three buds per branch) were collected from the same branches at the beginning of the bioassay before larvae were placed onto branches.

Sample Preparation. To analyze water-soluble flavonoids, six buds were extracted for 1 hr with 70% aqueous acetone (3×1 ml) in Eppendorf tubes. Aqueous acetone (70%) was earlier found to be a suitable and efficient solvent for (birch) leaf water-soluble phenolics (Hagermann, 1988; Salminen, 2003). We had three replicates of samples because each pair of two buds (one from both branches) was extracted in one tube. Three frass samples were extracted similarly, but the volume of the extraction solvent was 3×250 µl. After extraction, acetone was evaporated from samples, and the water phases were filtered for HPLC analysis. To analyze lipophilic flavonoid compounds, the non-water-soluble residues of the frass and bud samples were dissolved in 95% ethanol (1 ml). All extracts were filtered through 0.45-µm PTFE filters (Titan PTFE, 13 mm i.d., Scientific Resources Inc., USA) before analysis.

HPLC–DAD, HPLC–ESI–MS, and NMR Analyses. Extracts were analyzed by using HPLC–DAD (at 280 and 349 nm) and HPLC–ESI–MS systems. The analytical HPLC system (Merck–Hitachi, Tokyo, Japan) with diode array detector is described by Salminen et al. (2004). Column and chromatographic

conditions were as described by Salminen et al. (1999), except that 0.1 M H_3PO_4 was replaced with 0.05 M H_3PO_4 . HPLC–ESI–MS analyses were conducted as in Salminen et al. (1999), except that the data system used was Analyst Software 1.1. NMR spectra were acquired using a Bruker Avance 500 spectrometer (equipped with a BBI–5–mm–Zgrad–ATM probe) operating at 500.13 MHz for ^1H . Spectra were recorded at 25°C, using acetone– d_6 (and a few drops of D_2O) as a solvent. Proton spectra were referenced internally to the TMS signal using a value of 0.00 ppm.

Isolation of Flavonoid Aglycones. For structural elucidation of flavonoid aglycones, 5 g of dried lipophilic white birch bud extract (extracted $\times 1$ for 1 hr, with 100 ml of 95% aqueous ethanol) was dissolved in methanol and fractionated on Sephadex LH–20 column (40 \times 3.8 cm i.d., Pharmacia, Umeå, Sweden), with methanol as eluent. Fractions that contained flavonoid aglycones (checked with HPLC–DAD) were evaporated to dryness under reduced pressure, dissolved in petroleum ether, and fractionated on a silica column (10 \times 2.0 cm i.d., 70–230 mesh, 60 Å) with 50–ml fractions of petroleum ether containing increasing amounts of ethyl acetate. After fractionation, the fractions were again evaporated to dryness and dissolved in methanol. Flavonoid aglycones were further purified with a semipreparative Diasorb–130–C16T column (250 \times 15 mm i.d., 6 μm , BioChimMac, Russia–Austria–Germany), using a step gradient of CH_3CN (from 30 to 70%) in 0.1% HCOOH (in water). Samples were injected onto the column through a 500– μl loop valve.

Compound Identification and Quantification. Flavonoids were identified mainly on the basis of their spectra (UV and mass spectra) and retention times, as reported in the literature (Ossipov et al., 1995, 1996; Valkama et al., 2003; Lahtinen et al., 2004; Salminen et al., 2004). In addition to previously identified compounds, apigenin, kaempferol 4',7–dimethyl ethers, and kaempferol 3,4'–dimethyl ether were isolated and identified based on spectral properties (retention time, UV and mass spectra). The ^1H NMR spectra of apigenin and kaempferol 4',7–dimethyl ethers were also analyzed and compared to the literature (Erdtman et al., 1966; Herrera et al., 1996). All these flavonoids have been identified previously from *B. pubescens* by Wollenweber (1975). The identification of new fecal flavonoid glycosides was performed with HPLC–DAD and HPLC–ESI–MS. The structure of a glycoside was confirmed if mass spectral fragmentation revealed the ion peak of the aglycone part in addition to the molecular ion peak. Flavonoid glycosides were quantified as the corresponding aglycones: naringenin derivatives as naringenins, apigenin derivatives as apigenins, kaempferol derivatives as kaempferols, and quercetin derivatives as quercetins. Since there was no variation in the compound composition of the frass samples analyzed, the results were calculated as the mean values of these three samples. The contents of bud compounds were similarly calculated as the means of three replicates.

RESULTS

Analysis and Identification of Flavonoids. In order to study all the various flavonoids in buds and frass, first we extracted water-soluble compounds (mainly flavonoid glycosides) followed by the ethanol-soluble ones (mainly flavonoid aglycones). Bud flavonoid aglycones, which have not been identified previously (Valkama et al., 2003; Lahtinen et al., 2004; Salminen et al., 2004), were isolated and identified. All main aglycones of *B. pubescens* are shown in Figure 1. The aglycones were various methyl ethers of naringenin, apigenin, and kaempferol, which Wollenweber (1975) reported from *B. pubescens* buds. We also detected traces of a quercetin monomethyl ether and a pentahydroxy flavone trimethyl ether (identified in Valkama et al., 2003). These compounds may be quercetin 3'-methyl ether (according to UV and mass spectra), which Wollenweber (1975) reported in his work, and kaempferol 4',6,7-trimethyl ether (according to UV, mass and ¹H NMR spectra). Their precise structures were not characterized because of the relatively small amounts in the buds. The

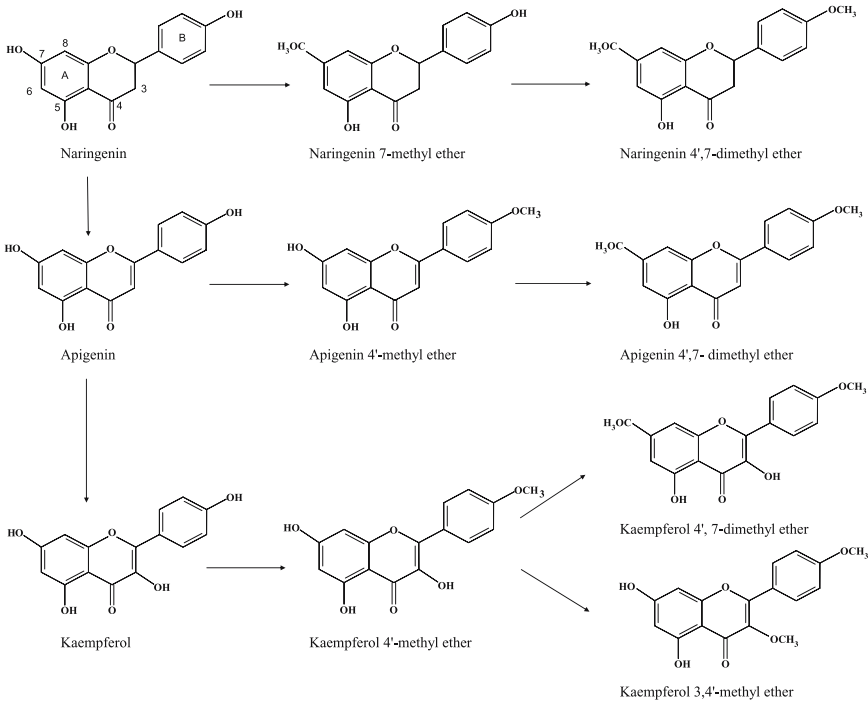


FIG. 1. Flavonoid aglycones identified in the buds of *B. pubescens*.

TABLE 1. DISTRIBUTION OF FLAVONOIDS IN THE LEAF DIET AND FRASS OF FIRST INSTAR *E. autumnata*: CORRELATION VALUES^a BETWEEN THE CONTENTS OF FLAVONOID AGLYCONES AND LARVAL PERFORMANCE

Fraction	Compound	In the leaf diet of first instars (µg/ml)	In the frass of first instars (µg/ml)	<i>r</i> Values for changes in instar duration	<i>r</i> Values for changes in mean RGR	
Flavonoid aglycones in ethanol fraction	Apigenin	11.7	–	0.49	–0.69	
	Apigenin 4'–methyl ether	57.5	–	0.14	–0.14	
	Apigenin 4', 7–dimethyl ether	70.9	–	0.77	–0.67	
	Kaempferol	14.7	–	0.57	–0.56	
	Kaempferol 4'–methyl ether	118.8	–	0.79	–0.65	
	Kaempferol 3, 4'–dimethyl ether	131.7	–	0.61	–0.47	
	Kaempferol 4', 7–dimethyl ether	83.0	–	0.44	–0.49	
	Naringenin	4.6	–	0.75	–0.63	
	Naringenin 7–methyl ether	285.0	traces	0.62	–0.51	
	Naringenin 4', 7–dimethyl ether	140.8	traces	0.93	–0.85	
	Pentahydroxyflavone trimethyl ether	4.2	–	0.17	–0.14	
	Quercetin derivative	32.0	–	0.45	–0.59	
	Flavonoids glycosides in water fraction	Apigenin glycosides	6.7	1.9		
		Apigenin 4'–methyl ether glycosides	–	24.7		
Apigenin 4',7–dimethyl ether glycosides		–	–			
Kaempferol glycosides		40.5	5.2			
Kaempferol 4'–methyl ether glycosides		–	7.2			
Kaempferol dimethyl ether glycosides		–	32.6			
Myricetin glycosides		8.7	–			
Naringenin glycosides		–	3.0			
Naringenin 7–methyl ether glycosides		–	45.9			
Naringenin 4',7–dimethyl ether glycosides		–	12.7			
Pentahydroxyflavone trimethyl ether glycosides	–	–				
Quercetin glycosides	126.8	16.8				

^aCorrelation values are calculated between the contents of flavonoid aglycones and changes in the duration of instar and in the mean relative growth rate (RGR). (Values base on the data from the experiment reported in Lahtinen et al., 2004).

distribution of flavonoids in the ethanol-soluble and water-soluble fractions of bud and frass samples is shown in Table 1.

Flavonoids in Ethanol-Soluble Fractions. The ethanol fraction of bud samples contained 12 flavonoid aglycones in variable amounts. The main compound was naringenin 7-methyl ether (285 µg/ml, Table 1) as in the ethanol extract of fully expanded white birch leaves (Valkama et al., 2003). Other predominant compounds in buds were naringenin 4',7-dimethyl ether, kaempferol 3,4'-dimethyl ether, and kaempferol 4'-methyl ether (contents >100 µg/ml). In contrast, as observed in the earlier study with fifth instars (Salminen et al., 2004), the ethanol fraction of frass samples was almost totally free of flavonoid aglycones. Only traces of naringenin mono- and dimethyl ethers were detected. This suggests that nearly all bud flavonoid aglycones were either absorbed into the larval tissues or transformed/degraded into other compounds in the larvae.

Flavonoids in Water-Soluble Fractions. In the water-soluble fractions of both bud and frass samples, the flavonoids were different flavonoid glycosides (Table 1). Bud extracts also showed traces of aglycones. The main glycosides in buds, as in fully expanded birch leaves (Ossipov et al., 1996), were quercetin glycosides. However, according to HPLC-DAD and HPLC-ESI-MS, the main flavonoid glycosides in the frass samples were glycosides of the main bud aglycones (naringenin 7-methyl ether, kaempferol dimethyl ethers, and apigenin 4'-methyl ether). These compounds were not present in the larval leaf diet (Table 1); thus, they are evidently formed in the larvae, as we have previously observed in fifth instar *E. autumnata* (Salminen et al., 2004).

DISCUSSION

Glycosylation in First Instars. Fifth instar *E. autumnata* have the ability to glycosylate foliar flavonoid aglycones into the corresponding glycosides (Salminen et al., 2004). The absence of lipophilic aglycones in the ethanol-soluble frass fraction and the presence of their glycosides in the water-soluble frass fraction (Table 1) confirms that first instars have a similar ability to handle bud flavonoids by glycosylating them. Glycosylation transforms lipophilic compounds into water-soluble ones, which are easier for the larva to excrete. Fifth instar *E. autumnata* excreted foliar flavonoid glycosides almost unaltered (Salminen et al. (2004) and led to the suggestion that all new nonfoliar glycosides in frass were formed from foliar aglycones. However, in the present study myricetin glycosides were not detected in frass samples (Table 1). Thus, it is possible that some of the content of new fecal glycosides also may be the degradation products of foliar flavonoid glycosides. If so, some dehydroxylation

reactions in the B-ring of myricetin glycoside molecules also would have occurred in first instars in addition to glycosylation.

Glycosylation and Larval Performance. In the hypothesis of this study, we suggested that the negative effects of aglycones on larval performance are due to their high levels in birch buds and/or the inability of first instars to glycosylate them. Even though we observed that neonate larvae have the ability to glycosylate, it was impossible to calculate the efficiency of glycosylation without trustworthy data on larval consumption, which in larvae this small are practically impossible to obtain. Because there are a variety of possible factors affecting glycosylation, there may be differences in the glycosylation rates of different aglycones. For example a decrease in number of free hydroxyl groups may diminish glycosylation because *O*-glycosides seem to be more common in *E. autumnata*'s frass than *C*-glycosides (Salminen et al., 2004). In addition, increased *O*-methylation may make molecules less prone to glycosylation (Wollenweber, 1994). Glycosylation may also be affected by the lipophilic character of aglycones; a more lipophilic molecule is more easily absorbed into larval tissues and may not be available for glycosylation in the larval digestive tract.

Lahtinen et al. (2004) found a strong negative correlation between the levels of individual birch bud flavonoid aglycones and the performance (RGR and instar duration) of first instar *E. autumnata*. The correlation coefficients in question are shown in Table 1. The seemingly most harmful compounds [both (absolute) *r* values >0.65] were naringenin 4',7-dimethyl ether, apigenin 4',7-dimethyl ether, and kaempferol 4'-methyl ether. Of these, the first two are highly lipophilic, highly methylated, and have only one free hydroxyl group in their structures. These compounds may be difficult for larvae to glycosylate and can cause negative effects on larval performance because they are not excreted as such.

The amounts of the glycosides naringenin 4',7-dimethyl and apigenin 4',7-dimethyl ether in frass indeed indicates some problems in their glycosylation, e.g., glycosides of apigenin 4',7-dimethyl ether were totally absent from the frass (Table 1). Although no direct correlation was obtained between the amounts of compounds in buds and in frass, the glycosides of the main bud aglycones [i.e., naringenin 7-methyl ether and kaempferol dimethyl ethers (3,4'- and 4',7-dimethyl ethers calculated together)] were the main compounds in frass and it is reasonable to suspect that the levels of aglycones in buds would predict the amounts of the corresponding glycosides in frass if the rate of glycosylation is constant for different aglycones. The level of the glycoside of naringenin 4',7-dimethyl ether in frass is relatively low (12.7 µg/ml) despite its high concentration in buds (140.8 µg/ml) (Table 1) and may indicate a low rate of glycosylation. On the basis of these results, we suggest that the glycosylation ability of first instar *E. autumnata* larvae can depend on the chemical character

of the aglycone and may be an important factor in the successful performance of first instar larvae.

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WHERE DID THE CHILI GET ITS SPICE? BIOGEOGRAPHY OF CAPSAICINOID PRODUCTION IN ANCESTRAL WILD CHILI SPECIES

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Abstract—The biogeography of pungency in three species of wild chili in the chaco and surrounding highland habitats of southeastern Bolivia is described. We report that *Capsicum chacoense*, *C. baccatum*, and *C. eximium* are polymorphic for production of capsaicin and its analogs, such that completely pungent and completely nonpungent individuals co-occur in some populations. In *C. chacoense*, the density of plants and the proportion of pungent plants increased with elevation. Above 900 m, all individuals in all populations except two were pungent; nonpungent individuals in at least one of the two polymorphic populations were likely a result of spreading by humans. The occurrence of pungent and nonpungent individuals in three species of ancestral *Capsicum* and the geographic variation of pungency within species suggest that production of capsaicin and its analogs entails both costs and benefits, which shift from one locality to another. Determining the selection pressures behind such shifts is necessary to understand the evolution of pungency in chilies.

Key Words—Capsaicin, capsaicinoid, PSM, fruit secondary metabolite, fruit chemistry, intraspecific variation, biogeography, spice, *Capsicum chacoense*, *Capsicum baccatum*, *Capsicum eximium*.

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INTRODUCTION

Geographically structured phenotypic variation in plant morphology, physiology, and chemistry has provided a window into the evolution and coevolution of plant–animal interactions (Berenbaum and Feeny, 1981; Berenbaum and Zangerl, 1998; Schemske and Bradshaw, 1999; Thompson and Cunningham, 2002; Fornoni et al., 2004; Siepielski and Benkman, 2004). Such variation is often used to infer constraints on the course of evolution and to discern the relative importance of various traits in the diversification of clades (Futuyma and Mitter, 1996; Berenbaum and Zangerl, 1998). The explicit examination of intraspecific variation in plant chemistry has led to major advances in our understanding of plant–pollinator and plant–herbivore interactions (Lerdau et al., 1994; Adler et al., 1995; Cronin and Hay, 1996; Zangerl et al., 1997; Berenbaum and Zangerl, 1998; Baldwin, 1998; Schemske and Bradshaw, 1999; McDonald et al., 1999; Redman et al., 2001; Cipollini et al., 2002; Rowell and Blinn, 2003; Fornoni et al., 2004). In contrast, studies of intraspecific variation in the chemistry of ripe fruit are almost nonexistent (Izhaki et al., 2002; Tsahar et al., 2002; Tewksbury, 2002), despite the importance of secondary metabolites in fruit–frugivore interactions (Cipollini and Levey, 1997; Cipollini, 2000).

In some taxa, the defining feature of fruit is chemical, and it is in these taxa where variation may have the largest impacts on fruit–frugivore interactions. This is exemplified in *Capsicum*, the wild chilies. *Capsicum* contains 23–27 wild species and more than 2000 cultivars, derived from 3 to 5 of the wild species (Andrews, 1995; DeWitt and Bosland, 1996). Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) and a series of homologous alkyl vanillylamides, called capsaicinoids, are responsible for the heat, or pungency, in chili pepper fruit (Iwai et al., 1979; Kawada et al., 1985; Govindarajan, 1986; Cordell and Araujo, 1993). Given that roughly a quarter of the world's population consume chilies each day (Cordell and Araujo, 1993), capsaicinoids are among the most widely used secondary metabolites in the world. To date, capsaicinoids have only been identified in the genus *Capsicum* (Govindarajan et al., 1987; Govindarajan and Sathyanarayana, 1991; Bosland, 1994). They are produced only in the fruit (Suzuki et al., 1980; Fujiwake et al., 1982) and are concentrated around the seeds and in the pericarp (Fujiwake et al., 1982; Suzuki and Iwai, 1984). Early taxonomies used the presence of capsaicinoids as a defining trait to characterize the genus *Capsicum* (Morton, 1938; Heiser and Smith, 1958), but not all *Capsicum* species produce capsaicinoids (D'Arcy and Eshbaugh, 1974).

Recent investigations support the hypothesis that capsaicinoid production is a monophyletic derived trait, as *Capsicum ciliatum*, a nonpungent species, appears basal to all pungent taxa (Walsh and Hoot, 2001), and pungency has been found in all taxa more derived than *C. ciliatum*. Furthermore, a single quantitative trait loci, *cap*, has been identified and accounts for 34–38% of the

phenotypic variation in capsaicinoid content (Blum et al., 2003). The only comprehensive theory advanced to explain the origin of pungency in *Capsicum* centers on an initial radiation from arid, high-elevation interior valleys of Bolivia (McLeod et al., 1982). The proposed ancestral species in this radiation was a form of *Capsicum chacoense*. More recent work using nuclear and chloroplast DNA supports a radiation of pungent taxa from drier mountainous regions, either in Bolivia or Peru (Walsh and Hoot, 2001), based on the earliest-branching pungent species (*Capsicum eximium* and *C. cardinasii*) and inconclusive bootstrap values separating the baccatum clade (*C. chacoense* and *C. baccatum*). Finally, the late discovery of a natural polymorphism for fruit pungency in an accession of *C. chacoense* from southeastern Bolivia (1959 collection by Paul Smith, USDA #PI260433, polymorphism discovered by P. Bosland, N.M. State) demonstrated that this species may not always have pungent fruit. Although early collectors have noted rare encounters with nonpungent plants (D'Arcy and Eshbaugh, 1974), details of this polymorphism have never been examined. The discovery and description of zones of polymorphism for capsaicinoid production may be a first step in understanding the origination and adaptive significance of capsaicinoids in wild fruit and their role in the radiation of the genus.

Our goal was to rediscover and map the occurrence of nonpungent *C. chacoense*, examine patterns of occurrence in light of natural and human-caused dispersal, and use this as a springboard to examine the biogeography of pungency in a broader taxonomic context. *C. chacoense*, *C. baccatum*, and *C. eximium* comprise three of the five most ancestral pungent *Capsicums* (Walsh and Hoot, 2001). All occur in southeastern Bolivia and northern Argentina, near to where the nonpungent form of *C. chacoense* was inadvertently collected in 1959. Our sampling and analysis focus on *C. chacoense*, and variation in capsaicinoid production across all three species in southeastern Bolivia, is examined.

METHODS AND MATERIALS

Field Sampling. We looked for *Capsicum* in the semiarid chaco and neighboring highland habitats in southwestern Bolivia (19°–22°S, 63°–65°W), sampling 29 natural populations of *C. chacoense*, 15 of *C. baccatum*, and 1 of *C. eximium* from February through April 2002–2005. These three species are easily distinguished by leaf and flower morphology, as well as plant stature. We examined all sampled populations for signs of obvious spatial patterning in the location and size structure of pungent and nonpungent plants that might be used to infer recent colonization of one form or another. Such patterns are described where present. In each population, ripe fruits were tasted from as many fruiting

TABLE 1. LOCALITY AND SAMPLING INFORMATION

ID	Location	Latitude	Longitude	Elevation (m)	Years ^a	n ^b	% P ^c	Density ^d
<i>C. chacoense</i>								
c1	Cerro, Colorado	-19.450	-62.372	396	2002	20	30	Low
c2	Ibasiriri	-19.564	-62.567	432	2002	56	55	Mid
c3	San Julian	-19.769	-62.700	490	2002-2004	41 (32-56)	29 (23-37)	Low
c4	Auga Blanca	-20.130	-62.870	533	2002	99	45	Mid
c5	Yuque	-20.263	-63.251	882	2003-2004	20.5 (20-21)	54 (35-71)	Mid
c6	Kurupaity	-19.628	-63.640	964	2002-2004	17 (10-30)	97 (90-100)	Mid
c7	Petrolero, lavertiente	-21.295	-63.300	371	2002, 2004	20.5 (16, 25)	81 (80-81.5)	Low
c8	Isiri	-21.346	-63.630	650	2003-2004	12.5 (8-17)	85 (75-94)	Low
c9	River ranch	-21.364	-63.635	664	2004	71	82	Mid
c10	Roadside 1	-21.395	-63.656	745	2004	21	100	Low
c11	Roadside 2	-21.445	-63.675	810	2004	17	100	Mid
c12	Roadside 3	-21.459	-63.694	949	2004	14	100	Mid
c13	Roadside 4	-21.456	-63.709	992	2004	16	100	Mid
c14	Palos, Blancos1	-21.43	-63.788	738	2002	130	100	High
c15	Palos, Blancos2	-21.446	-63.783	758	2003	70	100	High
c16	Tres Aguadas	-21.520	-63.781	855	2003-2004	100 per year	100	High
c17	Santa Ana	-21.554	-64.588	1925	2002	100	100	High
c18	Camino, Padkaya	-21.647	-64.623	1764	2002	45	100	High
c19	Calamuchita	-21.698	-64.624	1688	2002	232	62	High
c20	Roadside 5	-21.237	-63.579	502	2004	6		High
c21	Roadside 6	-21.348	-63.637	651	2004	8		High
c22	San Antonio	-21.289	-63.456	386	2005	2		High
c23	Capiatindi	-19.632	-62.608	451	2005	2		High
c24	Mariqui	-19.838	-62.931	583	2005	5		High

c25	Aguarati	-20.127	-63.119	700	2005	2	13 (6-25)	92 (88-100)	Low
c26	Yuque2	-20.290	-63.255	885	2005	1	10	60	Low
c27	Camatindi, Palavecino	-20.328	-63.280	813	2005	4	10	30	Low
c28	Cuevo	-20.448	-63.557	1096	2005	2	10	100	Low
c29	Ibibobo	-21.540	-62.992	343	2005	5			
<i>C. baccatum</i>									
b1	Kurupaity	-19.628	-63.640	964	2002-2004				
b2	Lagunillas	-19.622	-63.674	920	2005				
b3	Muyupampa	-19.883	-63.747	1222	2005				
b4	La Herradura	-19.500	-63.526	870	2005				
b5	Abapo chaco	-18.619	-63.269	594	2004				
b6	Gutierrez	-19.417	-63.530	978	2005				
b7	Roadside 5	-21.237	-63.579	502	2004				
b8	San Miguel	-20.070	-63.885	1016	2005				
b9	Monteagudo	-19.792	-63.993	1174	2005				
b10	Villa Montes	-21.268	-63.519	406	2005				
b11	Carapari	-21.826	-63.744	814	2005				
b12	Entre Rios	-21.517	-64.176	1234	2005				
b13	Cuevo	-20.448	-63.557	1096	2005				
b14	Yuque2	-20.290	-63.255	885	2005				
b15	North Yuque	-19.458	-63.240	828	2005				
<i>C. eximium</i>									
e1	Tarija	-21.417	-64.759	2027	2005		10	90	Low

^aYears sampled.

^bAverage number of plants sampled for pungency each year (range, if more than 1 yr).

^cAverage percent of plants pungent (range if multiple years). Percent pungent and plant density were calculated only for populations where >10 individuals were sampled.

^dDensity was estimated as follows: low is <10 plants per hectare; mid is 10-100 plants per hectare; high is >100 plants per hectare.

plants as possible to determine the ratio of pungent to nonpungent plants. This is an accurate method of capsaicinoid detection because humans can detect these compounds at <1.5 ppb (Scoville, 1912), a concentration far lower than is found in any pungent wild chili (see Results), and capsaicinoids are produced before fruit ripening. In field-testing plants for pungency, false positives are virtually impossible (if a plant lacks capsaicin, it will never taste pungent), but false negatives are possible if a particularly mild pungent plant is tasted after a more pungent plant. To guard against false negatives, multiple fruits from any plant that was determined to lack pungency were sampled, and we had multiple people check the pungency of the fruits. If anyone tasted capsaicin, the plant was considered pungent. In addition, some populations were sampled in multiple years to determine the stability of the ratios of pungent to nonpungent plants (Table 1). Voucher specimens were taken from each population and deposited at the Museo Historia Natural “Noell Kempff Mercado” and the Bolivian National Herbarium.

In one population (Figure 1, c3), permanent tags were affixed to all plants, and these were sampled in multiple years to verify that individual plants consistently produced either pungent or nonpungent fruit. In addition, seeds from pungent and nonpungent plants in five populations were collected and grown under identical conditions to quantify biogeographic variation in the concentrations of various capsaicinoids.

The density of individuals in all populations were estimated based on the area searched and the number of plants encountered. Because of differences in terrain and plant detectability among sites, all populations were classified into broad density classes. Low-density populations had <10 plants per hectare, medium-density populations had between 10 and 100 plants per hectare, and high-density populations had >100 plants per hectare. GPS locations of all populations were determined at the time of collection. In two populations (Table 1, c3 and c19), the location of all plants also was mapped, allowing us to examine fruit chemistry in a spatially explicit format.

Capsaicinoid Profiles of C. chacoense. To verify that nonpungent plants completely lacked capsaicinoids and to test for genotypic differences in capsaicinoid concentrations between populations of *C. chacoense*, seeds from five *C. chacoense* populations were collected and grown in a greenhouse to avoid environmental effects on capsaicinoid production (Harvell and Bosland, 1997). All plants were grown in 4-in. pots filled with uniform clay particles (Turface®, Profile Products LLC, Buffalo Grove, IL, USA) and kept moist through daily watering. The nutrient solution used was a standard 20–10–20 (NPK), rotated weekly with a 17–5–17 blend. Greenhouse temperatures averaged 23.4°C during the day and 17.4°C at night, under a 13:11 light/dark cycle. Fruit production started 90 days after germination. Fruits from pungent and nonpungent plants were analyzed from each population separately. Within

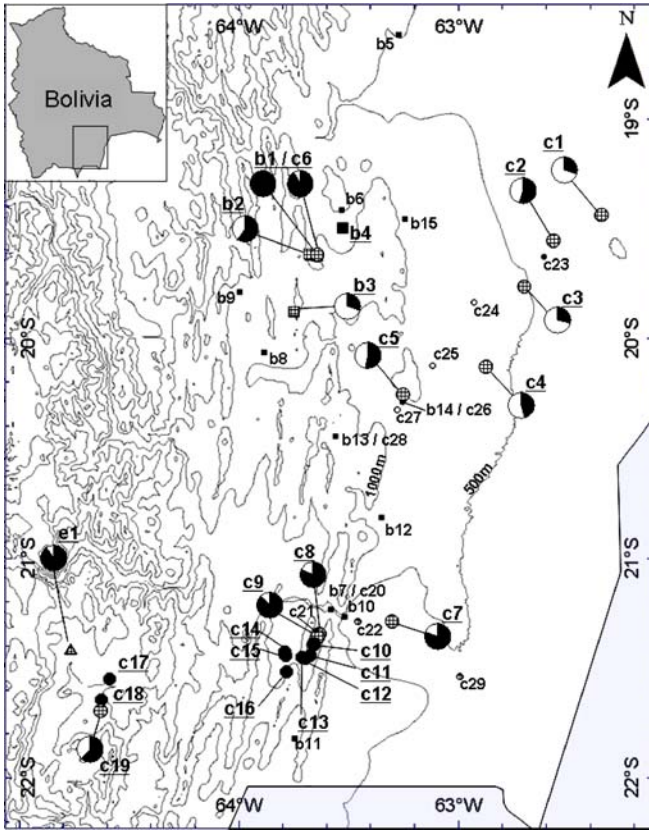


FIG. 1. Pungency distribution in sampled populations of *C. chacoense* (circles), *C. baccatum* (squares), and *C. eximium* (triangle) in southwestern Bolivia. Large symbols indicate populations where ≥ 10 plants were sampled for pungency; small symbols indicate the location of populations in which < 10 plants were sampled. Black symbols are completely pungent populations, hatched symbols are polymorphic populations, and white symbols indicate populations where no pungent plants were sampled. Pie charts indicate percent of plants pungent (black slice) in all polymorphic populations with ≥ 10 plants sampled. Contour lines represent 500-m elevation changes. Letter-number combinations refer to population data in Table 1.

each pungency by location combination, fruits from multiple plants with different maternal genotypes were bulk-sampled, creating a single, homogenous sample for analysis (10–30 genotypes used for each location by pungency combination). All fruit samples were harvested when fully mature, dried 48 hr at 65°C directly after harvest, and ground to a uniform particle size (skin, pulp, and seeds). This powder was then digested in 100% acetonitrile solution for 4 hr

at 85°C, following Collins et al. (1995). Total capsaicinoid concentrations (mg/g dry weight of the chili) and concentrations of capsaicin, dihydrocapsaicin, and nordihydrocapsaicin were measured for each population using an Agilent 1100 high-performance liquid chromatography (HPLC), equipped with an autosampler and an Agilent XDS Zorbax C-18 Column. HPLC conditions and mobile phases followed established protocols for the analysis of individual capsaicinoids (Collins et al., 1995). Concentrations were derived by comparing peak area of samples to external standards of 1 mg/ml for capsaicin and dihydrocapsaicin (Sigma-Aldrich Co.).

Analysis. The relationship between the percent of pungent individuals in a population and elevation (log transformed) was examined using linear regression. Linear regression was also used to examine the effect of elevation on the concentration of total capsaicinoids, as well as the three individual capsaicinoids that were resolved. In this analysis, we corrected for multiple tests on the same data by using sequential Bonferroni adjustment, with a total of four tests (Rice, 1989). The relationship between population density and elevation was examined by using analysis of variance. In the one population where spatial structure was found in the location of nonpungent plants, we mapped the structure and tested for significance using a chi-squared test. Mapping of populations was performed using ArcGIS 9.0.

RESULTS

A total of 1481 *C. chacoense* plants in 28 populations, 93 *C. baccatum* plants in 15 populations, and 10 *C. eximium* plants in a single population (Table 1) were sampled. In all three species, populations composed of plants that produced only pungent fruit and other plants that produced only nonpungent fruits were found (Figure 1). These were termed polymorphic populations.

Biogeography and Habitat Associations of C. chacoense, C. baccatum, and C. eximium. The proportions of pungent plants in all populations in which 10 or more plants were sampled are reported, totaling 21 *C. chacoense* populations, 4 *C. baccatum* populations, and 1 *C. eximium* population. The smaller number of polymorphic *C. baccatum* and *C. eximium* populations prevent detailed analysis of patterns of polymorphism, but the biogeographies of all three species are described, detailing overlapping distributions, where appropriate.

C. chacoense populations were found primarily in xeromorphic chaco forest at lower elevations (Figure 1, c1–c5, c7), grading into montane dry forest in the inter-Andean valleys at higher elevations to the west (Figure 1, c14–c16), and extending to the transitional zones around Tarija (Figure 1, c17–c19). In the lower chaco forests, *C. chacoense* were found in stands dominated by *Schinopsis*, *Aspidosperma*, *Ziziphus*, and *Prosopis*. Other species producing

fleshy fruit concurrent with *C. chacoense* included *Celtis*, *Vallesia*, *Capparis*, and *Ravenna*. In localities where *C. chacoense* was found at intermediate elevations (Figure 1, c8–c16), the vegetation was similar to lowland chaco, with increasing moisture toward the west (Figure 1, c16–c18). Here, chilies were found in woodlands with extensive *Prosopis*, *Schinopsis*, and *Bromelia*. Between these two dry ecoregions, and along the eastern flank of the Andes, *C. baccatum* was found, located in moister habitat (Ribera et al., 1994). In the transition between the wet yungas and the dry chaco, *C. chacoense* and *C. baccatum* co-occurred (Figure 1, b1/c6, b14/c26, b13/c28, b7/c20). At higher elevations around Tarija, *C. chacoense* (Figure 1, c17–c19) and the lone population of *C. eximium* (e1) were found in a more open habitat characterized by *Prosopis nigra* and other leguminous trees. In these habitats, *Commiphora*, *Celtis*, *Cassia*, and *Capparis* were all seen fruiting at the same time as *Capsicum*.

Pungency, Plant Density, and Elevation. The proportion of pungent plants, the capsaicinoid content of pungent fruits, and the density of chili plants all increased with elevation for *C. chacoense* (Figure 2A and B). In all but two populations higher than 800 m, greater than 90% of the plants were pungent, creating a bounded association between proportion of plants pungent and elevation (Figure 2A). This relationship can be decomposed into populations below 800 m, where a significant increase in pungency with increased elevation was found (Figure 2; adj $r^2=0.53$, $P=0.01$; 10 populations < 800 m), and a zone of consistent high incidence of pungency above 800 m (Figure 2A).

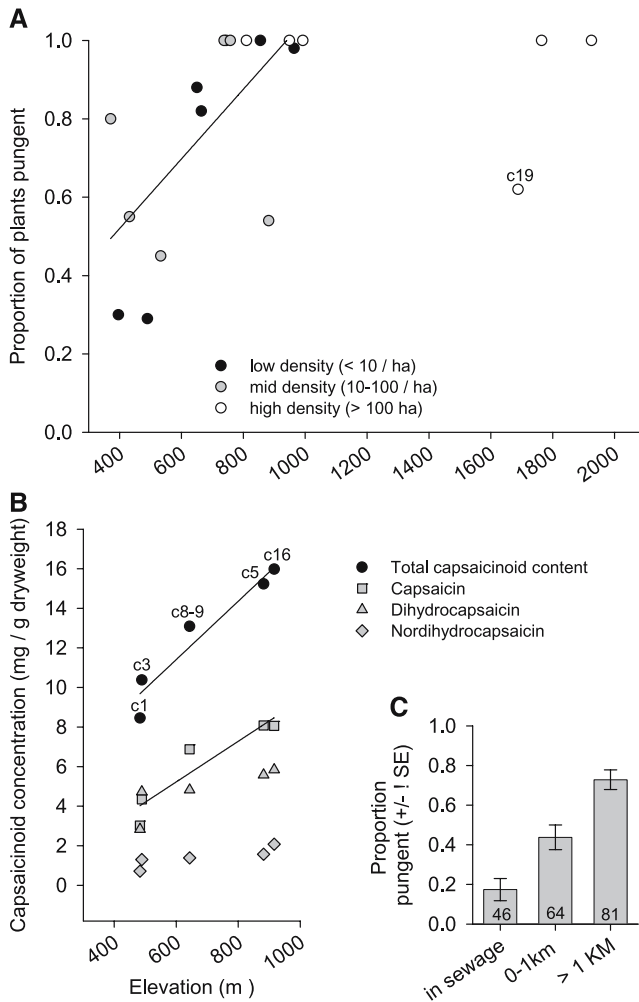
Population density also increased with elevation (Figure 2A; $F_{2,16}=6.8$, $P=0.007$); the mean elevation of low-density populations (<10 plants per hectare) was 530 m ($N=5$, $SE=73$ m), the mean elevation of mid-density populations (10–100 plants per hectare) was 778 m ($N=8$, $SE=75$ m), and high-density populations (>100 plants per hectare) averaged 1288 m in elevation ($N=6$, $SE=228$ m). Thus, compared to lower elevation populations, higher-elevation populations generally had more plants, and more of these were pungent.

In the five populations in which capsaicinoid production was analyzed, nonpungent plants were completely free of capsaicinoids, and total capsaicinoid content increased significantly with increasing elevation (adj $r^2=0.90$, $P=0.009$). All three capsaicinoids generally increased in higher-elevation populations (Figure 2B), with capsaicin showing the largest change with increasing elevation (adj $r^2=0.83$, $P=0.021$). This change was marginally significant after correction for multiple tests (Rice, 1989). Increases in dihydrocapsaicin and nordihydrocapsaicin with elevation (adj r^2 's=0.56 and 0.63, respectively) were not significant (P 's > 0.05).

In pungent plants, capsaicin was the most common of the capsaicinoids in four out of five populations (Figure 2B), averaging 6.07 ± 0.46 mg/g dry mass in

a ripe chili or $46.7 \pm 1.5\%$ of the total capsaicinoid content. Dihydrocapsaicin was the next most common, averaging 4.76 ± 0.24 mg/g dry mass or $37.8 \pm 0.9\%$ of the total capsaicinoid content, and nordihydrocapsaicin made up most of the remainder, averaging 1.4 ± 0.09 mg/g dry mass or $11.0 \pm 0.4\%$ of the total capsaicinoid content. In populations c3 where we sampled the same plants repeatedly over 4 yr, all plants consistently produced either pungent or nonpungent fruit ($N=25$ plants sampled across 3 yr, $N=15$ across 4 yr).

Dispersal by Humans. In one of the high-elevation polymorphic populations of *C. chacoense* (c19), the distribution of nonpungent plants appeared



highly clumped around two large (>1.4 m) nonpungent plants growing in a sewage outflow drainage for the town of Calamuchita. We sampled this population extensively to examine this pattern (232 plants sampled for pungency). Nonpungent plants in this population ($N = 88$) were concentrated around the two large plants (Figure 2c; $\chi^2_{2,189} = 35.1$, $P < 0.005$), and pungent plants became more and more dominant at increasing distance from these two large nonpungent plants (Figure 2c). Except for these two large individuals, all other nonpungent plants were small (<60 cm in height). This clumped pattern was not evident in other populations, and in the one low-elevation population in which we marked and mapped plants explicitly (c3, 123 plants), we found no clumping of nonpungent or pungent plants.

DISCUSSION

Genetic regulation of pungency occurs at a single locus, *C*, in *Capsicum annum* where accessions recessive for *c* fail to produce capsaicinoids (Blum et al., 2002). The gene required for production of capsaicinoids in cultivated chilies is *pun1* (Stewart et al., 2005). This understanding of the genetic control of pungency in *C. annum*, a highly derived species, may not apply to wild populations of *C. chacoense*, *C. baccatum*, and *C. eximium*, where our discovery of polymorphism for pungency demonstrates that pungency is not consistently present in more ancestral taxa of *Capsicum*. Of the five most ancestral wild species (Walsh and Hoot, 2001), *C. chacoense*, *C. baccatum*, and *C. eximium* are the only species with relatively wide ranges. *C. chacoense* is found throughout the chaco in Paraguay and Argentina (McLeod et al., 1982)

FIG. 2. (a) Proportion of plants in *C. chacoense* populations that are pungent, as a function of the elevation, for all populations with ≥ 10 plants sampled. Two high-elevation polymorphic populations are labeled. Linear fit is included for all populations <1200 m ($r^2 = 0.47$, $P = 0.0034$). (b) Concentration (mg/g dry mass) of total capsaicinoids (black circles), and the three individual capsaicinoids found in all three populations: capsaicin (gray squares), dihydrocapsaicin (gray triangles), and nordihydrocapsaicin (gray diamonds), as a function of the population elevation, for five populations grown from seed in identical greenhouse conditions (population labels indicated above black circles). Linear fit lines indicate significant relationships between elevation and the concentration of total capsaicinoids, and the primary capsaicinoid, capsaicin. (c) The distribution of nonpungent plants in relation to two large plants found in a sewage discharge drainage basin is shown for population c19. Bars are arcsine square-root-transformed percentage of plants pungent, error bars indicate one standard error of the mean, and number of plants sampled is indicated within the bars.

and as far west as Tarija and Sucre. According to McLeod et al. (1982, 1983), *C. chacoense* may be ancestral to *C. baccatum* and *C. eximium*. *C. baccatum* is also found in Paraguay and Argentina, but extends further north and west than *C. chacoense*—being found in Brazil and Peru as well (Eshbaugh, 1970), where it may have given rise to *C. tovarii*, a species endemic to southern Peru (Eshbaugh et al., 1983). Based on hybridization studies, *C. tovarii* is tightly linked to the *C. baccatum* complex (Tong and Bosland, 1999). *C. eximium* is a higher-elevation species, extending from the mountains in Argentina through the highlands in Bolivia, Peru, and Ecuador (Eshbaugh, 1976). *C. eximium* may be the progenitor of *C. cardinasii*, an endemic found only in the La Paz region in Bolivia with which it crosses readily (Eshbaugh, 1976; Walsh and Hoot, 2001).

If this interpretation is correct, the three most ancestral species of pungent chilies all have both pungent and nonpungent forms. Furthermore, while both *Capsicum cardenasii* and *C. tovarii* are reported as pungent, systematic sampling of these species has not occurred; they may be polymorphic, too. This interpretation fits well with McLeod et al. (1982) suggestion of a “nuclear area” of inter-Andean valleys northeast of Sucre, where divergent selection on ancestral *C. chacoense* led to both the purple-flowered group (*C. eximium*) and the white-flowered group (*C. baccatum*). According to this hypothesis, *C. chacoense* gave rise to *C. eximium*, which expanded into the dry highlands, and the white-flowered group (*C. baccatum*), which occupies semimoist lowlands. McLeod et al. further suggest that *C. baccatum*, through eventual migration down river to the Rio Grande, then into the moist tropics of the Amazon basin, radiated to form the moisture-loving *C. annum* group found in Brazil. This hypothesis relies on the importance of ecotones and heterogeneity in the physical environment in the creation of geographically explicit selection pressures (Stebbins and Major, 1965; Stebbins, 1972). Molecular data are currently insufficient to test this hypothesis (Walsh and Hoot, 2001), but the existence of polymorphic populations in *C. chacoense*, *C. baccatum*, and *C. eximium* refocuses attention to this area as a zone where early evolution of pungency may have occurred.

A more complete understanding of the early evolution of capsaicinoid production will require additional information on the link between nonpungent species, such as *C. ciliatum* (D’Arcy and Eshbaugh, 1974) and this clade of polymorphic species that appears to be ancestral to pungent *Capsicum* (Walsh and Hoot, 2001). Still, the finding that the first pungent *Capsicum* may all exhibit polymorphisms for capsaicinoid production has several implications for the ecology and evolution of these secondary metabolites.

The clinal variation in capsaicinoid production seen in *C. chacoense* and the existence of polymorphic populations of *C. baccatum* and *C. eximium* both suggest that capsaicin production is not uniformly beneficial. Furthermore, *C.*

chacoense populations are diffuse and widely scattered in the lowland chaco, and yet consistently exhibit polymorphisms for pungency, with at least 25% of the plants pungent in all populations in which at least 10 plants were sampled (Table 1). This pattern suggests either some form of frequency-dependent selection on pungency that prevents the pungent or nonpungent form from becoming fixed in the population, or some form of oscillating selection pressure, in which conditions favor pungent plants at some times and nonpungent plants at others, regardless of the frequency of the two phenotypes (Futuyma, 1998).

Teasing apart these possibilities will require a greater knowledge of the selective pressures acting on the entire fruit phenotype and the costs and benefits of secondary metabolite production (Berenbaum, 1995). Although the array of potential selective pressures on fruit secondary metabolites has been outlined (Cipollini and Levey, 1997; Cipollini, 2000), the full array of adaptive hypotheses has not been addressed in any system. The work that comes closest is that on emodin in *Rhamnus* fruit (Izhaki, 2002; Izhaki et al., 2002; Tsahar et al., 2002). This is the only system in which the effects of intraspecific variation in fruit secondary metabolites have been studied systematically. Variation in emodin concentrations among plants is correlated with nutrient concentrations (Izhaki et al., 2002). It affects both invertebrate and vertebrate fruit consumers (Tsahar et al., 2002, 2003) and shows intra- and interannual variations in its effect on consumers (Tsahar et al., 2002). These complex selective environments suggest that multiple adaptive functions may be the norm for secondary metabolite production (Schmitt et al., 1995; Cipollini and Levey, 1997), but a general theory of the pressures governing this variation has yet to appear and is unlikely until we place the adaptive benefits into a mechanistic framework that includes not only the benefits, but also the costs and potential trade-offs of secondary metabolite production (Berenbaum, 1995).

The discovery of three basil lineages all with polymorphisms for the production of a major secondary metabolite creates an unprecedented opportunity to examine both the costs and benefits of secondary metabolite production and to test these theories in three closely related taxa. Furthermore, in *C. chacoense*, nonpungent plants dominate populations in low-elevation chaco, and, among pungent plants, capsaicinoids (particularly capsaicin) occur at relatively low concentrations. As elevation increases, plant density increases, pungent plants dominate, and capsaicinoid concentrations become higher. These correlated patterns further suggest shifts in the relative costs and benefits of capsaicinoid production across elevation, and they may point to developmental or allocation trade-offs during fruit maturation. Because the pungent forms of all three of these species are harvested and consumed by locals, and *C. baccatum* (var. *baccatum*) has been domesticated (*C. baccatum* var. *pendulum*), human movement of genotypes might obscure evolutionary relationships. This applies equally to nonpungent forms of these species, as nonpungent plants are

indistinguishable from the pungent forms, except by tasting the fruit. We see possible evidence of the influence of humans in Calamuchita (c19) where nonpungent plants were concentrated around two large nonpungent individuals in the sewage outflow, suggesting that the large nonpungent plants may have arrived through human consumption of nonpungent chilies from elsewhere, followed by a gradual radiation from these two founders. This interpretation is speculative, but *Capsicum* are typically long-lived plants in the wild (Tewksbury et al., 1999), providing sufficient opportunity for establishment and subsequent local radiation to occur. More rigorous explanations will have to wait for information on relatedness and gene flow between populations. The existence of nonpungent forms at high elevations in two locations (c5 and c19) does suggest that whatever selective forces are responsible for the general shift from polymorphic to completely pungent populations with increasing elevation are not uniform: nonpungent plants can grow and reproduce in some high-elevation locations.

The clinal variation in pungency and percent of pungent individuals suggest either a lack of gene flow that prevents mixing of the pungent and nonpungent forms across this gradient, or a shift in selective pressures such that both pungent and nonpungent forms are maintained in the lowland chaco, but selection favors pungent plants at most high-elevation locations. We view the former explanation as unlikely for the entire gradient, given the clinal pattern across elevation and the fact that the distances between polymorphic and completely pungent populations are often small. For example, population c9, in which 18% of plants sampled lacked pungency, is less than 30 km from populations c14 and c15, in which 200 plants were sampled and all were pungent. Between these two populations, we found small populations of chilies that were totally pungent (Figure 1; populations c10–c13). The major dispersers of chili seeds in Bolivia are *Elaenia parvirostris* and *Turdus amaurochalinus*, both austral migrants (Jahn et al., 2002). Thirty kilometers is not an unreasonable distance for a migrant bird to carry seeds, and given the existence of suitable chili habitat between these populations, there appears to be ample opportunity for gene flow between populations, even for a predominantly self-pollinated species such as *C. chacoense* (McLeod et al., 1982).

If changes in capsaicinoid production are instead due to changes in selective pressures acting on fruit phenotypes, it presents a rare opportunity to identify and characterize the selective pressures that favor the production of a major secondary metabolite. We know that birds readily eat both pungent and nonpungent chilies (Tewksbury and Nabhan, 2001; Tewksbury unpublished data). Many aspects of the biotic and abiotic environment likely vary across this 1000-m altitudinal gradient, any one of which might shift in favor of pungent plants at higher elevations. For example, nonpungent fruits in polymorphic populations are much more likely than pungent fruits to be damaged by fungi

(Tewksbury and Levey, unpublished data), and the risk of damage may vary across elevation. In addition, in one polymorphic population (c3), capsaicinoids appear to protect seeds from seed predators when fruits fall directly to the ground (Tewksbury and Levey, unpublished data). All of these results suggest complex interactions among different aspects of fruit phenotype. Fruit removal rates, invertebrate seed predation, and fungal pathogen load may all change with elevation and interact with plant density, which varies by more than an order of magnitude from diffuse low-elevation populations to dense high-elevation populations. Thus, there are potentially many biotic and abiotic selective pressures that could influence the chemical phenotype of chili fruits across this gradient.

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DIEL PERIODICITY IN THE PRODUCTION OF GREEN LEAF VOLATILES BY WILD AND CULTIVATED HOST PLANTS OF STEMBORER MOTHS, *Chilo partellus* AND *Busseola fusca*

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Abstract—The volatile chemicals produced by four poaceous plant species, blue thatching grass, *Hyparrhenia tamba*, Napier grass, *Pennisetum purpureum*, sorghum, *Sorghum bicolor*, and maize, *Zea mays*, which are host plants for the lepidopterous stemborers, *Chilo partellus* and *Busseola fusca*, were collected by air entrainment and analyzed by gas chromatography. The total quantities of volatiles collected hourly, over a 9-hr period, from *P. purpureum* and *H. tamba* showed an approximately hundredfold increase in the first hour of the scotophase. Thereafter, the amount decreased rapidly to levels present during photophase. Although onset of scotophase also triggered an increase in quantities of volatiles collected from two cultivars of *S. bicolor* and two out of three cultivars of *Z. mays*, these increases were less dramatic than in the two wild grasses, being only up to 10 times as much as in the last hour of photophase. Analysis showed that up to 95% of the increase in volatiles at the onset of the scotophase was due to just four compounds, the green leaf volatiles hexanal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexen-1-yl acetate, with the latter dominating the volatile profile. Volatiles from *P. purpureum* were also collected at 10-min intervals for 70 min spanning the transition from light to dark. The vast increase in production of the green leaf volatiles in this species occurs in the first 10 min of the scotophase followed by a rapid decline within the next 20 min. The relevance of these results to the control of stemborers in a “push–pull” strategy is discussed.

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Key Words—Stemborer, maize, sorghum, diel periodicity, green leaf volatile, oviposition, host location.

INTRODUCTION

Maize, *Zea mays* L., and sorghum, *Sorghum bicolor* Moench, are essential food and cash crops for millions of people in the largely mixed crop–livestock farming systems of sub-Saharan Africa. Lepidopterous stemborers, predominantly the indigenous *Busseola fusca* Fuller (Noctuidae) and the introduced *Chilo partellus* (Swinhoe) (Crambidae), are major constraints to efficient production causing reported losses of 20–40% in maize in the region (Seshu-Reddy and Sum, 1992), the latter being more injurious (van den Berg et al., 1991). Stemborers are difficult to control, largely because of the cryptic and nocturnal habits of the adult moths and the protection provided to the immature stages by the stem of the host plant (Ampofo, 1986; Seshu-Reddy and Sum, 1992). A “push–pull” system for controlling stemborers in African smallholder maize and sorghum production has been established (Khan et al., 2000, 2001). This involves the use of a trap crop (pull) surrounding the plot and repellent (push) plants as intercrops between the rows of sorghum or maize. A number of wild hosts can be used as the trap crop, including Napier grass, *Pennisetum purpureum* Schumach, which are considerably more attractive to gravid female stemborer moths than the crop itself (Ndemah et al., 2002; Rebe et al., 2004). Although the semiochemicals mediating the repellent effects of intercrops such as *Melinis minutiflora* Beauv. and *Desmodium uncinatum* (Jacq.) have been identified (Khan et al., 2000; Khan and Pickett, 2004), the basis for the differential attraction/oviposition between wild and cultivated hosts is not understood.

Many insects show different sensitivities to host-derived semiochemicals throughout the day, and this sensitivity can be under the control of circadian clocks (reviewed by Lazzari et al., 2004). Most oviposition by gravid females of *C. partellus* takes place during the first 2 hr of scotophase (Päts, 1991), and this behavior may be an example of such effects. However, it may also be that this insect and other stemborer species showing similar behavior have adapted to take advantage of the release of volatile cues by host plants. Physiologically active volatile chemicals of host plants have been identified (Birkett et al., unpublished data), and the current investigation into the temporal pattern of their emission was undertaken to determine whether a transient release of volatile chemicals could be attracting moths at the time of day appropriate for host location.

METHODS AND MATERIALS

Plants. Plant species used in this study were maize, *Z. mays* (Western Seed Company Hybrids 502 and 909, and a UK cultivar, Earliking), sorghum, *S. bicolor* (var. Serredo and Gadam), Napier grass, *P. purpureum*, and blue thatching grass, *Hyparrhenia tamba*. Seeds were planted individually for maize and sorghum and in a group for *H. tamba* in plastic pots filled with compost. *P. purpureum* was grown from cut stems planted in compost, 2–3 in a pot. All plants were grown in an insect- and pathogen-free glasshouse under controlled conditions with a 16-hr photoperiod, 60% relative humidity, and 27–30°C during photophase and 22–25°C during scotophase. Supplementary lighting, comprising sodium lamps (SON-T, 600 W) that gave photosynthetically active radiation (PAR) of approximately 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ at bench height, was used, and the lights were switched off at 20:30 hr, approximately 30 min after sunset but approximately 30 min before civil twilight, so that the light–dark transition was relatively sharp. Plants used in experiments were 19–27 d old, although the ages of plants from the same species were within 1–2 d of each other.

Collection of Headspace Volatiles from Intact Plants. Volatile compounds were collected via entrainment of intact plants by using portable equipment developed at Rothamsted (Agelopoulos et al., 1999; Birkett et al., 2003). To avoid causing plant stress by transfer, all entrainments were performed at the site where the plants were being grown. Part of the plant was enclosed in an open-bottomed glass cylinder (100 mm OD \times 600 mm), and the bottom was closed with two semicircular aluminum plates that fitted closely together, except for a hole in the middle that fitted around the plant. To accommodate stems of different shapes and sizes, the holes comprised circular openings of 2 cm (for *H. tamba*) and 1 cm diam (for *P. purpureum* and *S. bicolor*), and an elliptical opening of 2 \times 1.3 cm (for *Z. mays*). Any large gaps between the plates and the stem(s) were packed with silanized and baked glass wool. Air was pumped into the vessel through an activated charcoal filter, via a port in one of the aluminum plates, at 1.2 l min^{-1} , and volatiles were collected on Tenax TA (60/80 mesh, 0.05 g) packed into a glass tube (80 mm \times 5 mm OD) inserted into a collection port at the top of the vessel. Air was drawn out through the collection tube by another pump at 1 l min^{-1} . The differential flow rates eliminated the risk that unfiltered air could be drawn into the vessel from outside, while obviating the need for an injurious tight seal around the stem(s). All connections were made with polytetrafluoroethylene (PTFE) tubing and ferrules, and as much as possible of the equipment, especially the glassware, was heated at 180°C for at least 5 hr before use. Glassware was washed in soapy water and rinsed with

TABLE 1. TIME COURSE OF TOTAL VOLATILE PRODUCTION BY WILD AND CULTIVATED HOST PLANTS

Time period	<i>P. purpureum</i>	<i>H. tamba</i>	<i>S. bicolor</i> (Gadam)	<i>S. bicolor</i> (Serredo)	<i>Z. mays</i> (909)	<i>Z. mays</i> (502)	<i>Z. mays</i> (Earlicking)
14:30–15:30	7.48 ± 1.66 ^a	124.73 ± 32.72 ^a	22.14 ± 1.60 ^a	66.57 ± 4.79 ^a	26.14 ± 5.72 ^a	40.97 ± 10.36 ^a	85.57 ± 16.34 ^a
15:30–16:30	6.85 ± 2.74 ^a	85.53 ± 29.86 ^{ab}	14.36 ± 1.79 ^a	71.36 ± 9.91 ^a	25.72 ± 4.47 ^a	35.97 ± 3.45 ^a	66.68 ± 10.72 ^{ab}
16:30–17:30	4.01 ± 0.83 ^a	58.75 ± 25.92 ^{ab}	18.75 ± 5.29 ^a	46.58 ± 8.75 ^a	22.13 ± 7.51 ^a	37.09 ± 7.24 ^a	67.84 ± 12.04 ^{ab}
17:30–18:30	3.67 ± 1.22 ^a	40.48 ± 11.90 ^{ab}	20.74 ± 6.68 ^a	47.57 ± 7.76 ^a	21.07 ± 6.87 ^a	33.65 ± 6.37 ^a	58.87 ± 6.35 ^{abc}
18:30–19:30	3.28 ± 1.27 ^a	32.09 ± 15.41 ^b	17.05 ± 4.99 ^a	53.68 ± 13.64 ^a	16.92 ± 9.45 ^a	39.6 ± 6.18 ^a	42.53 ± 9.26 ^{abc}
19:30–20:30	2.93 ± 0.98 ^a	23.60 ± 5.63 ^b	14.36 ± 4.39 ^a	37.66 ± 6.11 ^a	24.11 ± 8.90 ^a	26.66 ± 4.77 ^a	32.9 ± 2.63 ^{abc}
20:30–21:30	633.00 ± 230.53 ^b	3237.2 ± 333.7 ^c	211.21 ± 109.49 ^b	353.82 ± 81.10 ^b	56.61 ± 9.50 ^a	57.53 ± 10.3 ^a	28.6 ± 8.97 ^{bc}
21:30–22:30	11.44 ± 5.67 ^a	51.74 ± 1.91 ^{ab}	7.78 ± 2.09 ^a	99.60 ± 21.80 ^c	5.99 ± 2.17 ^a	24.67 ± 6.63 ^a	19.19 ± 8.24 ^c
22:30–23:30	10.46 ± 4.30 ^a	38.36 ± 13.17 ^{ab}	10.27 ± 4.19 ^a	101.41 ± 22.3 ^c	6.36 ± 2.77 ^a	23.71 ± 3.45 ^a	22.03 ± 9.26 ^c

Results are in ng g⁻¹ h⁻¹, estimated (mean ± SE, *N* = 5 except for *Z. mays* (502), where *N* = 3). Within each species, means bearing the same superscript are not significantly different, *P* < 0.001. Scotophase began at 20:30.

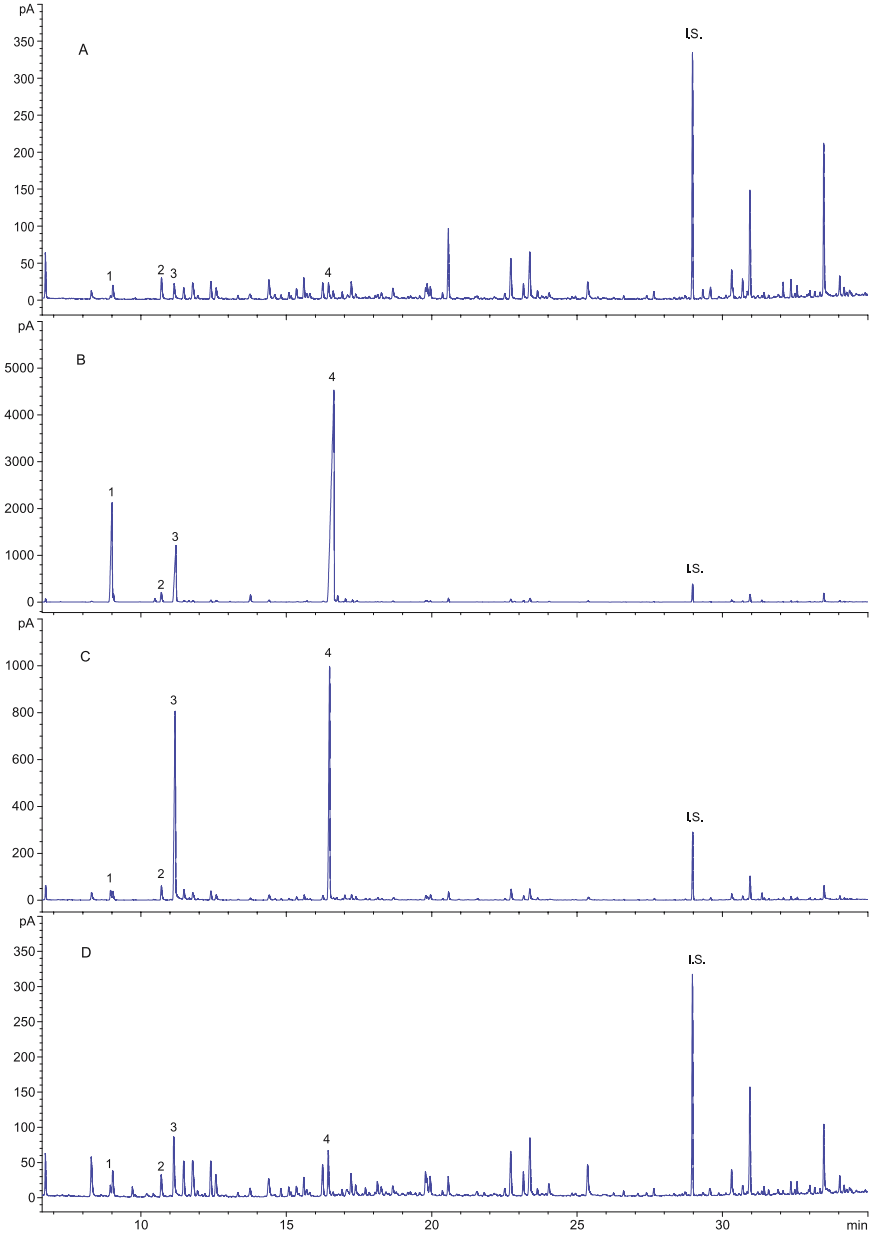


FIG. 1. Gas chromatograms (HP-1 column) of 1-h entrainments of the volatiles from *P. purpureum* collected (A) in the last hour of the photophase, (B) in the first hour of the scotophase, (C) in the second hour of the scotophase, (D) in the third hour of the scotophase. Numbered peaks: 1, hexanal; 2, (*E*)-2 hexenal; 3, (*Z*)-3-hexen-1-ol; 4, (*Z*)-3-hexen-1-yl acetate. (Different y-axes for each chromatogram).

TABLE 2. TIME COURSES FOR THE PRODUCTION OF GREEN LEAF VOLATILES BY *P. purpureum*, *H. tamba*, *S. bicolor*, AND *Z. mays*

Compound	14:30–	15:30–	16:30–	17:30–	18:30–	19:30–	20:30–	21:30–	22:30–	23:30–
	15:30	16:30	17:30	18:30	19:30	20:30	21:30	22:30	23:30	
(a) <i>P. purpureum</i>										
Hexanal	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	5.48 ± 1.76 ^b	0.02 ± 0.01 ^a	0.03 ± 0.01 ^a	nd ^a
(<i>E</i>)-2-Hexenal	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	6.52 ± 2.16 ^b	nd ^a	nd ^a	nd ^a
(<i>Z</i>)-3-hexen-1-ol	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	51.79 ± 15.93 ^b	2.14 ± 0.90 ^a	0.54 ± 0.19 ^a	0.57 ± 0.57
(<i>Z</i>)-3-Hexen-1-yl acetate	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	528.35 ± 205.26 ^b	1.32 ± 0.37 ^a	0.57 ± 0.23 ^a	0.57 ± 0.23 ^a
(b) <i>H. tamba</i>										
Hexanal	0.09 ± 0.09 ^a	0.09 ± 0.09 ^a	0.15 ± 0.15 ^a	0.05 ± 0.05 ^a	0.09 ± 0.09 ^a	0.05 ± 0.05 ^a	16.88 ± 4.38 ^b	0.06 ± 0.03 ^a	0.06 ± 0.03 ^a	0.06 ± 0.03 ^a
(<i>E</i>)-2-Hexenal	0.20 ± 0.07 ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	12.53 ± 2.94 ^b	nd ^a	nd ^a	nd ^a
(<i>Z</i>)-3-Hexen-1-ol	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	77.00 ± 15.55 ^b	9.34 ± 6.08 ^c	1.85 ± 1.46 ^{ac}	3.01 ± 0.96 ^a
(<i>Z</i>)-3-Hexen-1-yl acetate	5.32 ± 1.89 ^a	10.80 ± 6.67 ^a	3.67 ± 1.21 ^a	3.25 ± 0.78 ^a	3.58 ± 1.55 ^a	2.16 ± 0.73 ^a	2957.7 ± 695.59 ^b	7.76 ± 0.87 ^a	3.01 ± 0.96 ^a	3.01 ± 0.96 ^a

(c) <i>S. bicolor</i> , Gadam											
Hexanal	0.12 ± 0.03 ^a nd ^a	0.26 ± 0.19 ^a nd ^a	0.44 ± 0.22 ^a nd ^a	0.25 ± 0.15 ^a nd ^a	0.29 ± 0.17 ^a nd ^a	0.19 ± 0.11 ^a nd ^a	11.70 ± 8.06 ^b 4.08 ± 2.37 ^b	0.05 ± 0.01 ^a nd ^a	nd ^a		
(<i>E</i>)-2-Hexenal	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	29.07 ± 20.90 ^b	0.49 ± 0.31 ^a	nd ^a		
(<i>Z</i>)-3-hexen-1-ol	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	58.23 ± 37.23 ^b	0.41 ± 0.10 ^a	nd ^a		
(<i>Z</i>)-3-Hexen-1-yl acetate	0.59 ± 0.09 ^a	0.56 ± 0.07 ^a	0.67 ± 0.13 ^a	0.78 ± 0.19 ^a	0.68 ± 0.08 ^a	0.71 ± 0.13 ^a			nd ^a		
(d) <i>Z. mays</i> , 909											
Hexanal	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	0.45 ± 0.18 ^a	nd ^a	nd ^a		
(<i>E</i>)-2-Hexenal	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	0.41 ± 0.13 ^a	nd ^a	nd ^a		
(<i>Z</i>)-3-Hexen-1-ol	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	13.82 ± 3.56 ^b	nd ^a	nd ^a		
(<i>Z</i>)-3-Hexen-1-yl acetate	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	nd ^a	35.84 ± 8.99 ^b	0.16 ± 0.02 ^a	nd ^a		

Results are in ng g⁻¹ h⁻¹, actual amounts (mean ± SE, N = 5). Within each species and compound, means bearing the same superscript are not significantly different, P < 0.001. Scotophase began at 20:30.
 nd: below the detection limits of 0.02 ng g⁻¹ h⁻¹.

distilled water and then acetone before conditioning. Tenax tubes were washed with redistilled diethyl ether and conditioned at 220°C with filtered nitrogen pumped through for 2 hr before use. After each collection, water vapor was removed from the Tenax tubes by passing filtered air through them for 30 min at approximately 500 ml min⁻¹.

In the first series of experiments, plant volatiles were collected for 1-hr periods for 9 hr, the last 6 hr of photophase, and the first 3 hr of scotophase. In these experiments, plants were inserted into the entrainment vessels 3 hr before collection of volatiles commenced, in order to minimize any effects due to the initial stress on the plants caused by manipulating them into the entrainment vessel. Each experiment was replicated five times with different plants, except for *Z. mays*, hybrid 502, for which there were only three replicates. When the entrainment was finished, the part of the plant entrained was excised and its fresh weight recorded.

A second series of short entrainment experiments was performed only with *P. purpureum* (five plants), since this is the main trap crop used in the “push-pull” strategy. In these, volatile collections were made for 10-min periods for 70 min starting 20 min before the transition from light to dark and 1 hr or more after inserting the plant into the vessel.

Analysis of Volatiles. Gas chromatography (GC) of the collected volatiles was carried out on a nonpolar (HP-1, 50 m × 0.32 mm ID × 0.52 μm film thickness) capillary column using an HP6890 GC (Agilent Technologies, UK) fitted with a flame ionization detector (FID). Volatiles collected on the Tenax absorbent tubes were transferred directly onto the GC column by thermal desorption using an Optic 2 programmed temperature vaporization (PTV) inlet (Anatune, UK), programmed for rapid heating from 30 to 220°C in 12 sec. Each Tenax tube was spiked with 100 ng *n*-tetradecane (in 0.5 μl redistilled hexane), as an internal standard, before inserting the tube into the PTV. Compounds of interest had already been identified by GC-mass spectrometry, and coinjection with solutions of volatiles collected in the same way but on Porapak Q and over a longer time period (Birkett et al., unpublished data).

Statistics. For analysis, a one-way analysis of variance (ANOVA) was applied. Least significant difference (LSD), at the 1% level, between means on the transformed scale, was used to assess comparisons of interest.

RESULTS

Nine-Hour Time-Course Entrainments. The results for the total amounts of volatiles collected in each hour on a per-gram fresh weight basis are shown in Table 1. Since it was not possible to determine response factors for all

components, the amounts shown are estimates based on the total area under the peaks compared with the peak area of the internal standard (100 ng). This assumes the same detector response to every compound. There was considerable plant to plant variation in the quantity of volatiles emitted (from the SE associated with the means), despite the plants being the same age and chosen for their similarity in size. Even so, the differences between the amounts emitted in the first hour of scotophase and the amounts emitted in the last hour of photophase are highly significant except in all three cultivars of maize.

Further analysis showed that the large increases in total volatile production are due to just four compounds (e.g., Figure 1): hexanal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexen-1-yl acetate. Table 2 shows the time course for the emission of each of these compounds by the four species, although only one cultivar for sorghum and maize. Here, results are absolute amounts (as opposed to the estimates in Table 1) of the four compounds since the response factor for each compound was determined relative to the internal standard, and the results corrected accordingly. In all four plant species, (*Z*)-3-hexen-1-yl acetate showed the largest increase relative to the other compounds, although in *P. purpureum* and *H. tamba*, the, albeit smaller, increases in (*Z*)-3-hexen-1-ol are slower to decline, still showing a slight elevation in the third hour of scotophase (Table 2).

TABLE 3. TIME COURSE OF VOLATILE PRODUCTION BY *P. purpureum* USING SHORT ENTRAINMENT TIMES

Time period	Total volatiles	Hexanal	(<i>E</i>)-2-Hexenal	(<i>Z</i>)-3-Hexen-1-ol	(<i>Z</i>)-3-Hexenyl acetate
20:10–20:20	4.99 ± 0.73 ^{ad}	nd ^a	nd ^a	nd ^a	nd ^a
20:20–20:30	3.81 ± 0.64 ^{ad}	nd ^a	nd ^a	nd ^a	nd ^a
20:30–20:40	1601.66 ± 165.33 ^b	161.26 ± 21.07 ^b	55.76 ± 13.97 ^b	155.05 ± 31.42 ^b	963.70 ± 122.40 ^b
20:40–20:50	504.55 ± 90.32 ^c	nd ^a	0.15 ± 0.05 ^a	7.76 ± 1.04 ^c	492.19 ± 103.74 ^c
20:50–21:00	56.96 ± 21.17 ^d	nd ^a	nd ^a	1.24 ± 0.27 ^{ac}	52.69 ± 20.75 ^d
21:00–21:10	5.28 ± 1.47 ^{ad}	nd ^a	0.15 ± 0.03 ^a	0.25 ± 0.07 ^a	2.06 ± 0.62 ^a
21:10–21:20	2.10 ± 0.29 ^a	nd ^a	nd ^a	nd ^a	nd ^a

Results are in ng g⁻¹, estimated for total volatiles and actual for compounds (mean ± SE, N = 5). Within each column, means bearing the same superscript are not significantly different, P < 0.001. Scotophase began at 20:30. nd: below the detection limits of 0.02 ng g⁻¹.

Interplant variation in actual amounts, within a species, was high, as reflected by the large values for SE.

Short Time Course. The detailed results for the estimated total volatile amounts and for the actual amounts of the four individual compounds emitted by *P. purpureum* are shown in Table 3. These results show that approximately 74% of the total volatiles collected in the first 50 min of scotophase are actually emitted in the first 10 min, and a further 23% in the next 10 min.

DISCUSSION

It has long been known that the time of release of floral volatiles varies greatly among plant species, and that this can be associated with behavior of the associated pollinator. The foliage of some tree species is also known to release large transient bursts of acetaldehyde during light to dark transitions (Holzinger et al., 2000; Karl et al., 2002). Furthermore, a number of volatile C₂–C₆ compounds, including (*Z*)-3-hexen-1-yl acetate, have been identified, together with a number of monoterpenes and their oxidation products, above a boreal coniferous forest, by using a ground-based chemical ionization mass spectrometer, and found to exhibit a clear diurnal variation (Sellegrri et al., 2004). Recently, Graus et al. (2004), using proton transfer reaction-mass spectrometry, have shown that, in addition to acetaldehyde, several C₆ compounds are released by gray poplar, *Populus × canescens* (Aiton) Smith, in considerable quantities after light–dark transitions. The first compound to show an increase in emission was hexenal (isomer not specified) after 30 sec, with the other aldehydes, alcohols, and esters appearing after a further 20 sec. However, whereas hexenal and acetaldehyde emissions only lasted for about 3 min, the high levels of the others lasted for up to 15 min. These C₆ compounds comprise some of the green leaf volatiles, which also include the compounds that have shown such large increases in the present study, hexanal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexen-1-yl acetate, and are normally associated with leaf damage, whether caused by herbivory or abiotically (Turlings et al., 1998; Ngi-Song et al., 2000).

Green leaf volatiles are produced from linolenic and linoleic acids through the lipoxygenase pathway (Paré and Tumlinson, 1996). They are liberated from membranes as a result of damage, by the action of a lipoxygenase enzyme that produces hydroperoxides initially. A hydroperoxide lyase enzyme then converts the hydroperoxides to hexanal (from linoleic acid) and (*E*)-2-hexenal (from linolenic acid), which undergo further reactions to give other C₆ aldehydes, alcohols, and esters (Hatanaka, 1993; Bate and Rothstein, 1998). The same pathway seems to be involved in the production of green leaf volatiles at the

change from light to dark conditions as indicated in the work of Graus et al. (2004), who showed that anoxic conditions at the light–dark transition eliminated production of the green leaf volatiles, the lipoxygenase enzyme requiring molecular oxygen (Hatanaka et al., 1987). If the reason for the production of green leaf volatiles on plant damage is attributable to release from storage pools, loss of the normal compartmentalization of the enzyme and the substrates in the intact cell, or simply the release of fatty acid substrates from the membrane, it is difficult to understand why the light–dark transition would do the same. It has been proposed that the sudden big change from a condition of saturating photosynthetic photon flux density to one of darkness causes significant disturbances in membranes, thereby inducing the typical wounding responses, in the absence of any visible leaf damage (Graus et al., 2004). The light–dark transition used in these studies was relatively sharp, which may cause a more dramatic effect on membranes than a gradual change. However, the conditions used here are similar to those at nightfall in Africa, near the equator, where related field studies have been conducted (Khan et al., 2000, 2001; Khan and Pickett, 2004).

A study on the induction of volatile emission from *Nicotiana tabacum* by caterpillars of *Heliothis virescens*, *Helicoverpa zea*, and *Manduca sexta* (De Moraes et al., 2001) showed that a number of herbivore-induced chemicals were produced only at night, and the production of other daytime volatiles was also increased during the scotophase. The compounds involved were mainly C₆ aldehydes, alcohols, and their esters, some of which are the same compounds as those showing large increases in production during the light-to-dark transition in the current study. Although, in the light of our results, it is possible that the increased production of these green leaf volatiles observed by De Moraes et al. (2001) was due to the onset of darkness rather than caterpillar induction, other plants of *N. tabacum* showed increases, albeit smaller, in the amounts of nocturnal volatiles produced when the caterpillars of *H. virescens* were removed from the plant several hours before dark. Clearly, the temporal emission of volatile chemicals by plants both as a normal response to the light–dark transition and as a response to biotic and abiotic damage needs further careful investigation.

Studies on the electrophysiological responses of *C. partellus* and *B. fusca* to components of the headspace volatiles of the same four species of host plant (as used in this study) identified the four green leaf volatiles that showed increases at light–dark transition as eliciting EAG responses in both insects' antennae (Birkett et al., unpublished data), although behavioral activity associated with these responses has not yet been determined. Gravid female stemborer moths are known to seek oviposition sites during the first 6 hr of scotophase, with most of the activity in the first 2 hr (Päts, 1991). Further behavioral studies are required, not only to determine the behavioral activity of

these green leaf volatiles but also to investigate the possible correlation between the production of large amounts of these compounds with time of oviposition flights by moths. The existence of such a correlation could explain why wild grasses are more attractive than the cultivated crop plants for egg laying since these plants produce much larger amounts of the green leaf volatiles at nightfall than the maize and sorghum. It is interesting to note that within the cultivated hosts, more oviposition by *C. partellus* takes place on sorghum than on maize (Kfir, 1992), which is also consistent with these results.

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EPICUTICULAR FACTORS INVOLVED IN HOST
RECOGNITION FOR THE APHID PARASITOID
Aphidius rhopalosiphii

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Abstract—In insect parasitoids, fitness is dependent on the host finding and recognition abilities of the female. Host recognition cues have been described for various host–parasitoid systems, but are still under investigation in aphid parasitoids. Our study aimed to clarify the respective role of physical and chemical cues in recognition of the aphid cuticle. Shed aphid exuviae were used as an elicitor in order to avoid any influence of color, movement, or volatiles present in a living aphid. We assessed the effect of chemical and heat treatments on the texture of the cuticle by using scanning electron microscopy and tested the recognition of treated cuticles by the parasitoid. We showed that recognition cues of the cuticle can be removed chemically (using combined treatments with n-hexane and methanol). Moreover, heat treatment destroyed the physical texture of the cuticle without significantly reducing parasitoid recognition. In a second step, we showed that epicuticular extracts deposited on pieces of paper triggered female attack behavior. First results concerning the chemical composition of the active extract are presented. This study shows that chemical compounds extractable by organic solvents mediate cuticle recognition by aphid parasitoids.

Key Words—Cuticular kairomones, chemical cues, oviposition behavior, Aphidiinae, *Sitobion avenae*, cuticular hydrocarbons, wax esters.

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INTRODUCTION

In insect parasitoids, fitness depends to a considerable extent on the host recognition capabilities of the adult female. The host recognition process involves both physical and chemical factors (Godfray, 1994). Host body size (Shirota et al., 1983; Kouamé and Mackauer, 1991), host cuticle texture (Arthur, 1981), shape (Vinson, 1985), and color (Ankersmit et al., 1981, 1986; Michaud and Mackauer, 1994, 1995; Battaglia et al., 2000), as well as host movements (Arthur, 1981; Mackauer et al., 1996; Dippel and Hilker, 1998) act as cues triggering the attack behavior of female parasitoids. It has also been frequently reported that host recognition is mediated by semiochemicals (Strand and Vinson, 1982; Grasswitz and Paine, 1992; Battaglia et al., 1993).

Several authors have pointed out the role of epicuticular factors in host recognition by aphidiine wasps (Pennacchio et al., 1994). For example, in three aphid parasitoid species, females are not able to distinguish between host and nonhost species at a short distance, and an antennal contact is needed for recognition at the species level (Le Ralec et al., 2005). Oviposition attempts of aphid parasitoids toward host exuviae have been occasionally observed (Michaud, personal communication; Outreman, personal communication). Exuviae are useful biological material in behavioral analyses because visual factors like movement, color, and global shape are excluded as well as semiochemicals secreted by living aphids. Moreover, from the perspective of chemical analysis, only cuticular compounds are extracted from exuviae without any contaminants from the internal body (Grasswitz, 1998). Exuviae from *Acyrtosiphon pisum* elicit attacks by *Aphidius ervi* even when coarsely crushed (Battaglia et al., 2000). This response is still observed under red light, indicating that visual cues are not involved. Nevertheless, the attack behavior is not observed if the parasitoid is prevented from touching the exuviae with its antennae. Battaglia et al. (2000) evoked the “possibility that a cuticular compound, which functions as recognition kairomone,” occurs in the cornicle secretion and on the cuticle of exuviae. When antennal contact is required for host recognition, nonvolatile chemical compounds are likely to be involved, and a role for the cuticle texture must be considered (Godfray, 1994). Moreover, *Aphidius* wasps present both chemo- and mechanoreceptors on the antennae (Battaglia et al., 2002). Scanning electron micrographs of aphid epicuticle show the occurrence of wax secretion in a bloom covering (Retnakaran et al., 1979; Pope, 1983). Besides its waterproofing function (Pope, 1983), the epicuticular wax layer can also play a key role in communication. Hydrocarbons of the wax layer of the insect cuticle may be involved in, e.g., species, gender, and nestmate recognition, as well as in chemical mimicry (Howard and Blomquist, 2005). Therefore, the integration of both chemical and physical factors is a tentative explanation for the mechanism of host recognition by aphidiine parasitoids.

In order to assess the respective roles of cuticular wax semiochemicals and/or texture in parasitoid host recognition, exuviae were treated in various ways. In the first step, potential semiochemicals were washed off with solvents. During the second step, cuticle texture was altered by using a heat treatment. The effect of the solvent extraction on cuticular surface texture was studied by scanning electron microscopy (SEM), and the consequences of heat treatment and solvent extraction on parasitoid behavior were analyzed. To further assess the role of chemical cues, we observed the behavior of the parasitoid towards a cuticular extract. Finally, the chemical composition of the extract was investigated by coupled gas chromatography–mass spectrometry (GC–MS).

Our biological model was the aphid–parasitoid system, *Sitobion avenae* Fabricius–*Aphidius rhopalosiphi* De Stefany Perez. *A. rhopalosiphi* is one of the most abundant Aphidiinae species in cereal fields in North Europe (Jones, 1972; Stilmant, 1997) and has shown potential for biological control of aphids (Levie et al., 2005).

METHODS AND MATERIALS

Insect Rearing. *A. rhopalosiphi* individuals were collected in winter wheat fields near Louvain-la-Neuve, Belgium, in summer 2000, and reared on *S. avenae* maintained on winter wheat (*Triticum aestivum* cv. Windsor). Colonies of both *A. rhopalosiphi* and *S. avenae* were kept in the laboratory under the following conditions: $19.5 \pm 0.6^\circ\text{C}$, 40–50% relative humidity, and 16:8 hr L:D photoperiod. Parasitoids were reared on synchronized L2 aphid colonies. In a framed wooden cage (50 × 50 × 30 cm) with fine mesh on the sides, pairs of parasitoids (one pair per 50 aphids) were released for 24 hr. Ten days later, mummies were removed from wheat with a scalpel and kept in Petri dishes in groups of 50 individuals each. To avoid possible influences of hunger or mate-searching behavior (Michaud, 1994), emergent females were given access to food (honey–water) and males. All females were naive (no access to host) when tested.

Collection of Exuviae. Sheets of paper were placed for 1–3 d under aphid colonies of mixed age. Exuviae falling from the colony were collected and carefully separated from dead aphids and waste material. They were weighed with a 0.1-mg-precision balance (Ohaus explorer).

Experiment 1: Effect of Solvent Washing of the Exuviae. Exuviae were washed in methanol (SoxM) or hexane (SoxH) for 4 hr by using a soxhlet. Exuviae were recovered and allowed to dry for 24 hr before use in behavioral assays. Unwashed exuviae were used as controls. In a second part of the experiment, cuticular compounds present on the surface of exuviae were extracted (1) for 4 hr at room temperature with only one solvent, methanol (M), or n-hexane (H), or (2) sequentially for 1.5 hr by two successive solvents—

n-hexane followed by methanol (H + M), or methanol followed by n-hexane (M + H), and finally (3) for 4 hr with a mixture of n-hexane/methanol (ratio 1:2 v/v). During the extraction process, exuviae were held in a glass bottle and shaken at 100 rpm. In all cases, 1 mg exuviae was added to 10 ml solvent.

Experiment 2: Effect of Heat Treatment of the Exuviae. Exuviae were heated for 16 hr in an oven at 80°C. At the same time, a control batch was kept at room temperature. Needles and grips were washed in n-hexane and methanol prior to use in each treatment to avoid chemical transfer between specimens. Exuviae of the same experiment came from the same collection.

Scanning Electron Microscopy Method. In order to investigate the surface of aphid epicuticle, exuviae were studied by two different SEM methods. First, exuviae were sputter-coated with fine gold and directly observed in a Philips XL20 SEM (INRA, Rennes). Second, using a SEM (Oxford CT1500 cryosystem) at the microbiology laboratory (MBLA unit, UCL), specimens were flash frozen (-212°C) in liquid nitrogen under vacuum for cryo-SEM, transferred to the preparation chamber, and then to the SEM chamber where the frozen samples were sublimated (-80°C) to remove ice particles. Specimens were viewed under 2–5 kV at -190°C to -170°C (SEM Phillips XL20). To normalize the observations, all illustrations reported here represent the surface of wing buds of a “fourth instar exuvia” (between L4 and adult instar of the aphid). To ensure that observed differences were not due to individual variation, at least 20 exuviae were observed for each treatment.

Behavioral Assays. Glass Petri dishes (diam. 5 cm, height 1.5 cm, Schott®) were prepared by washing them with ethanol and deionized water and placed on a light table (2500 lx). Sixteen exuviae per dish were regularly disposed 1 cm apart in four rows of four exuviae each. Exuviae were maintained within a droplet of deionized water that dried at room temperature for 30 min before use. One parasitoid female was released at the center of the Petri dish and observed for 15 min. The behavioral items noted were “encounter with exuviae” (ENC), “antennal contact on exuviae” (ANT), and “abdominal bending towards exuviae” (ABD). In order to minimize variability, each set of experiments was completed during the same working day. Between 20 and 30 females were tested per treatment, each female being tested against one substrate only. The temperature during experiments was $23 \pm 1^\circ\text{C}$.

Experiment 3: Assay of the Cuticular Extract Activity. Exuviae were extracted as described above with a mixture of n-hexane/methanol (1:2, v/v) for 15 min. The extract was filtered and concentrated using a rotavapor (R110, Büchi, Switzerland) to 2 ml. The extract was deposited at the bottom edge of a filter paper (Whatman No. 1, 3 × 3 cm) held vertically, the solvent moving up the paper by absorption. The paper was allowed to dry at room temperature for 2 hr before the behavioral test. Small pieces (1 × 1 mm) were cut from the filter paper by using a scalpel parallel to the front of migration. Sixteen pieces of

paper were deposited in a glass Petri dish, and a behavioral test was conducted as described above except that the tests stopped after 5 min. New scalpel blades and clean grips were used for each treatment.

Chemical Analysis of the Cuticular Extract of Exuviae. Exuviae (20 mg) were extracted with an n-hexane/methanol (1:2, v/v) mixture for 15 min (as described above). After evaporation of the solvent in a rotavapor, the crude extract was dissolved in 2 ml of n-hexane/methanol, and 1 μ l was analyzed by GC-MS. The GC-MS investigations were performed on a Hewlett Packard HP 5989 Mass Spectrometer coupled with an HP 6890N gas chromatograph equipped with an HP-5 (cross-linked 5% phenylmethylpolysiloxane) column (30 m \times 0.25 mm I.D.; film thickness 0.25 μ m). The operating conditions were fixed as follows: split-splitless injector at 280°C; carrier gas, helium at 1 ml min⁻¹; temperature program, from 50°C to 300°C at 15°C min⁻¹, then at 300°C for 25 min. The mass spectra were recorded in the electron impact mode at 70 eV (source temperature, 230°C; scanned mass range, 35 to 700 amu). The detected peaks were identified by their retention time data and their characteristic fragmentation patterns. The mass spectra of the compounds were also compared with those of the NBS75K.L and Wiley275K.L computer MS Libraries. Four batches of 20 mg exuviae were analyzed by the described method.

Statistical Analysis. Data were analyzed using SAS System (SAS Institute, Cary, NC, 1999). PROC UNIVARIATE was first used to test the distribution of all behavioral observations. Data were log transformed in case of significant deviation from normality. To compare the number of behavioral events, variance analyses were performed using PROC GLM with Scheffé's multiple comparison method. To represent the proportion of exuviae rejected for oviposition after antennal analysis, a rejection ratio (Rej) was computed as follows: $1 - (\text{ABD}/\text{ANT})$ (where ABD = number of abdominal bendings, ANT = number of antennal contacts). As each female rejected a proportion of exuviae after antennal contact, this ratio was calculated for each female. Since these data cannot be normalized by any transformation, the ratios were analyzed by using the Kruskal-Wallis test with multiple comparisons following Siegel and Castellan (1988). The effect of cuticular extract was compared to the control using chi-square tests.

RESULTS

Experiment 1: Chemical Treatments. SEM observations revealed that the cuticular surface of the control exuviae is coated by a bloom of epicuticular waxes (Figure 1). The surface became smooth after soxhlet extraction (Figure 1), whereas the ornamentation remained intact when methanol was used on its own at room temperature (Figure 2). The SEM studies revealed no differences between individual exuviae that received the same treatment.

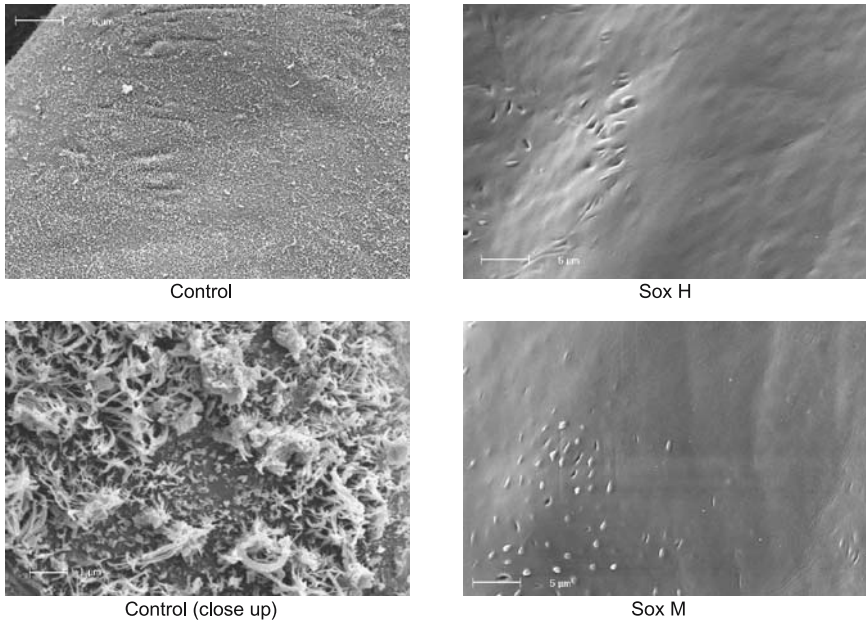
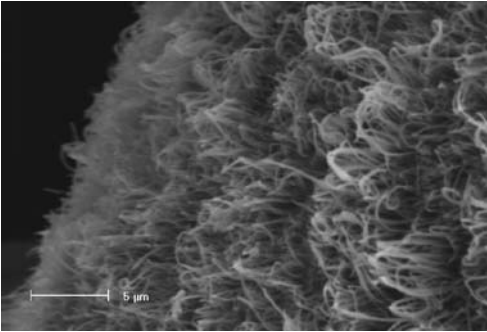


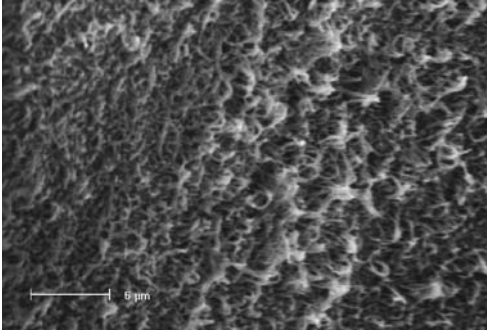
FIG. 1. Cuticular surface of the wing bud of fourth instar exuviae after chemical treatments in Soxhlet apparatus. Sox H = exuviae treated with n-hexane in soxhlet, Sox M = exuviae treated with methanol in soxhlet.

Analysis of variance showed a significant effect of washing exuviae with hot solvents on the behavioral response of the parasitoid (Table 1). The number of encounters ($F_{2,66} = 21.98$; $P < 0.001$), antennal contacts ($F_{2,66} = 35.50$; $P < 0.001$), and abdominal bendings ($F_{2,66} = 14.99$; $P < 0.001$) were clearly reduced by chemical treatment. The rejection ratio followed the same trend with a higher level of rejection after chemical treatment. The rejection ratio could not be tested statistically due to the high number of females that made no antennal contact. In this experiment, extraction with methanol inhibited recognition stronger than the n-hexane extraction.

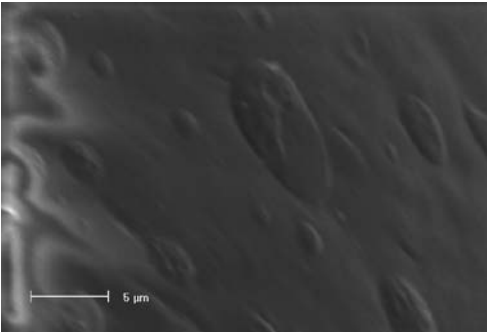
At room temperature, solvent extraction had no significant effect on exuviae recognition (Table 2). However, successive chemical treatments with two solvents had a significant effect on host acceptance compared to control. The number of abdominal bendings towards exuviae was strongly reduced when both solvents were used sequentially or in a 1:2 mixture ($F_{5,108} = 19.57$; $P < 0.001$). Again, the rejection ratio followed the same trend with a strong increase in rejection for treatments employing both solvents.



Control



M(rt)



Heat

FIG. 2. Cuticular surface of the wing bud of fourth instar exuviae after chemical treatment at room temperature or after heat treatment. M(rt) = exuviae treated with methanol at room temperature, Heat = exuviae heated in drying oven at 80°C.

TABLE 1. MEAN NUMBER OF BEHAVIORAL ITEMS OBSERVED ON SOXHLET-EXTRACTED EXUVIAE

	<i>N</i>	Enc	Ant	Abd	Rejection ratio
Sox M	24	9.3 ^b (1.5)	1.8 ^c (0.6)	0.17 ^c (0.09)	0.91
Sox H	22	14.6 ^b (2.3)	4.8 ^b (1.0)	1.18 ^b (0.3)	0.76
Control	23	34.8 ^a (4.2)	21.4 ^a (2.9)	6.87 ^a (1.6)	0.61

Standard errors are given in brackets. Means of the same column sharing the same letter are not significantly different ($\alpha = 0.05$). Enc = encounter, Ant = antennal contact, Abd = abdominal bending, Sox H = exuviae treated with n-hexane in soxhlet, Sox M = exuviae treated with methanol in soxhlet.

Experiment 2: Heat Treatment. Heating exuviae up to 80°C caused a “melting” of the epicuticular waxes that led to a “smoothing” of their typical microstructures. Indeed, when heated, the lipid surface constituents became amorphous with some spots that we considered as accumulated material (Figure 2). Heat treatment did not significantly affect the behavioral responses of the parasitoids ($F_{1,36} = 0.04$; $P = 0.847$ for encounter, $F_{1,36} = 0.41$; $P = 0.526$ for antennal contact, $F_{1,36} = 2.00$; $P = 0.166$ for abdominal bending) (Table 3).

Experiment 3: Activity of the Cuticular Extract. The parasitoids were stimulated to attack pieces of filter paper containing the cuticular extract (Figure 3). More than 75% of the females ($N = 20$) showed repeated abdominal bendings towards the extract. Only one female ($N = 17$) showed a single abdominal bending event on the control paper. Both the top ($\chi^2_2 = 28.3$, $P < 0.001$) and the bottom ($\chi^2_2 = 204.0$, $P < 0.001$) portion of the filter paper had a significant effect on parasitoid behavior compared to the control filter paper.

TABLE 2. MEAN NUMBER OF BEHAVIORAL ITEMS OBSERVED ON EXUVIAE TREATED WITH SOLVENTS AT ROOM TEMPERATURE

	<i>N</i>	Enc	Ant	Abd	Rejection ratio
H + M	29	24.4 ^b (2.4)	11.4 ^d (1.6)	1.0 ^b (0.4)	0.91 ^A
M + H	10	16.0 ^b (2.0)	6.4 ^{cd} (1.1)	0.6 ^b (0.2)	0.91 ^A
[HM]	15	34.0 ^{ab} (4.3)	13.2 ^{bcd} (2.6)	0.3 ^b (0.2)	0.97 ^A
Hrt	18	34.2 ^{ab} (4.1)	23.6 ^b (2.8)	9.5 ^a (1.7)	0.60 ^B
Mrt	17	33.9 ^{ab} (3.9)	26.2 ^b (3.3)	11.4 ^a (2.2)	0.57 ^B
Control	20	51.1 ^a (4.3)	41.1 ^a (3.8)	15.4 ^a (2.1)	0.63 ^B

Standard errors are given in brackets. Means of the same column sharing the same letter are not significantly different ($\alpha = 0.05$). Enc = encounter, Ant = antennal contact, Abd = abdominal bending, H + M = exuviae sequentially treated with n-hexane and then methanol, M + H = exuviae sequentially treated with methanol and then n-hexane, [HM] = exuviae treated with a n-hexane/methanol (1:2) mixture, Hrt = exuviae treated with n-hexane, Mrt = exuviae treated with methanol.

TABLE 3. MEAN NUMBER OF BEHAVIORAL ITEMS OBSERVED ON HEATED AND CONTROL EXUVIAE

	N	Enc	Ant	Abd	Rejection ratio
Heat	18	31.8 ^a (5.8)	20.9 ^a (3.9)	6.4 ^a (1.3)	0.69 ^A
Control	20	32.2 ^a (4.7)	23.4 ^a (3.6)	9.3 ^a (1.5)	0.60 ^A

Standard errors are given in brackets. Means of the same column sharing the same letter are not significantly different ($\alpha = 0.05$). Enc = encounter, Ant = antennal contact, Abd = abdominal bending. Heat = exuviae heated in drying oven.

Chemical Composition of the Cuticular Extract. The cuticular extract composition consists of compounds belonging to four major classes of compounds: approximately 20 hydrocarbons, 3 wax esters, 2 alcohols, and 2 aldehydes. The list of the detected compounds is presented in Table 4. The hydrocarbon fraction consists of a homologous series of n-alkanes (C25 to C31), branched monomethyl alkanes (X-MeC25 to X-MeC31), and one dimethyl alkane (11,13-DiMeC29). The wax esters detected at the end of the run (Table 4) were medium-chain fatty acids (C16) esterified to long-chain alcohols (C18 to C22). The extract is also characterized by the occurrence of two long-chain aldehydes (C28 and C30). The identification of the octacosanal (C28) has

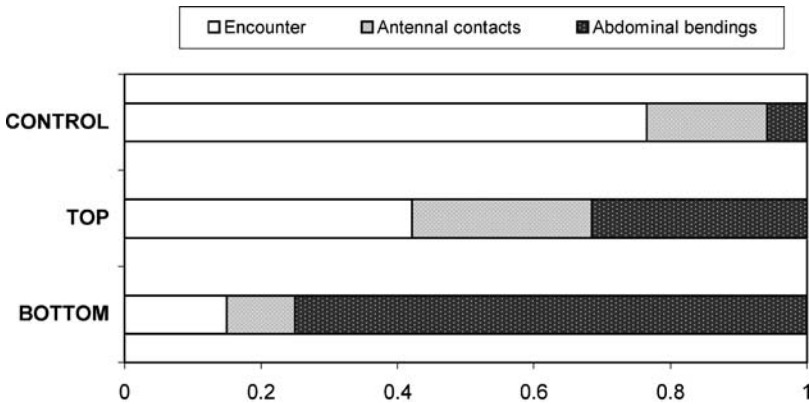


FIG. 3. Behavioral response of the females to pieces of paper covered with the cuticular extract. Encounter = proportion of females that did not react when encounter the extract, Antennal contacts = proportion of females that showed antennal drumming, Abdominal bendings = proportion of females that showed abdominal bendings. “Top” and “bottom” refer to the part of the filter paper that received the cuticular extract, the front of migration of the extract being from bottom to top. “Control” refers to filter paper that received pure solvent.

TABLE 4. CUTICULAR COMPOUNDS IDENTIFIED IN HEXANE/METHANOL EXTRACTS

RT (min)	Compound	Diagnostic MS ions	Area % (mean \pm SE) ^d
15.89	n-Pentacosane	352	3.08 \pm 0.12
16.21	2-Methylpentacosane	351, 323	0.55 \pm 0.20
16.27	3-Methylpentacosane	351, 337	0.31 \pm 0.01
16.40	n-Hexacosane	366	0.70 \pm 0.02
16.72	2-Methylhexacosane ^b	—	0.17 \pm 0.06
16.91	Heptacosane	380	11.22 \pm 0.09
17.07	11-Methylheptacosane ^b	—	0.33 \pm 0.03
17.16	5-Methylheptacosane	337, 85	0.25 \pm 0.02
17.27	3-Methylheptacosane	365	0.21 \pm 0.03
17.41	n-Octacosane	394	0.61 \pm 0.02
17.96	n-Nonacosane	408	10.99 \pm 0.33
18.15	11-Methylnonacosane	407, 168, 280	1.06 \pm 0.03
18.26	5-Methylnonacosane	365, 85	0.12 \pm 0.06
18.32	11,13-Dimethylnonacosane	168, 224, 239, 295	0.23 \pm 0.05
18.58	n-Triacontane	422	0.67 \pm 0.11
18.79	11-Methyltriacontane ^b	—	0.33 \pm 0.11
18.89	Octacosanal	408, 390	0.92 \pm 0.07
19.30	n-Hentriacontane	436	4.57 \pm 0.19
19.38	Unidentified alcohol	—	14.61 \pm 0.70
19.54	11-Methylhentriacontane	168, 308	1.02 \pm 0.12
20.59	Triacontanal	418	1.27 \pm 0.24
21.11	Unidentified hydrocarbon	—	0.92 \pm 0.04
21.27	Unidentified alcohol	—	13.57 \pm 0.66
24.83	Hexadecanoic acid octadecyl ester	257, 508	2.50 \pm 0.12
29.20	Hexadecanoic acid eicosanyl ester	257, 536	20.97 \pm 0.56
33.94	Trihexanoin ^c	285, 383, 99	5.32 \pm 0.80
35.49	Hexadecanoic acid docosanyl ester	257, 564	3.51 \pm 0.09

RT = retention time.

100% = total area of detected peaks.

^dMeans and standard errors calculated of four cuticular extracts.

^bMethyl position inferred from Kovats index only.

^cTentative interpretation.

been confirmed by comparison with the mass spectra of synthesized compounds kindly provided by Prof. Morris (Horticulture and Food Research Institute of New Zealand, Auckland, New Zealand).

DISCUSSION

This study used aphid exuviae in order to better understand the role played by semiochemicals and/or texture associated with wax secretions in host

recognition by the parasitoid *A. rhopalosiphi*. The results confirm that epicuticular factors are involved in host recognition. When these factors are chemically removed, the exuviae lose their activity in terms of eliciting responses from the parasitoid. Secondly, the results show that the tactile recognition of the epicuticular wax layer by the parasitoid is not involved in host recognition. The microstructure of the epicuticular wax layer is destroyed by the heat treatment, but these exuviae retain their activity for the parasitoid. We observed that the wax layer was still present on the heated cuticle, although its microstructure was destroyed. However, soxhlet extraction took away epicuticular waxes. The behavioral study revealed significant activity when the wax layer was still present regardless of its physical state. Rather, the kairomones embedded within epicuticular waxes seem to play a role in recognition of the host cuticle. The parasitoid's response was lost when the host cuticle received specific chemical treatments. First, hot chemical treatment was efficient, using n-hexane or methanol. Second, at room temperature, the solvents had to be combined to destroy the recognition response. Long extraction time (4 hr) in the soxhlet apparatus led to complete extraction of epicuticular constituents, some of which were not fully extracted at room temperature. With simple macerations at room temperature, two solvents of different polarities (hexane and methanol) were necessary for complete extraction of the active factor. At the 1:2 ratio, n-hexane and methanol are fully miscible and form a mixture that can be considered as a novel solvent with novel properties, such as e.g., polarity and boiling point. The use of the two solvents, sequentially or in mixture, enabled us to extract the active compounds from the cuticle. This suggests that the activity is due to several compounds with different polarities, rather than to a single compound. Moreover, heat treatment of the exuviae reduced the parasitoid's response but did not completely inactivate the cuticle. This suggests that the kairomonal activity of the exuviae is due more to a (mixture of) contact chemical(s) rather than to a mixture of short-range volatiles, as these would certainly almost be destroyed or removed by heating. Finally, the presence of cuticular kairomones was unequivocally proven by the parasitoid's responses towards pieces of paper impregnated by the extract. This represents the first direct evidence of the occurrence of cuticular kairomone(s) that elicit aphid parasitoid attacks. The results also show that a rough fractionation of the extract can be made by using the absorption capacity of the filter paper, since the bottom of the filter elicits a stronger reaction of the parasitoid than the top.

Hydrocarbons are known to be commonly involved in insect communication (Chapman, 1998). For example, chemical mimicry can often be attributed to cuticular hydrocarbons of similar composition (Dettner and Liepert, 1994; Liepert and Dettner, 1996; Allan et al., 2002). Methylene chloride extract of the cuticle of *S. avenae* revealed n-alkanes ranging from 23 to 35 carbon atoms and

several methyl-branched homologues (Hebanowska et al., 1989). However, these results were obtained by extracting whole aphids for 2 weeks, and contamination from internal body contents cannot be excluded (Grasswitz, 1998). In our study, the compounds extracted by the hexane/methanol mixture were not exclusively hydrocarbons. Wax esters as well as long-chain alcohols were also present in the extract. The cuticular extract also contained two aldehydes that are not often reported in studies on insect cuticles (Howard and Lord, 2003) and, to our knowledge, never on aphid cuticles. Moreover, we cannot exclude that other molecules such as sugars were extracted from the cuticle but not detected by the GC-MS technique. In further studies, the extract will be fractionated, and the different fractions will be assayed for recognition activity in order to identify the active compounds by a biologically guided chemical analysis.

In the experiments presented here, all oviposition attempts on exuviae were preceded by antennal contacts. This is not the case when a parasitoid faces a living aphid (Battaglia et al., 1993). *A. rhopalosiphi* usually starts its oviposition sequence on aphids without antennal contact, approaching the host with the antennae bending backwards (van Baaren et al., 2004). The differences in the behavioral sequence may be due to the nature of the stimulus involved. Both short-range (color, movement) and contact (kairomones) cues are present on the aphid, whereas on the cuticle, the chemical contact kairomones are the only remaining cues. These kairomones can be recognized antennal contact and also during ovipositor contact. The ovipositor of the Aphidiinae consists of three pairs of valvulae (Le Ralec and Rabasse, 1988; Le Ralec et al., 2001). At rest, the valvulae 1 and 2 are protected inside the third ones. During the stinging, the third valvulae weigh upon the host cuticle and separate from each other allowing the penetration of the valvulae 1 and 2. The third valvulae have been shown to wear both mechano- and chemoreceptors that could receive information from the cuticle of the host (Le Ralec and Rabasse, 1988). This means that the parasitoid may use two sequential tools to acquire information about the cuticular chemical cues of its host.

It has been shown that host feces or host secretions may play a role in the host-searching process and act as cues for location of host colonies (Weseloh, 1981). With regard to Aphidiine wasps, several authors have demonstrated a role for aphid honeydew in the host-location process (Budenberg, 1990). A similar mechanism seems to be involved in the recognition of exuviae: chemical traces inform the parasitoid of the host presence. If exuviae recognition by the parasitoid is advantageous to host location, the ecological significance of the attacks of exuviae is unclear. Indeed, the attack of an exuvia should be costly in time and energy for the parasitoid, and this behavior should vanish by natural selection. A possible explanation could come from the "Neo-Hopkins principle" (Jaenike, 1983; Corbet, 1985): the response to cuticular compounds could come

from the chemical environment experienced by the parasitoid at the emergence from the mummy. This conditioned chemosensory responsiveness can influence the host-searching and host-recognition behaviors of the adult. This effect on host location has already been shown for *A. rhopalosiph* (van Emden et al., 2002) but remains to be tested at the host-recognition level. From an applied point of view, the identified chemical stimulus could provide opportunities to manipulate parasitoid behavior in order to enhance oviposition in artificial rearing systems (Battaglia et al., 1995).

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LEAF EPICUTICULAR WAX CHEMICALS
OF THE JAPANESE KNOTWEED *Fallopia japonica*
AS OVIPOSITION STIMULANTS FOR *Ostrinia latipennis*

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Abstract—Extraction, fractionation, and gas chromatography–mass spectrometry analyses guided by bioassays have shown that *n*-alkanes and free fatty acids in leaf epicuticular wax of the Japanese knotweed *Fallopia (Reynoutria) japonica* stimulate oviposition in the Far-Eastern knotweed borer, *Ostrinia latipennis* (Lepidoptera: Crambidae). *n*-Alkanes made up 48.1% of the total amount of epicuticular wax, and their carbon chain length was in the C₁₆–C₃₃ range, with *n*-nonacosane (*n*-C₂₉) most abundant, followed by *n*-C₂₇, *n*-C₂₅, and *n*-C₃₁. Free fatty acids with C₉–C₂₂ accounted for 22.3%, and hexadecanoic acid was predominant. A mixture of authentic *n*-alkanes and fatty acids of the composition found in the epicuticular wax, a mixture of *n*-alkanes, and a mixture of fatty acids significantly enhanced oviposition. Thus, it was demonstrated that both *n*-alkanes and free fatty acids in leaf epicuticular wax of *F. japonica* are naturally occurring oviposition stimulants for *O. latipennis*.

Key Words—*Fallopia japonica*, *Ostrinia latipennis*, alkane, fatty acid, oviposition stimulant.

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INTRODUCTION

The chemical composition of the epicuticular wax on the aerial surface of a plant varies among species, varieties within a species, parts within a plant, and different stages of development (e.g., Städler, 1986; Eigenbrode and Espelie, 1995; Shepherd et al., 1999, and references therein). Such variations provide substantial information by which many insect herbivores in the Lepidoptera, Diptera, and Coleoptera evaluate potential host plants. Knowledge of the kairomones in epicuticular wax may be useful in conjunction with pest management programs for improved monitoring of female populations in the field, possible disruption of egg-laying behavior, and genetic manipulation of host plants to create stimulant-free varieties (Metcalf, 1985).

The genus *Ostrinia* (Lepidoptera: Crambidae) includes important agricultural pests, such as the European corn borer *O. nubilalis*, the Asian corn borer *O. furnacalis*, and the adzuki bean borer *O. scapularis*. Although other members of *Ostrinia* have attracted little attention, the genus actually contains 21 closely related species with diverse host plant ranges (Mutuura and Munroe, 1970; Ishikawa et al., 1999; Ohno, 2003). Studies on the kairomones of respective host plants could provide clues to pest management, in addition to information on how host plant recognition systems have changed in association with the diversification of host plants during species divergence. Among the many *Ostrinia* species, however, only oviposition stimulants for *O. nubilalis* on maize have been studied to date (Udayagiri and Mason, 1997).

As a first step toward the comprehensive clarification of oviposition attractants/stimulants for the genus *Ostrinia*, we started by analyzing stimulants on the host plant of an oligophagous species, since it is likely that oligophagous species use more host-specific compounds as cues for host selection than polyphagous species, and hence should show a clearer behavioral response in bioassays. The Far-Eastern knotweed borer *O. latipennis*, a species of current interest, is oligophagous and usually uses either the giant knotweed *Fallopia (Reynoutria) sachalinensis* or the Japanese knotweed *F. japonica* as a host plant. The present study was conducted to elucidate oviposition stimulants in the leaf epicuticular wax of *F. japonica*.

METHODS AND MATERIALS

Insects. Egg masses of *O. latipennis* were collected from leaves of the giant knotweed *F. sachalinensis*, at Towa (39.9°N, 141.2°E) in July 2002. Larvae were reared as broods in clear plastic jars filled with 200 g of a commercial diet for insects (Insecta LF™, Nosan Corp., Yokohama, Japan). The environmental conditions were 24 ± 1°C, a 15-hr light–9-hr dark photoregime,

and 60% R.H. After pupation, females and males were segregated, based on the morphology of the abdominal terminal segments.

Extraction of the Leaf Epicuticular Wax. Twenty *F. japonica* plants naturally growing at five different sites on the Yayoi campus of Tokyo University were selected, and three leaves at upper, middle, and lower positions of each plant were collected. The area and weight of each leaf were measured, and these 60 leaves were dipped individually into 400 ml of dichloromethane for a period of 5 sec. The epicuticular wax solution was concentrated under a stream of nitrogen to a volume of 5 ml and stored at -20°C prior to use.

Fractionation. The leaf dichloromethane extract was analyzed for lipid classes by spotting an aliquot on a 5×20 cm TLC plate (Silica gel 60 F₂₅₄, Merck Japan Limited). The sample was developed using hexane/diethyl ether/formic acid (80:20:1 v/v/v). After drying, the developed plate was stained with iodine vapor or with 5% H₂SO₄ in 95% ethanol according to Nelson et al. (2002). Six distinct bands with R_f values of 0.03 (B1), 0.10 (B2), 0.19 (B3), 0.38 (B4), 0.68 (B5), and 0.74 (B6) were visualized. Band positions were compared with standards of nonacosane and octadecanoic acid.

Chemicals. All *n*-alkanes (>99% purity) from C₁₉ through C₃₁ (*n*-C₁₉–*n*-C₃₁), except *n*-C₂₇, were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Tridecyl acetate, *n*-C₂₇, tridecanoic acid (13:0), hexadecanoic acid (16:0), heptadecanoic acid (17:0), octadecanoic acid (18:0), and (*Z*)-9-octadecenoic acid (18:1) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dodecanoic acid (12:0) was from Nacalai Tesque Inc. (Kyoto, Japan). Other fatty acids used in the present study were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

Bioassay. Bioassays were carried out in the same environmental conditions as outlined above. Before the test, about 20 pairs of newly emerged females and males were placed into a fabric screen cage (20 × 20 × 20 cm) for arbitrary mating for a period of two nights to obtain mated females. Leaves of the Japanese knotweed, their epicuticular extract, TLC fractions (B1–B6), and the blends of chemicals identified were assayed by no-choice and two-choice tests. One-milliliter test solution containing materials equivalent to 15-cm² fresh leaf and the same amount of solvent were applied to cut halves of a filter paper circle (9-cm diam) as the treatment and control, respectively. No-choice test: Clear plastic cups (9-cm diam and 4.5 cm high) were used for the no-choice test. The treated or control filter paper was introduced into each plastic cup. Two-choice test: Fabric screen cages were used for the two-choice test. The halves of the control and treatment filter paper were taped together and stapled onto the ceiling of the fabric screen cage 2 hr before the start of scotophase. In both tests, one female was introduced into the container to avoid any possible influence on oviposition by other females. The following morning, numbers of egg masses and/or eggs were counted. Each test was replicated more than 20

times. A female usually laid 1–6 egg masses the night after copulation, and each egg mass included 60–140 eggs. After each test, the *bursa copulatrix* was removed from the females and dissected to check for the presence of a spermatophore, which is an index of successful mating. Only those data obtained using mated females were used for statistical analyses.

Chemical Analysis. Epicuticular wax, B3, and B6 were dried under a stream of nitrogen and redissolved in hexane containing 1 ng/ μ l of tridecyl acetate as an internal standard. An aliquot (2 μ l) of the solution was injected into a gas chromatograph (GC-17A, Shimadzu, Kyoto, Japan) fitted with an FID, a split/splitless injector, and a fused silica capillary column (DB-Wax or DB-35ms, 30 m \times 0.25 mm i.d., film thickness 0.25 μ m, J&W Scientific, Folsom, CA, USA). The oven temperature was controlled as follows: DB-Wax—maintained at 100°C for 2 min, programmed to increase at 15°C/min to 220°C, then 5°C/min to 230°C, and held at 230°C for 40 min; DB-35ms—100°C for 2 min, increased at 20°C/min to 310°C, then at 2°C/min to 330°C, and held at 330°C until all components eluted. The injector and detector temperatures were 220°C (DB-Wax) or 310°C (DB-35ms). The carrier gas was nitrogen. The quantity of each compound was calculated based on the peak area and calibrated by comparing it with that of tridecyl acetate.

Epicuticular wax, B3, and B6 were analyzed by gas chromatography–mass spectrometry (GC-MS) directly or after derivatization with *N,O*-bis(trimethylsilyl)-trifluoroacetamide according to the method of Shepherd et al. (1995). A QP-5050A GC-MS (Shimadzu) system equipped with a DB-wax or DB-35ms column was run under the same temperature conditions as described for the GC analysis. Helium was used as the carrier gas. The mass spectrometer was used in the electron impact mode (70 eV) and scanned over the mass range 40–600 *m/z*. The interface and ion source temperatures were 250 and 270°C, respectively.

Chemicals were tentatively identified by matching their mass spectra with those in the NIST library using the software CLASS-5000 (Shimadzu). Fatty acids and alkanes were further verified by comparison of the diagnostic ions and the GC retention time with those of the respective authentic standard.

RESULTS

Influence of Leaf and Its Epicuticular Wax on Oviposition. The no-choice test showed that fresh leaves of *F. japonica* significantly increased both the number of egg masses and the number of eggs laid by *O. latipennis* females. The epicuticular wax derived from the leaves had similar effects (Table 1). In the two-choice tests, the epicuticular wax-treated filter papers received significantly more egg masses than the solvent-treated ones (Table 2).

TABLE 1. NUMBERS (MEAN \pm SD) OF EGG MASSES AND EGGS LAID BY FEMALES OF *Ostrinia latipennis* IN NO-CHOICE TESTS

Treatment	Number of mated females	Egg masses	Eggs
Leaf of <i>Fallopia japonica</i>	20	3.0 \pm 0.9 ^a	289 \pm 32 ^a
Control		1.8 \pm 0.4	137 \pm 72
Epicuticular extract	19	3.7 \pm 0.7 ^b	375 \pm 71 ^b
Control		2.0 \pm 0.4	140 \pm 61
Alkanes ^c + fatty acids ^d	22	3.1 \pm 1.0 ^a	281 \pm 40 ^a
Control		1.9 \pm 0.3	149 \pm 51
Alkanes ^c	19	3.0 \pm 0.8 ^a	268 \pm 78 ^a
Control		2.0 \pm 0.3	115 \pm 38
Fatty acids ^d	20	2.8 \pm 1.8 ^a	199 \pm 48 ^a
Control		1.8 \pm 0.3	111 \pm 23

^aSignificantly different from the control by *t* test at 5% level.

^bSignificantly different at 1% level.

^cAlkanes listed in Table 4 were mixed accordingly.

^dFatty acids listed in Table 3 were mixed accordingly.

Fractionation of Active Chemicals. When separated by TLC, the epicuticular wax resolved into six bands (B1–B6). Among them, only B3 and B6 significantly increased the oviposition of *O. latipennis* (Table 2).

GC-MS Analyses. The yield of epicuticular wax recovered from leaves, expressed as per fresh leaf weight, was 447 \pm 34 μ g/g ($N = 3$). Alkanes were the

TABLE 2. NUMBERS OF EGG MASSES (MEAN \pm SD) LAID BY FEMALES OF *Ostrinia latipennis* IN TWO-CHOICE TESTS

Test material	Number of mated females	Treatment	Control
Epicuticular extract	18	2.1 \pm 0.6 ^a	0.9 \pm 0.2
TLC band 1 ($R_f = 0.03$)	20	1.8 \pm 0.6 ns	1.5 \pm 0.6
TLC band 2 ($R_f = 0.10$)	19	2.0 \pm 0.7 ns	1.6 \pm 0.5
TLC band 3 ($R_f = 0.19$)	22	2.6 \pm 0.7 ^a	1.0 \pm 0.3
TLC band 4 ($R_f = 0.38$)	21	1.4 \pm 0.5 ns	1.6 \pm 0.4
TLC band 5 ($R_f = 0.68$)	20	1.8 \pm 0.5 ns	1.6 \pm 0.5
TLC band 6 ($R_f = 0.74$)	20	2.4 \pm 0.5 ^a	0.8 \pm 0.3
Alkanes ^b + fatty acids ^c	21	2.3 \pm 0.5 ^a	0.9 \pm 0.2
Alkanes ^b	20	2.1 \pm 0.7 ^a	0.8 \pm 0.2
Fatty acids ^c	19	2.3 \pm 0.7 ^a	1.0 \pm 0.3

ns = no significant difference.

^aSignificantly different from the respective control by *t* test at 5% level.

^bAlkanes listed in Table 4 were mixed accordingly.

^cFatty acids listed in Table 3 were mixed accordingly.

TABLE 3. MAJOR COMPONENTS IN TLC BAND 3 FROM EPICUTICULAR LIPID OF LEAVES OF JAPANESE KNOTWEED *Fallopia japonica*^a

Component	Diagnostic ion ^a	Composition ^b (%)
Nonanoic acid (9:0)	230(M), 215, 145,132, 117, 129, 75, 73	2.4
Decanoic acid (10:0)	244(M), 229, 145,132, 117, 129, 75, 73	1.7
Hendecanoic acid (11:0)	258(M), 243, 145,132, 117, 129, 75, 73	1.5
Dodecanoic acid (12:0)	272(M), 257, 145,132, 117, 129, 75, 73	4.5
Tridecanoic acid (13:0)	286(M), 271, 145,132, 117, 129, 75, 73	3.0
Tetradecanoic acid (14:0)	300(M), 285, 145,132, 117, 129, 75, 73	8.6
Pentadecanoic acid (15:0)	314(M), 299, 145,132, 117, 129, 75, 73	3.1
Hexadecanoic acid (16:0)	328(M), 313, 145,132, 117, 129, 75, 73	39.2
Heptadecanoic acid (17:0)	342(M), 327, 145,132, 117, 129, 75, 73	3.8
Octadecanoic acid (18:0)	356(M), 341, 145,132, 117, 129, 75, 73	20.0
9-Octadecenoic acid (18:1)	354(M), 339, 145,132, 117, 129, 75, 73	6.9
Nonadecanoic acid (19:0)	370(M), 355, 145,132, 117, 129, 75, 73	2.2
Eicosanoic acid (20:0)	384(M), 369, 145,132, 117, 129, 75, 73	1.4
Docosanoic acid (22:0)	412(M), 397, 145,132, 117, 129, 75, 73	1.7

^a Diagnostic ions are based on the trimethylsilyl derivatives.

^b Percentage of each compound in the B3 fraction.

TABLE 4. MAJOR CHEMICAL COMPONENTS OF TLC BAND 6 FROM EPICUTICULAR LIPID OF LEAVES OF JAPANESE KNOTWEED *Fallopia japonica*

Component	Diagnostic ion	Composition ^a (%)
Hexadecane (<i>n</i> -C ₁₆)	226(M)	0.2
Heptadecane (<i>n</i> -C ₁₇)	240(M)	1.4
Octadecane (<i>n</i> -C ₁₈)	254(M)	0.7
Nonadecane (<i>n</i> -C ₁₉)	268(M)	4.8
Eicosane (<i>n</i> -C ₂₀)	282(M)	3.6
Heneicosane (<i>n</i> -C ₂₁)	296(M)	7.8
Docosane (<i>n</i> -C ₂₂)	310(M)	4.6
Tricosane (<i>n</i> -C ₂₃)	324(M)	8.7
Tetracosane (<i>n</i> -C ₂₄)	338(M)	4.6
Pentacosane (<i>n</i> -C ₂₅)	352(M)	9.8
Hexacosane (<i>n</i> -C ₂₆)	366(M)	3.5
Heptacosane (<i>n</i> -C ₂₇)	380(M)	10.9
Octacosane (<i>n</i> -C ₂₈)	394(M)	2.9
Nonacosane (<i>n</i> -C ₂₉)	408(M)	24.9
Triacontane (<i>n</i> -C ₃₀)	422(M)	1.9
Hentriacontane (<i>n</i> -C ₃₁)	436(M)	9.0
Dotriacontane (<i>n</i> -C ₃₂)	450(M)	0.5
Tritriacontane (<i>n</i> -C ₃₃)	464(M)	0.3

^a Percentage of each compound in the B6 fraction.

most abundant components (48.1%), and free fatty acids followed (22.3%). B3 and B6 had R_f values similar to those of octadecanoic acid and nonacosane, respectively. These two bands were characterized by GC-MS directly or after the formation of trimethylsilyl derivatives. As expected, B3 were fatty acids. Their chain lengths were from C_9 to C_{22} , and even carbon numbers were predominant. Among them, 16:0 was most abundant followed by 18:0 and 14:0 (Table 3). Alkanes were the only chemicals in B6. They had chain length in the range of C_{16} – C_{33} , with odd carbon numbers abundant. Among these alkanes, n - C_{29} dominated, followed by n - C_{27} , n - C_{25} , n - C_{31} , and n - C_{23} (Table 4).

Effects of Alkanes and Fatty Acids. A synthetic mixture of n -alkanes and fatty acids was prepared according to the composition found in epicuticular wax of the leaves of *F. japonica* and was tested by both no-choice and two-choice experiments. Treatment with the mixture caused a significant increase in oviposition in both bioassays (Tables 1 and 2). When a mixture of n -alkanes and a mixture of fatty acids were tested independently, each exhibited similar oviposition-stimulating activity (Tables 1 and 2).

DISCUSSION

A gravid lepidopteran female usually exhibits an obvious sequence of behavior to obtain epicuticular information from a potential host plant as soon as she alights on it (Renwick and Chew, 1994), demonstrating that sensory cues on the plant's surface are crucial for host acceptance. The no-choice test in the present study showed that the leaf epicuticular wax of *F. japonica* significantly increased the numbers of egg masses and eggs laid by *O. latipennis* females, thus indicating significant stimulatory effects on oviposition. The two-choice bioassay showed that females of *O. latipennis* prefer to lay their eggs on filter paper substrates treated with leaf epicuticular wax or fractions thereof. Observations of oviposition behavior and wind tunnel experiments are necessary to determine whether the apparent preference is attributable to attractiveness or oviposition-stimulatory effects.

n -Alkanes with chain lengths from C_{16} to C_{33} were the most abundant components in leaf epicuticular wax of *F. japonica*. An analysis of the steam volatile oil from *F. japonica* leaf showed similar results: n -alkanes in the range C_{10} – C_{30} were predominant (Miyazawa and Kameoka, 1976). A mixture of authentic n -alkanes of the natural composition exhibited significant oviposition-stimulating effects on *O. latipennis*. Similarly, in *O. nubilalis*, n - C_{26} , n - C_{27} , n - C_{28} , n - C_{29} , and n - C_{33} alkanes had significant oviposition-stimulating effects when applied individually to filter paper substrate (Udayagiri and Mason, 1997). In the corn earworm *Helicoverpa zea*, several n -alkanes (n - C_{25} and n - C_{32}), 2-methyl (C_{28} , C_{29} , C_{30} , and C_{32}), and 3-methyl-isomers (C_{29} and C_{31}) were

shown to be oviposition stimulants (Breedon et al., 1996). In the diamondback moth *Plutella xylostella*, both paraffin and a mixture of C₁₆ through C₂₉ *n*-alkanes caused marked increases in the relative acceptability of an aqueous homogenate of cabbage leaf (*Brassica oleracea*) and sinigrin, demonstrating obvious synergistic effects (Spencer, 1996).

The present study shows that a mixture of synthetic fatty acids of natural composition also enhanced oviposition in *O. latipennis*. Several aliphatic acids, 8:0, 9:0, 10:0, 11:0, 12:0, 13:0, linolenic acid (18:3), and hexadecatrienoic acid (16:3), together with β -bergamotenoic, curcumenoic, and farnesoic acids, are oviposition stimulants for *H. zea* (Breedon et al., 1996). Five saturated acids (8:0, 9:0, 10:0, 11:0, and 12:0) and two unsaturated acids [18:1 and linoleic acid (18:2)] significantly enhanced oviposition in the spruce budworm *Choristoneura fumiferana* (Grant et al., 2000). Moreover, 18:1, 20:0, 22:0, and 24:0 fatty acids caused a large increase in oviposition in the cowpea weevil *Callosobruchus maculatus* (Parr et al., 1998). In mango pulp weevil *Sternonchetus frigidus*, fractions containing several fatty acids (12:0, 14:0, 12-methyl-pentadecanoic acid, 14-methyl-heptadecanoic acid, 16-methyl-8-octadecenoic acid, and 9,12-octadecadienoic acid) stimulated oviposition (De Jesus et al., 2003). Furthermore, 18:1 and 18:2 fatty acids provide the orientation of the flight of gravid females of the navel orangeworm *Amyelois transitella* toward the source of an odor in a wind tunnel (Phelan et al., 1991).

In our previous study (Li and Ishikawa, 2004), five fatty acids (16:0, 18:0, 18:1, 18:2, and 18:3) were found in larval frass of *O. latipennis* and three other congeners, *O. furnacalis*, *O. scapularis*, and *O. zealis*, and a mixture of these fatty acids deterred the oviposition of females of all four species. Among these acids, 18:1 was recently shown to be the active deterrent (Li and Ishikawa, 2005). The apparent inconsistency between the previous and present studies can be explained if we assume that 18:1 has different functions depending on its concentration. At low concentrations, as found on the surface of Japanese knotweed, 18:1 exerts a stimulatory effect, whereas the same compound exhibits an opposite effect at high concentrations as found in larval frass. A similar case has been reported in the cowpea weevil *C. maculatus* (Parr et al., 1998). The substance 18:1 is the main component in epicuticular wax of mung seeds, a preferred host of the weevil. The fatty acid mixture as found in the epicuticular wax of mung seeds stimulated oviposition; however, an increase of 18:1 in the mixture resulted in deterrence.

n-Alkanes and free fatty acids are major constituents in epicuticular wax of numerous plant species and are biosynthesized through a highly conserved pathway within the plant kingdom (Kolattukudy, 1996; Ohlrogge and Jaworski, 1997). These chemicals have been shown to attract and/or stimulate oviposition of many insect species in Lepidoptera and Coleoptera (Eigenbrode and Espelie, 1995). In contrast, species-specific secondary metabolites are key factors

attracting and stimulating oviposition of oligophagous moths (Renwick and Chew, 1994; Honda, 1995). Consistent with this, we also found that *F. japonica* leaves still significantly stimulated oviposition even after the extraction of epicuticular wax (Li, unpublished data). Further analyses and the identification of these chemicals need to be carried out. For these analyses, however, a more discriminative bioassay has to be devised because the present one is not ideal in that a substantial number of eggs are laid on the control.

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HARD CLAMS (*Mercenaria mercenaria*) EVALUATE PREDATION RISK USING CHEMICAL SIGNALS FROM PREDATORS AND INJURED CONSPECIFICS

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Abstract—Hard clams, *Mercenaria mercenaria*, are sessile, filter-feeding organisms that are heavily preyed upon by blue crabs, which find their clam prey using chemical cues. Clams may evade blue crabs by reducing their pumping (feeding) behavior when a threat is perceived. The purpose of this study was to determine the type of signals that clams use to detect consumers. Clams decreased their pumping time in response to blue crabs and blue crab effluent, but not to crab shells, indicating that chemical signals and not mechanical cues mediated the response of clams to distant predators. Because predator diet can influence prey evaluation of predatory threats, we compared clam responses to blue crabs fed a steady diet of fish, clams, or that were starved prior to the experiment. In addition, we used injured clams as a stimulus because many organisms detect predators by sensing the odor of injured con- or heterospecifics. Clams reduced feeding in response to injured conspecifics and to blue crabs that had recently fed. Clams reacted similarly to fed crabs, regardless of their diet, but did not respond to starved blue crabs. Because blue crabs are generalist predators and the threat posed by these consumers is unrelated to the crab's diet, we should expect clam reactions to blue crabs to be independent of the crab's diet. The failure of clams to react to starved blue crabs likely increases their vulnerability to these consumers, but clam responses to injured conspecifics may constitute a strategy that allows animals to detect an imminent threat when signals emanating from blue crabs are not detectable.

Key Words—Blue crab, chemical cue, clam, diet, flume, foraging, predator avoidance, predator–prey interaction, risk evaluation.

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INTRODUCTION

Predators often have profound impacts on prey populations and on the organization and function of communities in general (Paine, 1966; Carpenter et al., 1985; Schmitz et al., 1997; Schmitz, 1998; Menge, 2000). The overall effect of predators on communities is determined by interactions between individual predators and prey (Lima, 1998, 2002). Therefore, the ability of predators to forage and the ability of prey to avoid consumers influence the magnitude of top-down forces in a given system (Menge, 2000; Werner and Peacor, 2003). Because decisions made by prey under the risk of predation have important consequences for both prey populations as well as entire communities, it is important to understand how prey evaluate and respond to predation risk (Lima and Dill, 1990; Werner and Peacor, 2003).

Although avoiding consumers is of great importance to prey, predator avoidance is often costly and results in decreased growth or fecundity (e.g., Lima and Dill, 1990; Peckarsky, 1996; Katz and Dill, 1998; Leonard et al., 1999; Nakaoka, 2000). Prey may minimize predator avoidance costs by using flexible avoidance strategies that balance the frequency or magnitude of predator avoidance responses with a perceived level of risk (Sih et al., 1985; Schmitz et al., 1997; Schmitz, 1998; Chivers and Smith, 1998; Katz and Dill, 1998; McIntosh and Peckarsky, 1999). Thus, prey require stimuli that accurately reveal the level of risk to determine when and how predator avoidance strategies should be employed.

Prey commonly use chemical signals to evaluate risk (Chivers and Smith, 1998; Katz and Dill, 1998) because chemical cues typically provide prey with accurate information concerning the location and intentions of predators (Chivers and Smith, 1998; Katz and Dill, 1998; Brown et al. 2000). This is particularly true in aquatic environments where visual or mechanical cues are often unavailable (Zimmer and Butman, 2000; Weissburg et al., 2002). Additionally, predators can more easily manipulate their posture or behavior to appear less threatening to prey than change their chemical signature (Katz and Dill, 1998; Brown et al., 2000).

Chemical cues indicative of danger may emanate from predators, from injured conspecifics, and sometimes from sympatric species (Petranka et al., 1987; Mathis and Smith, 1993; Chivers and Smith, 1998; Katz and Dill, 1998). Prey may use one or combinations of these signals to evaluate risk (Chivers and Smith, 1998; Katz and Dill, 1998; Bryer et al., 2001; Smith and Belk, 2001) and respond differently to chemical signals depending on other factors such as time of day (e.g., Peckarsky, 1996). Signals released from predators provide the most accurate indication of a predatory threat, and prey may minimize their predator avoidance costs by exclusively responding to these signals (reviewed by Katz and Dill, 1998). Although cost effective, an avoidance strategy in which prey

respond only to predator odors may increase their vulnerability when these chemicals are difficult to detect or when predators reach prey prior to the arrival of their chemical signals (e.g., when olfactory predators find prey by searching upstream). In contrast, chemical cues released by injured conspecifics may provide a stronger but less reliable indication of danger (reviewed by Katz and Dill, 1998). Yet, prey may overutilize predator avoidance tactics and incur high costs if they depend on less reliable signals (Lima and Dill, 1990; Katz and Dill, 1998).

Some prey limit their responses to predators that have eaten conspecifics or closely related species (e.g., Chivers and Smith, 1998; Katz and Dill, 1998; Chivers and Mirza, 2001; Mirza and Chivers, 2001; Smith and Belk, 2001; Brown and Dreier, 2002; Madison et al., 2002), and this predator detection strategy has been hypothesized to minimize predator avoidance costs. However, prey that depend on predator diet cues before initiating antipredator measures may be vulnerable to generalist predators that switch diets frequently (Bryer et al., 2001; Chivers and Mirza, 2001). Bryer et al. (2001) and Chivers and Mirza (2001) hypothesized that prey responses that are dependent on predator diets should only occur in systems where the threat posed by a predator is directly related to that predator's most recent foraging activity.

In this study, we examined the effects of a generalist predator's diet on the response of a common prey organism using blue crabs *Callinectes sapidus* and hard clams *Mercenaria mercenaria* as model organisms. Blue crabs *C. sapidus* are important predators and scavengers in southeastern estuaries (Eggleston et al., 1992; Micheli, 1997) and are the primary consumer of juvenile hard clams *M. mercenaria* in these areas (Micheli, 1995, 1997). Blue crabs are also a threat to adult clams, as they can nip their siphons and decrease their feeding efficiency, growth, and fecundity (Peterson, 1986; Coen and Heck, 1991; Irlandi, 1994). Clams release attractive chemicals into the water as they feed, and blue crabs follow these waterborne chemical odor plumes to locate their clam prey (Weissburg and Zimmer-Faust, 1993; Weissburg et al., 2002). Irlandi and Peterson (1991) found that clams responded to the presence of predators by reducing their feeding time and hypothesized that feeding reductions would make clams less apparent to consumers. Indeed, caging predators near clam beds decreases clam mortality (Smee and Weissburg, in press), but clam growth and reproductive output are diminished by long-term exposure to predators (Nakaoka, 2000). Thus, clam responses to predators are adaptive and costly.

We hypothesized that clams detect approaching blue crab predators by using chemical signals or hydrodynamic signals or both. We exposed clams to both chemical and hydrodynamic signals from blue crabs to verify the type of cue that clams used to detect blue crabs. The results indicate that clams were responding to chemical cues emanating from blue crabs, and we conducted a second experiment to determine the nature of these signals. In the second

experiment, we compared changes in clam behavior when exposed to blue crabs that had been fed different diets and to injured conspecifics. Our results suggest that prey respond to a generalist predator regardless of diet, presumably because the dietary history of such a predator does not predict the risk to its potential prey. Furthermore, there are limits to prey perceptual abilities that may result in increased predation risk. For example, prey may be unable to detect starved predators, although a highly motivated consumer increases the chance that potential prey may be attacked.

METHODS AND MATERIALS

Animal Capture and Maintenance. Animals were collected from Wassaw Sound, GA, USA, and associated tributaries. Hard clams *M. mercenaria* were hand dug with clam rakes and fingers in the intertidal zone, and blue crabs *C. sapidus* were captured with commercially purchased crab pots. After capture, animals were returned to the Skidaway Institute of Oceanography (SkIO) near Savannah, GA, and housed in flow-through sea tables supplied by water pumped from the Skidaway River. Sea table water was filtered through both gravel and sand filters, and the water temperature and salinity in the sea tables ranged from 25 to 30°C and from 25 to 30 ppt, respectively. Clams acclimated in the sea tables for at least 6 hr prior to behavioral assays (see below) and were used in behavioral assays within 48 hr after removal from the field. Blue crabs were kept in the sea tables for at least 1 wk prior to use in the behavioral assays. Crabs were fed with a daily diet of either fish (*Menhaden* sp.) or clams (*M. mercenaria*) or were starved during the 1-wk acclimation period. We returned each clam or crab to the field after a single use (except for a few clams that were injured as part of the experiment or used for food; see below).

Experimental Arena. Experiments were conducted in a paddle-driven racetrack flume at SkIO (4.8-m-long working section \times 1 m wide \times 0.33-m water depth). The upstream bend of the flume is divided into five 23-cm channels to reduce secondary circulation. Flow is further conditioned by honeycomb baffling (5 cm thick with 7-mm openings) at the downstream end of this bend and by a polyvinyl chloride (PVC) flow straightener (10 \times 4.5-cm openings) placed at the end of the working section to prevent back-flow. The working section contains a false bottom (0.30-m diam. \times 0.15 m deep) located 2.3 m downstream from the entrance point of the working section and is in the center of the flume to minimize wall effects. Both the working section and false bottom of the flume were filled to a uniform depth of 1 cm with commercially purchased sand (grain size 0.04 ± 0.04 cm). The flume was supplied by the same water source as the sea tables and had similar temperature and salinity. Flume water passed through both gravel and sand filters as

well as a 10- μm filter bag. Flow speed was maintained at 3 cm sec^{-1} in all experiments. This flume produces stable and reproducible boundary layers at current speeds ranging from 1 to 15 cm sec^{-1} . See Ferner and Weissburg (2005) for a detailed flume description and characterization of the flow environment boundary.

Behavioral Assays. Experiments utilized changes in clam pumping (feeding) behavior as assays for the ability of clams to detect predation risk. Although previous investigators have assumed that clams are actively pumping only when their siphons are extended (e.g., Irlandi and Peterson, 1991), we performed preliminary experiments to verify this supposition. We visualized the excurrent from clams by carefully pipetting a 0.1% solution of fluorescein dye above the excurrent siphon of a clam. Thirty-six clams that had their siphons extended were tested in this manner, and all were releasing an excurrent. We tested 15 clams with open shells but withdrawn siphons, and only three were pumping. Thus, we concluded that siphon extension was indicative of pumping.

Behavioral trials consisted of challenging clams to detect and respond to blue crab predators, injured clams, predator-conditioned water, and predator shells. We judged clam responses to predation risk by determining if clam feeding (no. of siphon extension observations) was significantly less in response to these treatments when compared to a control that lacked predators or injured conspecifics. In each assay, we placed five clams in the false bottom of the flume and allowed them to acclimate for 30 min. Clam density in these experiments was five clams per 0.07 m^2 and mimics densities observed in natural habitats (Walker, 1987; Smee and Weissburg, unpublished data). We introduced predators, crushed conspecifics, or predator-conditioned water at the conclusion of the 30-min acclimation period by placing a tethered crab, injured clam, or the nozzle (see below) from our delivery system 0.5 m upstream from the clam bed. We recorded the siphon position of each clam (extended or not) prior to introduction of the predator treatments and at 5-min intervals after introduction for 30 min. Thus, each clam could have been observed feeding (pumping) a maximum of seven times, and we used the total number of observations in which clams were pumping as our measure of clam pumping time. That is, the response of each clam in a trial was measured by a single number between 0 and 7, which indicated how many times we observed an individual clam pumping.

The order of treatments and controls in these experiments was randomly assigned each day, and each treatment and the control were replicated at least five times (5 trials \times 5 clams per trial = 25 clams for each treatment and control). Each clam and predator was used only once. Clams that neither pumped nor burrowed were excluded from the analysis, and we excluded approximately 25% of the clams from the experiment by using this criterion.

Including inactive clams in our analysis would have enhanced our results, but we excluded them because we could not clearly determine the causes of clam inactivity.

Characterization of Predator Cues. Preliminary observations suggested that clams pumped significantly less when tethered blue crabs were placed upstream. We hypothesized that potential predators created hydrodynamic signals, chemical signals, or both that mediated the response of clam prey. Therefore, we conducted two experiments to determine the cue that clams use to detect predators. We tested responses of clams to hydrodynamic cues by placing an empty predator shell 0.1 m upstream from the clams and comparing clam pumping between this treatment and the control. Qualitative flow visualization with dye indicated that the turbulence created by the predator shells dissipated within the first 0.25 m downstream, although we could not exclude the possibility that a more exacting analysis of flow would reveal that perturbations induced by the shell extended farther downstream. Thus, we placed the predator shell 0.1 m upstream from the clam bed to ensure that the clams were in its turbulent wake.

To determine if clams were detecting chemical signals from predators, we designed a chemical delivery system to transport blue crab effluent to the experimental clams. The delivery system pumped water out of the flume and into a container ($0.31 \times 0.24 \times 0.36$ m) that was left empty (control) or that housed a blue crab that had recently eaten clams. The water from the container was released into the flume 0.5 m upstream from the clam bed via a 0.076-m diam PVC pipe oriented parallel to the flow. Water moved through the delivery system at a velocity of 3 cm sec^{-1} , which matched the free stream flow velocity in the flume. The large diameter pipe was selected because it was of similar size to a blue crab, which allowed us to simulate water passing over the crab at a rate similar to that occurring in experiments with clams exposed to a live predator.

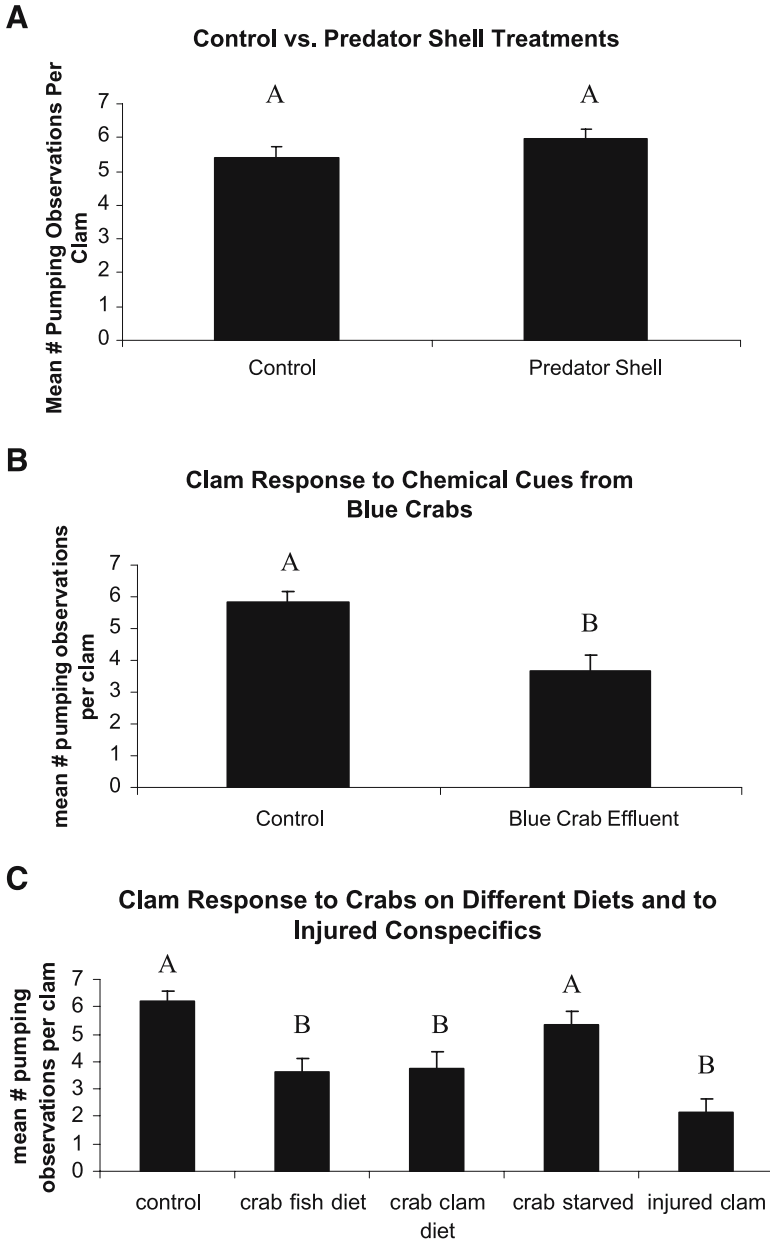
We realize that the flow diversion method may only crudely approximate the flux of chemicals experienced by a prey organism directly upstream of a crab predator. Although our approach replicates the rate of water movement over the animal, mixing in the delivery system and introduction through a pipe will probably change the chemical signal dynamics relative to that produced by water flowing over an individual crab. However, the flow diversion method we employed is a more realistic alternative than prey soaks or body washes because the volumetric rate at which water passes over the crabs in the diversion system is roughly equal to that passing over a crab in the flume. In contrast, soaks or body washes concentrate predator metabolites using arbitrarily determined volumes and time periods, and so produce unknown metabolite concentrations that will not be experienced by naturally foraging animals.

Effects of Predator Diet and Response of Clams to Injured Conspecifics. In this experiment, we measured the responses of clams when presented with odors from injured conspecifics as well as crabs that were fed with different diets prior to behavioral assays. The injured clam treatment was prepared by striking a clam with the blunt edge of a kitchen knife, removing the top valve, and making multiple lacerations on the visceral mass. This treatment mimicked crab feeding and insured that clam metabolites were released into the water. To measure the impact of crab diet on clam responses, collected blue crabs were fed with a daily diet of fish or clams for 1 wk or were starved for 1 wk prior to the experiment. We allowed clams to acclimate in the flume using the same methodology previously described, then placed a tethered blue crab or injured clam 0.5 m upstream from the clam bed, and monitored clam feeding.

Data Analysis. We initially examined the percentage of times that adjacent clams were feeding simultaneously to determine if interactions occurred between clams. Clams in control trials pumped during 87% of our observations. Thus, the proportion of time that two adjacent clams should be pumping simultaneously is 0.87^2 (0.76), assuming that adjacent animals do not influence each other. Adjacent clams ($N = 25$ pairs) in control trials pumped simultaneously in 70% of our assays, a value not significantly different from the random expectation (Sokal and Rohlf, 1995).

Since clams were not influencing each other, observations of pumping behavior of individual clams (number of siphon extensions observed for each clam) were arcsine transformed to meet analysis of variance (ANOVA) assumptions and were then compared using a nested ANOVA that examined the effects of predator treatment and trial nested within treatment (Sokal and Rohlf, 1995). The use of a nested ANOVA allowed us to determine if variations in clam responses were affected by variability in cue quality or quantity across replicate treatments, which is a source of uncontrolled variation in the experiments. The P value for the nest effect was greater than 0.20 in all experiments, indicating that clams in different groups were reacting similarly to the same treatments. The lack of a significant nest effect permitted us to lump trials within treatments and test the significance of the main effect by using the pooled error variance (Sokal and Rohlf, 1995). The absence of a nest effect suggests that cues from predators and injured conspecifics were roughly similar between replicate trials.

Experiments using tethered predators, predator effluents, and predator shells were conducted at different times over a period of several months. Therefore, each experiment was analyzed separately because it would be inappropriate to compare treatments to one another under these conditions. Separate control experiments were performed for each experiment to account for any variation in animal or general experimental conditions. Trials that used different predator diets or injured conspecifics were intermingled and, on a daily



basis, were presented in random order to test clams. After completing the nested ANOVA, a Tukey–Kramer *post hoc* analysis was employed to test for pairwise differences between treatments (Sokal and Rohlf, 1995).

RESULTS

Characterization of Predator Cues. Data from experiments using the predator shells indicated that there were no significant differences in clam pumping between the predator shell treatments and the controls ($F_{1,83} = 2.56$, $P > 0.11$, Figure 1A). Although not significant, we observed a higher clam-pumping rate in trials with predator shells. Thus, turbulence generated by the predator shell did not alter clam pumping, which suggested that clams were not using a hydrodynamic cue to detect predators.

In contrast to the results obtained with empty predator shells, clam pumping was significantly reduced ($\approx 40\%$) when clams were exposed to water released from the delivery system that had passed over a blue crab as compared to water passing through the empty system without blue crabs present ($F_{1,35} = 8.69$, $P < 0.01$, Figure 1B). Additionally, clam feeding was affected similarly by predator-conditioned water and (nonstarved) predators placed directly in the flume (see below). The failure of clams to cease pumping in response to hydrodynamic signals, combined with the positive response to predator-conditioned water delivered under environmentally realistic conditions, suggested that clam responses to predators were chemically mediated.

Effects of Predator Diet and Response of Clams to Injured Conspecifics. The data revealed that clam feeding decreased by 40% when exposed to blue crabs that had recently been fed and by 65% in the presence of injured conspecifics as compared to controls that lacked predators or injured clams ($F_{4,84} = 10.28$, $P < 0.001$, Figure 1C). Starved blue crabs caused a slight (15%) but insignificant reduction in clam feeding. Additionally, *post hoc* analysis revealed that clams pumped significantly more in the presence of starved blue crabs than those that were recently fed, and clam responses to crab predators

FIG. 1. Mean number of pumping observations per clam (\pm SE). Letters denote means that are significantly different based on a Tukey–Kramer *post hoc* test. Each clam could have been observed pumping a maximum of seven times during the 30-min observation period. (A) Control vs. predator shell placed 0.1 m upstream, $N = 42$ and 43 clams, respectively. (B) Control vs. blue crab effluent released from our delivery system, $N = 16$ and 21 clams, respectively. (C) Clam pumping in the presence of crabs fed with different diets and injured conspecifics. Sample sizes for each treatment are 24, 16, 15, 19, and 15 for the control, crab fed with fish, crab fed with clams, starved crab, and injured clam treatments, respectively. Differences in sample size result from exclusion of inactive clams from analyses.

were similar regardless of their diet. Although not significantly different from responses to fed crabs, clams reduced their feeding time almost 40% more after detecting an injured conspecific than a crab that had recently eaten (Figure 1C). Thus, clam feeding was affected more by the presence of injured clams than by the odors of fed predators, although both caused significant reductions in clam feeding as compared to controls and starved crab treatments.

DISCUSSION

Our results indicate that clams use chemical signals to detect upstream blue crabs and respond to these predators by reducing their feeding (pumping) behavior. Other bivalves (e.g., mussels) also use chemical cues to detect predators and respond by changing their morphology (e.g., Leonard et al., 1999) or behavior (e.g., Cote' and Jelnikar, 1999). Previous studies have shown that blue crabs depend on chemical cues to locate clam prey (Weissburg and Zimmer-Faust, 1993; Finelli et al., 2000; Weissburg et al., 2002). The modulation of the blue crab–clam predatory interaction by chemicals is perhaps unsurprising given that the water in our study area is extremely turbid, and chemical cues are likely the only signals that can be detected from a distance in this habitat. Both blue crabs and their prey use the same sensory modality to detect each other, so the conditions that affect the transmission of chemical signals will affect the sensory abilities of both organisms. Thus, the outcome of interactions between these organisms may differ considerably between areas that enhance chemical signaling as compared to those that impede it.

In nature, clam feeding rates may be influenced by other factors (e.g., food availability, temperature) that were not considered in the present study. Reactions to predators may change in the field depending on a variety of factors besides the perceived level of risk. Still, long-term exposure to predators has been shown to significantly decrease clam growth in the field (Nakaoka, 2000). In a related field study, Smee and Weissburg (in press) found that clam survival was significantly higher in clam plots with predators caged nearby as compared to control plots with empty cages. These studies indicate that clam reactions to predators, while costly, reduce mortality and suggest that clams react to predators across a range of natural conditions. Therefore, the clam reactions to predators and injured conspecifics observed in the present study should be indicative of the cues used by clams to avoid predation in the field.

Clams only responded to cues released by blue crabs if the crabs had recently been fed and not if they had been starved for 1 wk. Clams responded similarly to fed crabs regardless of whether the crab's diet consisted of fish or clams prior to behavioral assays. In addition, clams altered their feeding behavior in the pres-

ence of injured conspecifics, suggesting that they also use these signals to detect predatory threats.

The ability of clams to react to injured conspecifics may compensate for their inability to detect hungry blue crabs. Prey organisms may benefit from living in close proximity to conspecifics or related species, as neighbors can provide for shared vigilance against consumers or early warnings of danger (Hamilton, 1971; Powell, 1974; Sullivan, 1984; Fitzgibbon, 1990; Aukema and Raffa, 2004). The benefit provided by neighbors is particularly strong in organisms that respond to the odors of injured conspecifics or heterospecifics, as consumption of neighbors reveals a predatory threat (e.g., Mathis and Smith, 1993). Hard clams are commonly found in dense beds and can reach densities in excess of 50 clams m^{-2} in our study area (Walker, 1987; Smee and Weissburg, unpublished data). Clams living in dense beds may be better able to avoid unapparent predators, as neighbors that are eaten may warn of imminent peril.

Responses of prey that are dependent on predator diets have been found in many predator-prey systems (Crowl and Covich, 1990; Chivers et al., 1996; Stabell and Lwin, 1997; Chivers and Mirza, 2001; Smith and Belk, 2001) but are notably absent from others (Petranka and Hays, 1998; Bryer et al., 2001). The existence of diet-dependent responses may be contingent on whether the recent dietary history of the predator is correlated with risk to prey. For instance, seasonally hunting predators may pose a risk for prey only at certain times, so that predator diet may predict the potential threat level to that prey species (Chivers and Mirza, 2001). Alternatively, Bryer et al. (2001) suggest that diet-dependent responses to predators may not be beneficial when prey are hunted by generalist predators, such that the risk level posed by the predator is unrelated to its foraging habits. Bryer et al. (2001) observed that slimy sculpin responses to brook trout predators were unaffected by the trout's diet. They reasoned that the threat posed by brook trout to sculpins is unrelated to the trout's foraging habits, and thus, it would not be advantageous for the sculpin to base risk evaluation on predator diet cues.

The response of clams to blue crabs in our experiment was not dependent on the crab's diet. Because blue crabs are generalist consumers and eat almost anything alive or dead (Virnstein, 1977; Eggleston et al., 1992; Micheli, 1995, 1997), the threat of predation by crabs is unrelated to the crab's recent foraging activity. As in the previous example with slimy sculpins, knowledge of a blue crab's diet provides no valuable information for their prey, suggesting that it is not advantageous for clams to rely on diet cues as their sole means of evaluating risk.

Cost-benefit analyses are often used to explain the variability in responses to predators across predator-prey systems. However, cost-benefit explanations currently are focused on response specificity as opposed to response sensitivity and may be inadequate when prey fail to detect actual predatory threats because predators have not recently fed (Howe and Harris, 1978). If predators stop

releasing chemical signals or release chemicals that are difficult to detect, then organisms may not adequately perceive the true risk level. Starved predators often show enhanced search responses relative to those that are well fed, as revealed by increases in the duration and frequency of search bouts in response to a given stimulus level or decreases in the threshold stimulus levels that are required to initiate or maintain search (Mackie and Shelton, 1972; Zimmer-Faust and Case, 1982). Therefore, the threat posed by a starved crab is equal to, or possibly greater than, that posed by a crab that has recently foraged. Thus, it would be prudent for clams to respond to starved crabs, and their failure to do so suggests that starvation renders blue crabs less detectable to clam prey. Prey may be more likely to depend on the odors of injured con- or heterospecifics to detect consumers when predators are commonly undetectable, although this hypothesis has not been empirically tested.

We have yet to develop risk-based models for the sensitivity of potential prey to cues derived from their consumers, and research that attempts to quantify the stimulus levels necessary to elicit prey reactions is lacking. Prey with low sensitivity thresholds may experience large costs (e.g., reductions in the opportunity to feed), particularly if prey use general metabolites to detect their predators, as these substances may come from a variety of sources. In contrast, prey with higher sensitivity thresholds may decrease predator avoidance costs but may also be more vulnerable to their enemies. Recent technological advances in our ability to characterize and identify chemical signals (e.g., Millar and Haynes, 1998), as well as our ability to examine chemical signal transport in aquatic systems (e.g., Webster and Weissburg, 2001; Weissburg et al., 2002), may allow us to investigate threshold sensitivity and its relationship to predation risk in a more thorough manner than has been previously attempted.

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HERITABLE VARIATION IN THE SEX PHEROMONE OF THE ALMOND MOTH, *Cadra cautella*

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Abstract—The sex pheromone of the female almond moth, *Cadra cautella*, constitutes a highly variable signal. Age and period of the L/D cycle had significant effects on the variation in titers of (*Z,E*)-9,12-tetradecadienyl acetate (*Z9,E12-14:Ac*) and (*Z*)-9-tetradecenyl acetate (*Z9-14:Ac*) and the ratio of *Z9,E12-14:Ac* to *Z9-14:Ac*. Across age classes, minimum and maximum mean titers (ng ± SE) of *Z9,E12-14:Ac* and *Z9-14:Ac* ranged from 8.7 ± 0.46 to 21 ± 1.0 and from 2.0 ± 0.14 to 2.9 ± 0.15 , respectively; the mean ratio of *Z9,E12-14:Ac* to *Z9-14:Ac* ranged from 5.2 ± 0.21 to 11 ± 0.40 . The titers of *Z9,E12-14:Ac* and *Z9-14:Ac* and the ratio of *Z9,E12-14:Ac* to *Z9-14:Ac* were highest at the onset of the scotophase and lowest at the onset of the photophase. Similarly, the titer of *Z9,E12-14:Ac* and the ratio of *Z9,E12-14:Ac* to *Z9-14:Ac* were highest in females sampled 1 d postemergence and declined over all age classes. The titer of *Z9-14:Ac* increased from day 1 to day 2, and then declined to levels equivalent to day 1. Analysis of pupal and adult mass demonstrated a positive correlation ($r = 0.874$, $P < 0.001$); however, the titer of neither *Z9,E12-14:Ac* or *Z9-14:Ac* nor the ratio of *Z9,E12-14:Ac* to *Z9-14:Ac* were significantly correlated with pupal mass. Age-related variations in pheromone titer and ratios were heritable. The narrow-sense heritability of the observed variation in 1-d-old females, 1 hr into the scotophase is 1.2 ± 0.32 , 0.75 ± 0.24 , and 0.46 ± 0.17 for the titer of *Z9,E12-14:Ac* and *Z9-14:Ac* and the ratio of *Z9,E12-14:Ac* to *Z9-14:Ac*, respectively. A significant additive genetic correlation was observed between that of *Z9,E12-14:Ac* and *Z9-14:Ac*, but not between that of either *Z9,E12-14:Ac* or *Z9-14:Ac* and the ratio of *Z9,E12-14:Ac* to *Z9-14:Ac*. The results of the genetic analyses suggest that the traits of titer of *Z9,E12-14:Ac* and *Z9-14:Ac* have a greater potential to respond to selection than the trait of ratio of *Z9,E12-14:Ac* to *Z9-14:Ac*.

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Key Words—*Cadra cautella*, pheromone titer, diel periodicity, sex pheromone, (*Z,E*)-9,12-tetradecadienyl acetate, (*Z*)-9-tetradecenyl acetate, signal variation, Lepidoptera, Pyralidae, heritability.

INTRODUCTION

Among moths (Lepidoptera), the behavioral mechanisms used in pair formation are typically the same ones that prevent hybridization (Cardé, 1986). In most species reproduction is preceded by long-distance attraction of mates and short-range courtship, both of which are mediated by pheromones (see Cardé and Haynes, 2004). Specificity in the chemical communication channel is predominately the result of species-specific male and female pheromone blend compositions and male response profiles and courtship behavior (Tumlinson et al., 1974; Cardé et al., 1977; Phelan and Baker, 1987, 1990; Löfstedt et al., 1991; McElfresh and Millar, 2001). Phenological and diel differences in reproductive behavior may also contribute to reproductive isolation by temporal isolation of heterospecific reproductively mature males and females (Teal et al., 1978; Greenfield and Karandinos, 1979; Haynes and Birch, 1986).

Species-specific pheromone communication channels have been interpreted as indirect evidence of the selective effects of competition for the communication channel and/or reinforcement of isolating mechanisms over evolutionary time (see Cardé and Baker, 1984). These paradigms have been challenged by the recognition concept (reviewed by Löfstedt, 1993; Linn and Roelofs, 1995) and theories involving intraspecific competition for mating opportunities (see West-Eberhard, 1984). As Löfstedt (1993) pointed out, “Regardless of which type of selection one advocates as a major evolutionary force the mechanism for change in a communication system constitutes a problem.” In other words, because natural selection should favor signaling systems that maximize the ratio of the received signal relative to background noise (Endler, 1992), stabilizing selection should minimize signal variation, yet directional selection is required for change. In apparent contradiction to these arguments, the sex pheromones of some dytrisian Lepidoptera are characterized by high coefficients of variation (e.g., the coefficient of variation for the proportion of the triene pheromone component in *Phthorimaea operculella* pheromone glands was 23% and 31% for a California and Nagoya, Japan, population; Ono et al., 1990).

Geographic variation in moth sex pheromones has been observed in many species [e.g., *Agrotis segetum* (see Löfstedt, 1993); *Hemileuca eglanterina* (McElfresh and Millar, 2001)]. Age, diel (Tang et al., 1992), and temperature effects (Ono, 1993, 1994) also influence quantitative and qualitative variation in sex pheromones. To further understand the basis of variation in moth

pheromone signals, we studied the pheromone biology of a stored-products pest, the almond moth, *Cadra cautella* (Walker) (Lepidoptera: Pyralidae). Specifically, we tested the null hypotheses that mean titer of (*Z,E*)-9,12-tetradecadienyl acetate (*Z9,E12-14:Ac*) and (*Z*)-9-tetradecenyl acetate (*Z9-14:Ac*) (the major and minor pheromone components of *C. cautella*; Brady et al., 1971; Read and Haines, 1979) and the ratio of *Z9,E12-14:Ac* to *Z9-14:Ac* were independent of: (1) female age, (2) the period of the L/D cycle during which females were assayed, (3) whether or not females had access to water as adults, and (4) the temperature that females were reared at as larvae. We also measured the intensity of association between pupal and adult mass, and between pupal mass and the titers and ratio of *Z9,E12-14:Ac* to *Z9-14:Ac*. Finally, because selection is only expected to act on heritable variation, we also examined the heritability of the variation observed in the titers of *Z9,E12-14:Ac* and *Z9-14:Ac*, and the ratio of *Z9,E12-14:Ac* to *Z9-14:Ac* under controlled ages and rearing conditions.

METHODS AND MATERIALS

Insect Rearing. The colony of *C. cautella* was initiated in 1989 with ca. 500 larvae and pupae obtained from Kansas State University, Manhattan, KS, USA. Since then, the colony has been maintained in continuous culture at or above 400 mating pairs. The insects were reared in a controlled environment rearing room kept at a mean temperature of 26°C (range 25–27°C), with a photoperiod of L/D 16:8 hr and 50–60% RH. These rearing conditions were periodically verified with a Hobo® sensor (Onset Computer Corporation, Bourne, MA, USA). Egg and larval stages were reared in 1-l jars on diet made from 3 kg poultry laying mash, 2 kg rolled oats, 100 g brewer's yeast, and 200 ml glycerin. Last instars of both sexes were harvested and held in groups in inflated Ziploc® plastic bags with Kimwipe® tissues inserted as pupation substrate. Adults were collected as they emerged and placed in 25 × 25 × 25 cm plastic gauze rearing cages where mating and oviposition occurred. Eggs were collected from these cages and used to seed 1-l jars for the next generation.

Pheromone Extraction. Male testes are easily seen through the dorsal larval cuticle, allowing for rapid sex determination of wandering last instar larvae. For pheromone analyses, female larvae were collected and individually reared in 30-ml plastic cups in a light box kept in the rearing room described above. Conditions in the light box were identical to the rearing room (verified with a Hobo), except that the photoperiod was offset (ca. 4 hr) from the photoperiod of the rearing room. The rearing protocol described above provided a continuous culture of adult moths with emergence occurring daily. As a consequence, the progeny of a cohort of adults emerged over a span of ca. 7 d.

Female moths were held between the thumb and index finger, and even, gentle pressure was applied to the abdomen to extrude the ovipositor and pheromone gland. The pheromone gland, and as few terminal abdominal segments as possible, were excised using a pair of fine forceps and a razor blade. Before each group of dissections, new razor blades were rinsed in ethyl acetate and allowed to dry in the fume hood for 30 min. After each gland dissection, the forceps were rinsed in hexane and wiped with a Kimwipe. Excised glands were individually extracted in 25 μl hexane containing 1 ng/ μl of the internal standard, 13:Ac (not present in female glands, $N = 10$). Pheromone glands were excised in the second hour of the scotophase (unless otherwise noted) and extracted for 1 hr (optimized extraction time maximizing the ratio of the amount of pheromone extracted to extraneous material), after which the solvent extract was transferred to a clean glass insert inside a Teflon-lined, screw-capped 4-ml glass vial and stored at -20°C until analysis.

Gas Chromatography. Extracts were concentrated under a stream of nitrogen to ca. 3–4 μl , from which ca. 1 μl was injected into a gas chromatograph (GC) in splitless mode (injector 250°C). A Hewlett-Packard 6890 Series GC equipped with split/splitless injectors, flame ionization detectors, and interfaced to a computer with HP Chem Station Rev. A.04.02 was used for all analyses. The GC was fitted with DB-1 and DB-5 capillary columns (J&W Scientific, Folsom, CA, USA, 30 m length, 0.25 mm ID, 0.25 μm film thickness). The carrier gas was helium (linear flow rate of 28 cm/sec), detector temperature was 250°C , and an oven temperature program of 50°C for 1 min, $10^{\circ}\text{C}/\text{min}$ to 250°C for 10 min was used.

Experiment #1: Effect of Age on Titer and Component Ratio. On the day they were to be extracted and 1 hr prior to the onset of their scotoperiod, individual female moths were moved to an identical light box kept in the rearing room. This enabled us to access moths for experiments without exposing the overall population of moths to light during their scotoperiod. This protocol was followed for all other experiments except for the 21°C treatment of Experiment #4 (see below). Emergence was checked daily, and all female moths emerging each day were randomly assigned to one of the following treatments: 1, 2, 3, 4, or 5 d postemergence, and the pheromone gland dissected and extracted on the appropriate day as described above.

Experiment #2: Effect of the L/D Cycle on Titer and Component Ratio. All female moths emerging on a day were randomly assigned to one of the following treatments: gland excision and extraction at the onset of the scotoperiod (9:00 A.M.), mid-scotoperiod (1:00 P.M.), end of the scotoperiod (5:00 P.M.), or mid-photoperiod (1:00 A.M.). All females were assayed 1 d postemergence.

Experiment #3: Effect of Access to Water on Titer and Component Ratio. All female moths emerging on a day were randomly assigned to the treatments 1, 3, and 5 d postemergence, corresponding to the number of days

postemergence that female pheromone glands were extracted. Half of the females assigned to a treatment were reared with access to a wick partially immersed in a 4 ml-glass vial filled with water, and the other half were reared with access to a wick partially immersed in an empty 4-ml glass vial.

Experiment #4: Effect of Larval Rearing Temperature on Titters and Component Ratio. Ten 1-l glass jars containing the diet described above were seeded with ca. equal volumes of eggs. Simultaneously, five jars were maintained at 26°C in the rearing room described above and the other five were held at 21°C in an environmental chamber with a photoperiod of L/D 16:8 hr and 50–60% RH (verified with a Hobo). The pheromone glands of 10 females per jar were extracted for analysis. There were slight differences in the developmental rates of the larvae (larvae reared at 26°C developed faster). All females were assayed 1 d postemergence.

Experiment #5: Correlation Between Female Pupal and Adult Mass, and Pupal Mass and Titters and Component Ratio. To determine the correlation between pupal mass and adult mass, an empty 30-ml plastic cup was weighed to the nearest 0.1 mg and then reweighed with the addition of one pupa ($N = 30$). On the day of emergence, a second plastic 30-ml cup was similarly weighed with and without the adult moth. To determine the correlation between the pupal mass and pheromone titters, pupae and emergent adult females were weighed, and the pheromone glands of the adults were extracted as described above, respectively.

Statistical Analyses. The Shapiro–Wilk and Kolmogorov–Smirnov goodness-of-fit tests and Bartlett’s test were used to test for homogeneity of variances and normality for the data from Experiments #1–3. In Experiments #1–3, the data were not normally distributed and had heterogeneous variances. Although we were able to use the Box–Cox transformation to identify transformations that normalized the data (done only in Experiment #1), the transformed data still had heterogeneous variance. Despite the variance heterogeneity, none of the comparisons involved a ratio greater than 10 (this is the level of acceptable variance heterogeneity using the F_{\max} test; Tabachnick and Fidell, 2001). Given the outcome of these statistical tests of normality and variance heterogeneity, we elected to use a multiresponse permutation procedure (MRPP), which is free from the assumptions of the normality and homogeneity of variance (Mielke and Berry, 2001), to analyze the data from Experiments #1–4. We tested the null hypotheses that mean titters of Z9,E12-14:Ac and Z9-14:Ac, and the ratio of Z9,E12-14:Ac to Z9-14:Ac were independent of (1) the number of days postemergence that females were assayed, (2) the period of the L/D cycle in which females were assayed, (3) whether or not females had access to water as adults, and (4) the temperature that females were reared at as larvae. Under the experimental design used to test all four hypotheses, the treatment effect may also have included day effects; however, because

the cohorts of females analyzed were progeny of the same group of adults, were reared under controlled conditions, and all extractions were performed by the same individual, we expect that any day effect would be small relative to the treatment effect. Pairwise comparison of treatment groups within an experiment were performed using MRPP, which has a multiple-range test function. The intensity of the associations between pupal and adult mass and between pupal mass and mean titers of Z9,E12-14:Ac, Z9-14:Ac, or the ratio of Z9,E12-14:Ac to Z9-14:Ac were measured using simple linear correlation. In each case, we tested the null hypothesis $H_0: \rho = 0$ against $H_A: \rho \neq 0$ for $r_{0.05(2),28} = 0.361$. MRPP tests were performed using Blossom Statistical Software, version 2001-8d, (USGS, Fort Collins Science Center; www.fort.usgs.gov/products/software/blossom/blossom.asp).

Genetic Analysis. A half-sibling breeding design was used to characterize the genetic basis of the observed variation in the titers and ratio of the pheromone components. Wandering last instar male and female *C. cautella* larvae were randomly chosen from the laboratory colony and kept in 180-ml plastic cups in groups of 10 for pupation (males and females were kept separately). Virgin adults were paired 1–2 d after emergence in 180-ml cups with ca. 50 g of artificial diet for 1 d. The next day, the male moth was removed and similarly paired with a new 1- to 2-d-old virgin female. The original, mated female was placed individually in a new cup with artificial diet (1 d after mating). The following day (2 d after mating), the original mated female was placed in a third cup of artificial diet (3 d after mating) where she remained until death. Each dam was similarly placed on new media the 2 d following mating. On the third day, each male was paired with its third and final virgin female. The progeny of each female were reared, and extracts were prepared and assayed from pheromone glands from 1-d postemergence females during the second hour of the scotophase as described above. In total, 32 sires were mated to three dams each yielding 96 families. From these 96 families, the pheromone gland contents of 926 progeny were analyzed (an average of 9.7 progeny/family) (Figure 1). The genetic variances and covariances were estimated by restricted maximum likelihood using PROC MIXED (Fry, 2004) of SAS (1988). Heritabilities were estimated as described by Roff (1997) for a half-sibling design. Standard errors for the narrow-sense heritabilities and genetic correlation were determined following Roff (1997) and Robertson (1959), respectively. The null hypothesis that the additive genetic correlation did not differ from zero ($r_A = 0$) was tested as described by Fry (2004). The additive genetic coefficient of variation (CV_A) describes the “evolubility” (Houle, 1992) of a trait and was estimated for each trait using the equation $CV_A = 100 \times [(V_A)^{1/2}/\bar{X}]$, where V_A is the additive genetic variance of the trait (see above).

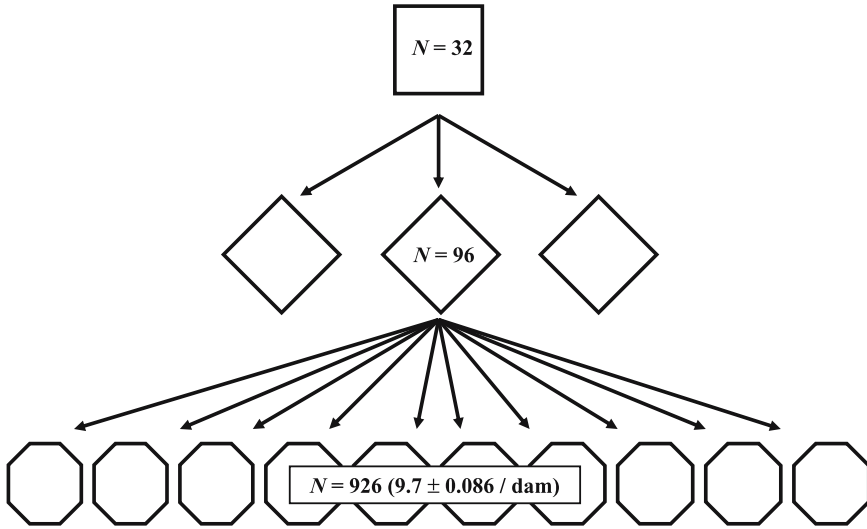


FIG. 1. The nested half-sibling design for genetic analysis of *C. cautella*. Thirty-two sires (square) were each mated to three dams (diamonds). From each dam, the pheromone glands of ten 1-d-old female offspring (octagons) were removed 1 hr into the scotophase and analyzed for the titers of Z9,E12-14:Ac and Z9-14:Ac and for the ratio of Z9,E12-14:Ac to Z9-14:Ac.

RESULTS

Analysis of Variation

Experiment #1: Effect of Age on Titer and Component Ratio. Mean titers of Z9,E12-14:Ac and Z9-14:Ac, and the ratio of Z9,E12-14:Ac to Z9-14:Ac varied significantly with female age (Figure 2). Female *C. cautella* had more Z9,E12-14:Ac 1 d postemergence than females from all other age classes. Similarly, females had more Z9,E12-14:Ac at 2 and 3 d postemergence than females from all older age classes. Unlike Z9,E12-14:Ac, the amount of Z9-14:Ac in female pheromone glands peaked at 2 d postemergence. Two- and 3-d-old females had more Z9-14:Ac than 1- and 5-d-old females, and 2-d-old females had more Z9-14:Ac than 4-d-old females. The ratio of Z9,E12-14:Ac to Z9-14:Ac was higher 1 d postemergence than for all other age classes, and at 2 and 3 d postemergence it was higher than at 4 and 5 d postemergence. In summary, the titer of Z9,E12-14:Ac and the ratio of Z9,E12-14:Ac to Z9-14:Ac decrease with increasing female age, whereas the titer of Z9-14:Ac initially increases with age and subsequently decreases.

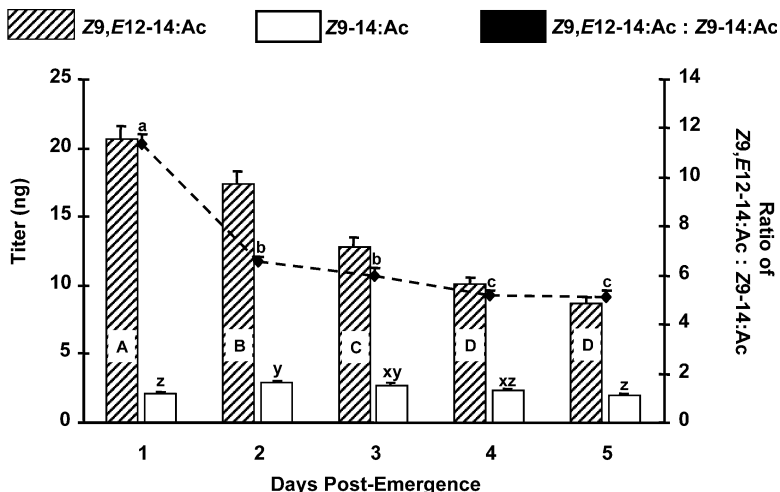


FIG. 2. The effect of age of newly emerged female *C. cautella* on mean titer (+SE) of Z9,E12-14:Ac (hatched bars) and Z9-14:Ac (open bars), and the ratio of Z9,E12-14:Ac to Z9-14:Ac (dashed line). Females were extracted 1 hr into the scotoperiod 1 d ($N = 97$), 2 d ($N = 101$), 3 d ($N = 105$), 4 d ($N = 101$), and 5 d ($N = 101$) postemergence. Hatched bars with different letters are significantly different ($P < 0.001$; except 1 vs 2 d postemergence, $P < 0.05$). Open bars with different letters are significantly different ($P < 0.001$; except for 1 vs. 3 d and 3 vs. 5 d postemergence, $P < 0.01$; and 2 vs. 4 d postemergence, $P < 0.05$). Filled diamonds from the dashed line with different letters are significantly different ($P < 0.001$; except for 3 vs. 4 and 5 d postemergence $P < 0.05$).

Experiment #2: Effect of the L/D Cycle on Titer and Component Ratio. Female *C. cautella* had Z9,E12-14:Ac and Z9-14:Ac present in their pheromone glands at all sampling times of the L/D cycle, but the mean titers of Z9,E12-14:Ac and Z9-14:Ac, and the ratio of Z9,E12-14:Ac to Z9-14:Ac varied significantly with L/D cycle (Figure 3). Females had more Z9,E12-14:Ac and Z9-14:Ac at the onset of the scotoperiod than at all other times. The amount of Z9,E12-14:Ac in female pheromone glands decreased throughout the scotoperiod and rose from the end of the scotoperiod to mid-photoperiod. Female Z9-14:Ac titers were higher mid-scotoperiod and at the end of the scotoperiod than mid-photoperiod. The mean ratio of Z9,E12-14:Ac to Z9-14:Ac was highest at the onset of the scotoperiod, decreased throughout the scotoperiod, and rose again to its maximum by mid-photoperiod. The only significant difference observed was between the ratio at mid-scotoperiod and mid-photoperiod.

Experiment #3: Effect of Access to Water on Titer and Component Ratio. The amount of Z9,E12-14:Ac decreased from 1, 3, to 5 d postemergence, whereas the amount of Z9-14:Ac was highest 3 d postemergence in females with and without access to water (Figure 4). The ratio of Z9,E12-14:Ac to Z9-

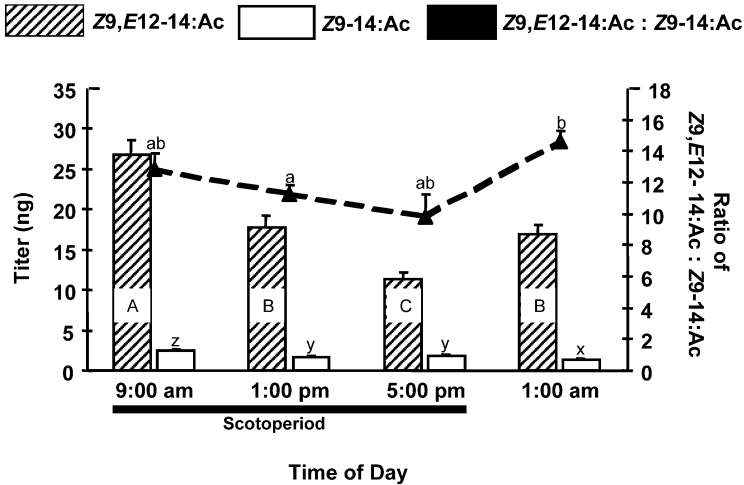


FIG. 3. The effect of L/D cycle on mean titers (+SE) of Z9,E12-14:Ac (hatched bars) and Z9-14:Ac (open bars) produced by female *C. cautella*, and on the ratio of Z9,E12-14:Ac to Z9-14:Ac (dashed line). Females were kept singly in cups within a light box on a 16:8 L/D cycle with the onset of the scotoperiod at 9:00 A.M. Female moths were extracted at the onset of the scotoperiod (9:00 A.M.) ($N = 58$), mid-scotoperiod (1:00 P.M.) ($N = 59$), end of the scotoperiod (5:00 P.M.) ($N = 60$), and mid-photoperiod (1:00 A.M.) ($N = 60$). Hatched bars with different letters are significantly different ($P < 0.001$). Open bars with different letters are significantly different ($P < 0.001$; except for 1:00 P.M. vs 1:00 A.M., $P < 0.05$). Filled triangles from the dashed line with different letters are significantly different ($P < 0.001$).

14:Ac decreased from 1 to 3 d postemergence. At 1, 3, and 5 d postemergence, there was no effect of access to water on the amount of Z9,E12-14:Ac ($P = 0.30$, $P = 0.57$, $P = 0.078$), Z9-14:Ac ($P = 0.30$, $P = 0.57$, $P = 0.17$), or the ratio of Z9,E12-14:Ac to Z9-14:Ac ($P = 0.18$, $P = 0.69$, $P = 0.82$) (Figure 4).

Experiment #4: Effect of Larval Rearing Temperature on Titer and Component Ratio. The mean titers of Z9,E12-14:Ac ($P = 0.28$) and Z9-14:Ac ($P = 0.87$) and the ratio of Z9,E12-14:Ac to Z9-14:Ac ($P = 0.61$) in the pheromone glands of females 1 d postemergence did not differ when larvae were reared at 21 or 26°C (Figure 5).

Experiment #5: Correlation Between Female Adult and Pupal Mass, and Pupal Mass and Titer and Component Ratio. Adult and pupal mass were positively correlated ($r = 0.87$, $P < 0.001$). The correlations between pupal mass and the amount of Z9,E12-14:Ac ($r = 0.082$) was not significant. The correlation between pupal mass and the amount of Z9-14:Ac ($r = -0.11$) and pupal mass and the ratio of Z9,E12-14:Ac to Z9-14:Ac ($r = 0.29$) also were not significant (Figure 6).

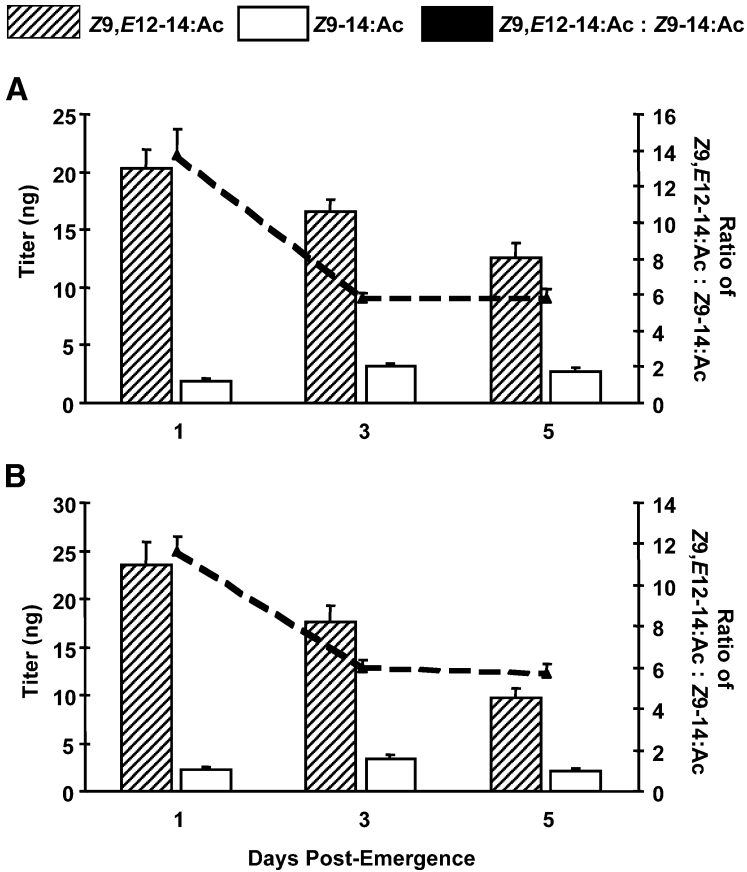


FIG. 4. The effect of access to water on mean titers (+SE) of Z9,E12-14:Ac (hatched bars) and Z9-14:Ac (open bars) produced by female *C. cautella*, and on the ratio of Z9,E12-14:Ac to Z9-14:Ac (dashed line). Titers and ratio 1, 3, and 5 d postemergence with access to water (A) and same without access to water (B). There was no effect of access to water on the amount of Z9,E12-14:Ac ($P = 0.30$, $P = 0.57$, $P = 0.078$), Z9-14:Ac ($P = 0.30$, $P = 0.57$, $P = 0.17$), or the ratio of Z9,E12-14:Ac to Z9-14:Ac ($P = 0.184$, $P = 0.692$, $P = 0.821$) 1, 3, and 5 d postemergence, respectively ($N = 30$).

Genetic Analyses

The considerable variation in both the titers and ratio of pheromone components among and within age classes and periods of the L/D cycle (Figures 2 and 3) and the absence of environmental effects in Experiments #3–4 (Figures 4 and 5) suggested a genetic basis to the observed variation in these traits.

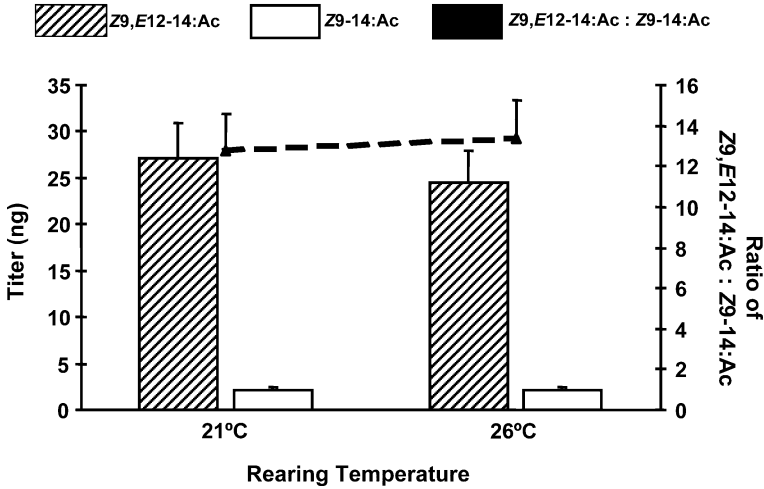


FIG. 5. Effect of larval rearing temperature on mean titers (+SE) of Z9,E12-14:Ac (hatched bars) and Z9-14:Ac (open bars) produced by female *C. cautella*, and on the ratio of Z9,E12-14:Ac to Z9-14:Ac (dashed line). There was no effect of rearing temperature on the titers of Z9,E12-14:Ac ($P = 0.28$) and Z9-14:Ac ($P = 0.87$), or on the ratio of Z9,E12-14:Ac to Z9-14:Ac ($P = 0.61$) ($N = 50$).

Heritability of Titer of Z9,E12-14:Ac and Z9-14:Ac. The mean titers of Z9,E12-14:Ac and Z9-14:Ac (\pm SE) among the F1 females were 25.73 ± 0.44 and 2.09 ± 0.047 ng ($N = 926$), respectively. Family means for Z9,E12-14:Ac and Z9-14:Ac (\pm SE) ranged from 12.66 ± 1.72 to 62.37 ± 3.62 and from 0.81 ± 0.11 to 7.24 ± 1.19 ng, and significant differences were found among families (Z9,E12-14:Ac and Z9-14:Ac, $P < 0.001$; ANOVA). The sire- and dam-based estimates of narrow-sense heritability (\pm SE) for Z9,E12-14:Ac are 1.20 ± 0.32 and 0.26 ± 0.10 , and for Z9-14:Ac are 0.75 ± 0.24 and 0.44 ± 0.09 , respectively (statistically significant, $h^2 > 2SE$). These moderate to high heritabilities (Arnold, 1994) suggest that a significant portion (26–100%) of the observed variation has an additive genetic basis.

Heritability of Ratio of Z9,E12-14:Ac to Z9-14:Ac. The mean ratio of Z9,E12-14:Ac to Z9-14:Ac (\pm SE) was 14.36 ± 0.19 ($N = 926$) among the F1 females. Family means ranged from $7.65 \pm 0.79:1$ to $23.04 \pm 3.55:1$, and differences were found among families ($P < 0.001$; ANOVA). The sire- and dam-based estimates of narrow-sense heritability (\pm SE) for this trait are 0.46 ± 0.17 and 0.56 ± 0.20 , respectively (statistically significant, $h^2 > 2SE$). These moderate heritabilities (Arnold, 1994) suggest that a significant portion (46–56%) of the observed variation has an additive genetic basis.

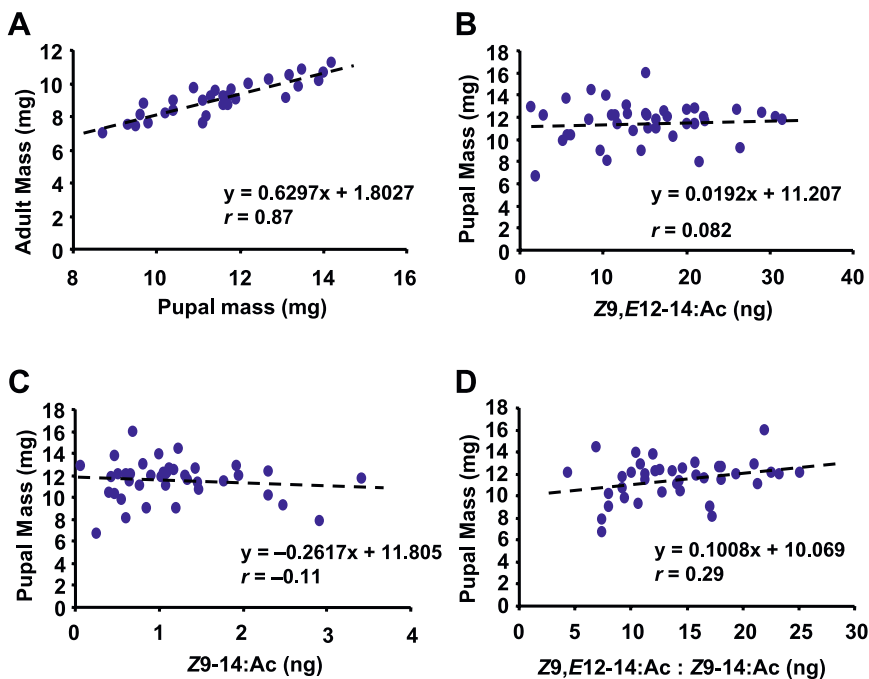


FIG. 6. Correlation analyses between adult mass and pupal mass (A); pupal mass and the major pheromone component Z9,E12-14:Ac (B), pupal mass and the minor pheromone component Z9-14:Ac (C), and pupal mass and the ratio of Z9,E12-14:Ac to Z9-14:Ac (D). Each dot represents one female moth ($N = 30$). The only correlation coefficient (ρ) significantly different than zero was observed between adult mass and pupal mass ($P < 0.001$).

Coefficients of Additive Genetic Variance and Genetic Correlations. The coefficients of additive genetic variance were high for both the sire- and dam-based estimates of all three traits (Table 1). The sire-based estimates of the genetic correlation (\pm SE) between the titers of Z9,E12-14:Ac and Z9-14:Ac, between the titer of Z9,E12-14:Ac and the ratio of Z9,E12-14:Ac to Z9-14:Ac, and between the titer of Z9-14:Ac and the ratio of Z9,E12-14:Ac to Z9-14:Ac are 0.85 ± 0.06 , 0.32 ± 0.2 , and -0.23 ± 0.34 , respectively (Table 1).

DISCUSSION

Analysis of Pheromonal Variation. The titers of Z9,E12-14:Ac and Z9-14:Ac, and the ratio of Z9,E12-14:Ac to Z9-14:Ac varied with age. In moths

TABLE 1. ESTIMATES OF PHENOTYPIC AND GENETIC VARIATION FOR THE TITERS OF Z9,E12-14:Ac AND Z9-14:Ac AND THE RATIO OF Z9,E12-14:Ac TO Z9-14:Ac^a

Trait	Mean (ng) ± SE	V _P	h ² ± SE	CV _A	r _A ± SE
Z9,E12-14:Ac (I)	25.73 ± 0.44	178.46	S _{IRE} 1.20 ^b ± 0.32 D _{AM} 0.26 ^b ± 0.10	55.99 25.94	
Z9-14:Ac (II)	2.09 ± 0.047	2.05	S _{IRE} 0.75 ^b ± 0.24 D _{AM} 0.44 ^b ± 0.09	59.76 44.37	0.85 ^c ± 0.06 0.32 ^d ± 0.20 -0.23 ^e ± 0.34
Ratio of I:II	14.36 ± 0.19	30.37	S _{IRE} 0.46 ^b ± 0.17 D _{AM} 0.56 ^b ± 0.20	25.91 28.78	

^aAll estimates are based on the analysis of 926 offspring from 32 sires mated to 3 dams.
^bSignificant heritabilities (h² > 2SE).
^cSire-based estimate of the genetic correlation between Z9-14:Ac and Z9,E12-14:Ac (r_A ≠ 0; P < 0.001).
^dSire-based estimate of the genetic correlation between Z9,E12-14:Ac and the ratio of Z9,E12-14:Ac to Z9-14:Ac (r_A = 0; P > 0.1).
^eSire-based estimate of the genetic correlation between Z9-14:Ac and the ratio of Z9,E12-14:Ac to Z9-14:Ac (r_A = 0; P > 0.5).

that mate soon after eclosion, the sex pheromone titer is generally low on the day of eclosion, increases for ca. 1 day, and then declines progressively until death (Schal et al., 1987; Tang et al., 1992; Kamimura and Tatsuki, 1993; Gemeno and Haynes, 2000). The data from our study are consistent with the general pattern of decreasing pheromone titer with age.

The diel periodicity of the titers of both the major and minor sex pheromone components of *C. cautella*, and the ratio of the two components peaked at the onset of the scotophase, decreased throughout the calling period (scotophase), and reached a minimum at the end of the scotophase. In this species, pheromone gland titers appear to decrease during calling and increase between calling periods. Similar diel periodicities of gland titer have been observed in other moth species (Konno, 1986; Ono et al., 1990; Hunt and Haynes, 1990; Delisle and Royer, 1994). However, in other moth species, pheromone titers have been observed to be synchronous with calling peaks (Pope et al., 1982; Delisle and McNeil, 1987; Tang et al., 1992; Kamimura and Tatsuki, 1993). The reduction in titers of both pheromone components during the scotoperiod suggests that the rate of release of pheromone from the gland exceeds the rate of biosynthesis, or that little biosynthesis occurs during the scotophase, or both.

The biosynthetic pathway used by female *C. cautella* to synthesize Z9,E12-14:Ac and Z9-14:Ac involves a Δ11 and Δ12 desaturase, and both pheromone components share a common precursor (Z9-14:CoA), upstream of the Δ12 desaturase (Jurenka, 1997). This suggests that the decrease in the ratio of

Z9,E12-14:Ac to Z9-14:Ac with age and photoperiod is due to reduced activity or titers of the $\Delta 12$ desaturase later in the photoperiod and in older moths.

Adult *C. cautella* have functional mouthparts and are capable of feeding. Ryne et al. (2002) speculated that water sources are limited in the habitat of *C. cautella*, and male and female *C. cautella* are attracted to traps baited with water (Chow et al., 1977; Ryne et al., 2002).¹ However, access to water had no effect on the titer of either Z9,E12-14:Ac or Z9-14:Ac, or the ratio of Z9,E12-14:Ac to Z9-14:Ac.

Variation in the temperature experienced during pupal and adult development can affect the age of first calling, the calling periodicity, and the calling duration in females, and the age of maximum responsiveness to pheromone, the selectivity of/preference for pheromone blends, and the periodicity of orientation behavior in males (reviewed in McNeil, 1991). Temperature was also observed to affect pheromone gland content (Raina, 2003), as well as the emission rate and blend ratios of pheromone from forcibly extruded pheromone glands (Liu and Haynes, 1994). Ono (1993, 1994) found that the ratio of the sex pheromone components of the potato tuberworm moth, *P. operculella* (Zeller) [(E,Z)-4,7-tridecadienyl acetate and (E,Z,Z)-4,7,10-tridecatrienyl acetate] varied with temperature [mean percent of (E,Z,Z)-4,7,10-tridecatrienyl acetate ranged from ca. 80% at 15°C to 38% at 35°C]. Contrary to these observations, the sex pheromone blend in our study species was not affected by temperature. One possible explanation may be the range of temperatures experienced. In our study, moths were reared at 21 and 26°C, whereas Ono (1993, 1994) reared moths at 15, 25, and 35°C. It is possible that a significant effect of temperature may be realized in moths reared at more extreme temperatures.²

As expected, adult mass was positively and significantly correlated with pupal mass. However, the pheromone titers of Z9,E12-14:Ac and Z9-14:Ac, and the ratio of Z9,E12-14:Ac to Z9-14:Ac were not significantly correlated with pupal mass. These results are consistent with those observed with other species (e.g., *Argyrotaenia velutinana*, Miller and Roelofs, 1980; *Holomelina lamae*, Schal et al., 1987).

Genetic Analysis of Pheromone Variation. According to Fisher's fundamental theorem, additive genetic variance for total fitness should rapidly be eliminated by selection (Fisher, 1930). An extension of this concept is that characters closely related to fitness should have low heritabilities (Falconer and Mackay, 1996). However, the heritability of a character is not completely

¹ The study of Chow et al. (1977) did not differentiate between *C. cautella* and *Plodia interpunctella*. The study of Ryne et al. (2002) identified specimens to species and sex, thus definitively demonstrating attraction of adult *C. cautella* to water.

² Our experimental design included a 31°C treatment. The survivorship of moths reared at this temperature was so low that we were unable to obtain enough females for analysis.

defined by the amount of additive genetic variance, but rather by the amount relative to total phenotypic variance. Consequently, characters with little additive genetic variance will have low heritabilities only when the amount of additive genetic variance is far lower than the total phenotypic variance. Theoretical arguments also suggest that additive genetic variance can exist for fitness components when negative genetic correlations with other fitness components exist. Despite this, the literature generally supports the prediction that the heritability of a character is negatively correlated with fitness consequences (Gustafsson, 1986; Roff and Mousseau, 1987; Mousseau and Roff, 1987). For example, Roff (1997) summarized published laboratory and field-based heritability estimates of life history, behavioral, and morphological traits. As predicted, heritability estimates increased with decreasing trait importance [mean heritabilities of life history, behavioral, and morphological traits were 0.28 ($N = 87$), 0.34 ($N = 27$), and 0.54 ($N = 240$), respectively].

Unlike dam-based heritability estimates, sire-based estimates are not influenced by maternal and common environmental effects or dominance variance and are consequently more accurate. The sire-based heritability estimates observed for the titers of Z9,E12-14:Ac and Z9-14:Ac were both greater than the mean for morphological traits (see above) and were all consistent with published estimates (Table 2). The heritability of the ratio of Z9,E12-14:Ac to Z9-14:Ac was intermediate to the observed mean of behavioral and morphological traits. One way to interpret these data is that variation in pheromone component titers and ratios may have minimal fitness consequences.

One of the primary goals of studies of genetic variation is to predict the potential for the evolution of the mean phenotype of a population (hereafter referred to as “evolvability” *sensu* Houle, 1992). In the short term this will be determined by the amount of additive genetic variance. To generate evolvability estimates, it is necessary to partition trait variance into additive genetic variance, V_A , and residual variance, V_R (Falconer and Mackay, 1996). Traditionally, narrow-sense heritability has been used to describe variance partitioning. Houle (1992) argued that measures of variation standardized by trait means are more accurate comparative measures of evolvability than measures standardized by total phenotypic variance (V_P) (narrow-sense heritability). The additive genetic coefficient of variation (CV_A) describes the amount of additive genetic variance standardized by the trait mean. Although the narrow-sense heritability of a trait describes the potential for a response to selection in absolute terms, the CV_A describes the potential in relative terms (Houle, 1992). The sire-based coefficients of variation observed for the titers of Z9,E12-14:Ac and Z9-14:Ac and for the ratio of Z9,E12-14:Ac to Z9-14:Ac were notably high (55.99, 59.76, and 25.91) [mean CV_A for developmental time, longevity and fecundity in *Drosophila melanogaster* are 2.47 ($N = 2$), 9.89 ($N = 7$), and 11.90 ($N = 12$); see Houle, 1992]. Comparison of the CV_A for all

TABLE 2. SIGNIFICANT NARROW-SENSE HERITABILITY ESTIMATES OF PHEROMONE TITERS AND RATIOS

Species	Design	Trait	$h^2 \pm SE$	Reference
<i>Pectinophora gossypiella</i>	Full-sibling	Titer of Z7, E11-16:Ac plus Z7, Z11-16:Ac	0.41 ± 0.09	Collins and Cardé, 1985
		Proportion of Z7, E11-16:Ac	0.34 ± 0.08	
<i>P. gossypiella</i>	Realized heritability	Proportion of Z7, E11-16:Ac	0.50 ± 0.04	Collins and Cardé, 1989
<i>Argyrotaenia velutinana</i>	Full-sibling	Ratio of E9-12:OAc to E11-14:OAc	0.64 ± 0.32	Sreng et al., 1989
	Realized heritability	Ratio of E9-12:OAc to E11-14:OAc	1.14; 1.56 ^a	
<i>P. gossypiella</i>	Realized heritability	Titer of Z7,E11-16: Ac plus Z7,Z11-16:Ac	0.71 ± 0.13	Collins et al., 1990
<i>Agrotis ipsilon</i> ^b	Full-sibling	Titer of Z9-14:Ac	1.15 ± 0.16	Gemeno et al., 2000
		Titer of Z11-16:Ac	0.83 ± 0.18	
		Ratio of Z9-14: Ac to Z7-12:Ac	1.09 ± 0.17	
		Ratio of Z11-16:Ac to Z7-12:Ac	0.67 ± 0.19	
<i>A. ipsilon</i> ^c	Full-sibling	Titer of Z11-16:Ac	0.56 ± 0.26	Gemeno et al., 2000
		Ratio of Z11-16: Ac to Z7-12:Ac	0.56 ± 0.26	
<i>Trichoplusia ni</i> ^d	Half-sibling ^f	Titer of Z7-14:Ac	1.13 ± 0.31	Gemeno et al., 2001
		Titer of Z9-14:Ac	0.65 ± 0.18	
<i>T. ni</i> ^e	Half-sibling ^f	Titer of 12:Ac	0.43 ± 0.19	Gemeno et al., 2001
		Titer of 11-12:Ac	0.44 ± 0.21	
		Titer of Z7-14:Ac	0.38 ± 0.18	
		Titer of Z9-14:Ac	0.77 ± 0.27	
<i>Plodia interpunctella</i>	Mother-daughter regression	Ratio of Z9,E12-14: Ac to Z9,E12-14:Ac	0.65 ± 0.14	Svensson et al., 2002
<i>Cadra cautella</i>	Half-sibling ^f	Titer of Z9-14:Ac	0.75 ± 0.24	Present study
		Titer of Z9,E12-14:Ac	1.20 ± 0.32	
		Ratio of Z9,E12-14: Ac to Z9-14:Ac	0.46 ± 0.17	

^aLow and high selection lines, respectively. Standard errors not given.

^bKentucky population.

^cFrench population.

^dPopulation N.

^ePopulation M.

^fSire-based estimates.

three pheromonal traits suggests that the titers of both pheromone components have an equivalent potential response to selection that is approximately twofold greater than that of the ratio of Z9,E12-14:Ac to Z9-14:Ac.

Individual traits are commonly not inherited independently, but rather two or more traits are often associated with each other (Lande, 1979; Lande and Arnold, 1983). We observed a strong, positive genetic correlation between the titer of Z9,E12-14:Ac and the titer of Z9-14:Ac (0.85 ± 0.06), suggesting that the genetic factors controlling the synthesis of the two pheromone components are either identical or closely linked. Neither of the genetic correlations between the titers of Z9,E12-14:Ac and Z9-14:Ac and the ratio of Z9,E12-14:Ac to Z9-14:Ac were significant (0.32 ± 0.20 and -0.23 ± 0.34 , respectively). The significant correlation between the pheromone titers, but not between the titer of either component and the component ratio, suggests that the pheromone titers and their ratio are determined by independent genetic factors and should consequently follow separate evolutionary trajectories. The existence of opposing pleiotropic effects in the genes for these traits would result in an overestimation of the degree of independence between these characters (Falconer and Mackay, 1996).

In *C. cautella*, the sex pheromone signal is extremely variable not only within and across-age classes but also throughout the L/D cycle. Although we observed more variability and higher gland titers and ratios of Z9,E12-14:Ac to Z9-14:Ac, the results generally agree with those of previous less comprehensive studies (Brady, 1973; Kuwahara and Casida, 1973; Coffelt et al., 1978; Coffelt and Vick, 1987; Barrer et al., 1987; Shani, 1990). The existence of a significant, positive genetic correlation between the pheromone component titers and the higher sire-based heritability and CV_A estimates for the pheromone component titers than for the ratio are all consistent with the hypothesis that pheromone component ratio has a lower potential response to selection than component titers.

Models of sex pheromonal signal evolution (see Cardé and Haynes, 2004) predict an asymmetry in the strength of selection experienced by males and females. In lepidopteran mating systems with female-biased investment in the zygote, selection is predicted to favor male response profiles broad enough to include most or all of the females in a population (see Phelan, 1997). Consequently, species with high signal variance should have male response profiles that are similarly broad, whereas male response profiles can be broad or narrow in species with low signal variance. These ideas can be extrapolated to make predictions about the evolvability of female signals and male response profiles. In general, signal and response profile variance should be positively correlated with their evolvability.

We observed high levels of heritable variation for both pheromone component titers and ratios. The high levels of heritable variation found in this

study may be a consequence of the release of this population from the “natural” selection regime, which includes the need for males to locate females over relatively long distances. Alternatively, such variation suggests that the paradigm that variation in pheromone titers and ratio has negative fitness consequences and is minimized by stabilizing selection may need reevaluation. Specifically, it suggests that male *C. cautella* response profiles should be similarly broad.

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THE MANDIBULAR GLAND SECRETIONS
OF THE LEAF-CUTTING ANTS *Atta sexdens sexdens*
AND *Atta opaciceps* EXHIBIT INTERCASTE
AND INTERCOLONY VARIATIONS

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Abstract—The mandibular gland secretions of worker castes from wild colonies of the leaf-cutting ants *Atta sexdens sexdens* and *Atta opaciceps* were analyzed quantitatively by gas chromatography–mass spectrometry. The secretions comprised a complex mixture of volatile, mainly oxygenated compounds, and their profiles exhibited considerable qualitative and quantitative variations among species and castes. The known alarm pheromone 4-methyl-3-heptanone was common to both species. The elevated relative proportions of this ketone found in the secretions of gardeners and generalists suggest that such castes are primarily responsible for the production and release of the alarm pheromone. Quantitative variations (but no qualitative differences) in the profiles of secretions of soldiers from different colonies of *A. sexdens sexdens* were detected, supporting the view that intraspecific colony recognition is mediated through mandibular gland secretions. Subsequent laboratory assays showed that, among the compounds identified by GC–MS, 4-methyl-3-heptanone elicited a strong alarm response in workers of *A. sexdens sexdens* and *A. opaciceps*.

Key Words—Alarm pheromone, *Atta sexdens sexdens*, *Atta opaciceps*, hymenoptera, formicidae, myrmicinae, attini.

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INTRODUCTION

Leaf-cutting ants possess a sophisticated mechanism of communication, the principal function of which is associated with the maintenance of the complex social structure of their highly territorial colonies (Jaffe, 1979; Whitehouse and Jaffé, 1995). The mandibular glands of the Formicidae constitute important sources of volatile organic compounds that mediate ant behaviors (Hölldobler and Wilson, 1990) such as alarm behavior, typically characterized by highly accelerated movements and accompanied by attack (Brown et al., 1970). However, for ants that live in small, less well-organized colonies, alarm pheromones may act rather as signals for evacuation because such insects are not well equipped for defense (Regnier and Wilson, 1969). In the majority of ant species belonging to the subfamily Myrmicinae, for example, *Atta* and *Acromyrmex*, the mandibular glands produce secretions containing mixtures of components that frequently include alcohols or ketones of low molecular weight and high volatility that could be involved in the communication of such a state of alarm (Blum, 1969).

The first alarm pheromone from leaf-cutting ants to be analyzed was a mixture of 4-methyl-3-heptanone, neral, and geranial isolated from workers of *Atta sexdens sexdens* (Butenandt et al., 1959). Moreover, 4-methyl-3-heptanone was found to be the component that elicited alarm pheromone activity in workers of *A. texana*, *A. robusta*, *A. bisphaerica*, *A. capiguara*, and *A. sexdens* (Moser et al., 1968; Blum, 1969; Riley et al., 1974; Hughes et al., 2001b). Recent studies (Hernández et al., 1999; Hughes et al., 2001b) have further revealed that 4-methyl-3-heptanone is commonly found in *A. laevigata*, *A. capiguara*, and *A. bisphaerica*, together with other ketones such as 2-heptanone, 4-methyl-2-heptanone, 2-octanone, and 3-octanone, and the related alcohols 2-hexanol, 2-heptanol, and 4-methyl-3-heptanol.

Colonies of *Atta* sp. are highly polymorphic and exhibit polyethism: Workers may be separated into four castes (Wilson, 1980), namely, minors (gardeners or nurses), generalists, foragers, and majors (defenders or soldiers). In the case of leaf-cutting ants, the composition of the alarm pheromone can vary significantly among the castes. Thus, for *Atta sexdens rubropilosa*, the mandibular gland secretions of the major castes consisted of a complex mixture with the citral isomers (geranial and neral) as the major components, whereas those of the gardeners and generalists were dominated by 4-methyl-3-heptanone, and practically no citral isomers were found (Do Nascimento et al., 1993). A similar situation was reported for *A. laevigata*, in which the secretions of the minor workers contained much higher levels of 4-methyl-3-heptanone than those of the major castes (Hughes et al., 2001b). Furthermore, for *A. capiguara* and *A. bisphaerica* the mandibular gland secretions can vary in composition among castes within the same colony (Hughes et al., 2001b).

Although such reports confirm the existence of a relationship between the nature of the alarm pheromone and the different castes of leaf-cutting ants, further investigations involving other species need to be made in order to establish a verifiable explanation for the variation of pheromone composition.

Whereas most studies have been conducted using single defined colonies of leaf-cutting ants (Moser et al., 1968; Schildknecht, 1976; Do Nascimento et al., 1993; Hernández et al., 1999, 2002), variations among colonies have been described by some authors. Thus, Whitehouse and Jaffé (1995) reported that specimens of *A. laevigata* responded in a much more aggressive manner to the presence of macerated heads of ants derived from a different colony compared to macerated heads of ants from their own colony. Moreover, Hughes et al. (2001b) detected small but consistent intraspecific differences in various colonies of *A. capiguara* and *A. bisphaerica*. Taken together, these findings suggest that the intercolony differences in the alarm pheromones of leaf-cutting ants can be detected by the ants. However, details of the variation in chemical composition of alarm pheromones among colonies and among castes of leaf-cutting ants is limited to a few species.

The mandibular gland secretion of *A. sexdens sexdens* is yellow in color, possibly due to the presence of degradation products of the citral isomers that are characteristic of this species. These aldehydes are released from the cephalic capsule of *A. sexdens sexdens* when the head of the ant is crushed and are readily recognized by their strong lemon smell, giving rise to the Brazilian name of “saúva-limão” for this ant (Blum et al., 1968). In contrast, the mandibular gland secretion of *A. opaciceps* is a transparent liquid, the chemical composition of which has not yet been studied. The aim of the present work was to identify the components present in the mandibular gland secretions of these two species of leaf-cutting ants and to determine any qualitative and/or quantitative variations among colonies and castes of the species. In addition, laboratory-based experiments were conducted to test the effects of mandibular gland compounds on workers of *A. sexdens sexdens* and *A. opaciceps*.

METHODS AND MATERIALS

Identification of Ants. Nests of leaf-cutting ants (two of *A. sexdens sexdens* and one of *A. opaciceps*), situated at different locations on the campus of the Federal University of Alagoas, were initially selected on the basis of the constant and discrete foraging activities of workers. Ten specimens from each nest were collected, representing the different worker castes, namely, two gardeners, two generalists, two foragers, and four soldiers. Specimens were stored separately in 70% alcohol in appropriately labeled glass containers and

deposited at the Centro de Pesquisa, Laboratório de Mirmecologia-CEPEC/CEPLAC, Itabuna-BA, Brasil. Specimens were identified and authenticated by Dr. Jacques H. C. Delabie (myrmecologist at CEPEC/CEPLAC).

Collection of Ants. Specimens of all castes of both species were collected at their nest entrances and along foraging trails. Collections were made as carefully as possible, using clean entomological forceps, in a manner that minimized the release of any volatile components. Specimens were transferred immediately to ice-cold glass tubes (4×2 cm i.d.) that were closed with rubber caps, packed in ice, and transported to the laboratory, where they were stored at 4°C until required for the preparation of extracts.

Preparation of Extracts. Based on the classification proposed by Della Lucia et al. (1993), castes were distinguished according to the width of the cephalic capsule. Mandibular glands and/or cephalic capsules from different castes of *A. sexdens sexdens* and *A. opaciceps* were separated with the aid of forceps under a Wild Leica M3B stereoscopic microscope equipped with a Wild Leica MPS05 camera. The glands or capsules were ground in 2 ml of hexane, and the extracts obtained were stored in sealed ampoules in the freezer until required for analysis. In order to provide satisfactory GC-MS analyses, extracts were prepared separately using 16 cephalic capsules of gardeners, eight cephalic capsules of generalists, two pairs of mandibular glands of foragers, or one pair of mandibular glands of soldiers.

Identification of Volatile Constituents. Extracts of mandibular glands and cephalic capsules were analyzed by GC-MS using a Shimadzu model 17A chromatograph coupled to a Shimadzu QP5050A mass-selective detector controlled by a Pentium 2 (200 MHz) computer running 5000 Class software. The volatile components of the extracts were separated on a fused-silica polydimethylsiloxane-coated column (30 m \times 0.25 mm i.d.) (0.5 μ m film thickness) and operated under the following conditions: carrier gas, helium at 1 ml/min; oven temperature, 30°C increasing at 8°C/min to 250°C; injector temperature, 200°C; injection mode, splitless; detector temperature, 270°C; ionization energy, 70 eV. Compounds were identified from their mass spectra and confirmed by comparison of retention time and mass spectrum with purchased or synthesized standards. 4-Methyl-3-heptanone and β -citronellol were used as external standards for quantification of components. Standards of *trans-p*-mentha-1[7]-8-dien-2-ol, *trans*-2-carene-4-ol, dihydrolinalool, (*E*)-myrtenol, limonene isomer, *cis*-4-decenal, δ -decalactone, and 2,3-dihydro-6-*trans*-farnesol were not available. These compounds were tentatively identified from matches with database or published mass spectra (Wiley 275 data base; McLafferty and Stauffer, 1989).

Quantitative Analysis of Volatile Constituents. The average amount of each component in a gland was calculated from its GC peak area expressed as a percentage of the total peak area of all components detected.

Ethological Assays. Bioassays were carried out in an olfactometer as described by Löfgren et al. (1983). The unfiltered output from an air pump was passed, via plastic tubing, through a Pasteur pipette containing a segment of filter paper impregnated with an appropriate test solution. The outlet of the pipette was loosely plugged with cotton wool to provide a uniform flow of stimulus-laden air. This air stream was passed over a moistened disc of filter paper, lining a glass Petri dish arena (19-cm diam). This arena was covered with red cellophane to prevent disturbance of the ants by visual stimulation from the surroundings.

Chemicals. Except for 4-methyl-3-heptanone, the synthetic standards listed in the tables were purchased (Sigma-Aldrich Co. Ltd., USA). 4-Methyl-3-heptanone was prepared from the corresponding alcohol by sodium hypochlorite oxidation, which gave a racemic mixture of 4-methyl-3-heptanone 99% pure by gas chromatography.

Bioassays of Alarm Pheromone. The responses of workers of *A. sexdens sexdens* and *A. opaciceps* to hexane solutions of synthetic compounds found in the secretion of the mandibular gland of generalists, the caste that produces compounds that elicit alarm behavior in nestmates (Wilson, 1980; Hernández et al., 1999; Hughes et al., 2001b; unpublished results), was observed. Ten ants (*A. sexdens sexdens* or *A. opaciceps*) were introduced into the arena and left for a 5-min acclimation period. Then the air stream from a pipette containing a filter paper impregnated with 10 μ l of the test solution passed over the arena. The concentration of each compound used in these bioassays was similar to that found in the mandibular gland secretions of generalists from each species. A piece of filter paper treated only with hexane was used as a control.

The criteria used to measure the responses of forager workers were similar to those reported by Hernández et al. (2002), as follows: (1) antennation: inspection of arena, followed by vigorous movement of antennae; (2) opening the mandibles: the ants attacked the outlet of the odor source (cotton swab) with their mandibles (biting). These behaviors were quantified and recorded continuously for 10 min.

Extracts were applied in randomized sequence, and each extract was tested separately 10 times. After each replication, the olfactometer was cleaned, the filter paper and cotton wool plug were changed, and 10 new insects were introduced into the arena. All of the bioassays were conducted at night (after 1800 hr) to coincide with the time that the ants would normally commence foraging. The temperature and relative humidity in the arena were $26 \pm 0.2^\circ\text{C}$ and $60 \pm 10\%$, respectively.

Statistical Analysis. Because the secretions of soldiers contained the largest number of individual components their compositions were used to assess proportional differences in the main constituents of the alarm pheromones between members of the two nests of *A. sexdens sexdens*. Each such constituent

was considered independently and, therefore, the percentage content of each component in the two colonies analyzed could be compared using one-way ANOVA (or F test) as well as by *t* test, both at the 5% level of significance. Tukey's test (at a significance level of 5%) was applied to compare means.

RESULTS

Identification of Volatile Components in Secretions from Different Castes of A. sexdens sexdens from Different Colonies. GC-MS analysis of extracts of the mandibular glands and cephalic capsules of different castes of workers from

TABLE 1. VOLATILE CONSTITUENTS OF THE MANDIBULAR GLANDS OF DIFFERENT CASTES OF *A. sexdens sexdens* (NEST 1)

Component	Composition (%) ^a				Source
	Gardeners	Generalists	Foragers	Soldiers	
4-Methyl-3-heptanone	100	53.5 ± 1.11	3.2 ± 0.83	4.6 ± 0.50	See Methods and materials
4-Methyl-3-heptanol	—	—	0.10 ± 0.07	0.1 ± 0.02	Aldrich
6-Methyl-5-hepten-2-one	—	—	—	0.3 ± 0.06	Sigma
Nonanal	—	6.9 ± 4.67	0.4 ± 0.25	—	Aldrich
<i>trans</i> -2-Caren-4-ol ^b	—	—	—	0.4 ± 0.02	MS
1-Decene	—	10.5 ± 3.98	—	—	Aldrich
<i>cis</i> -4-Decenal ^b	—	—	—	0.1 ± 0.03	MS
Decanal	—	—	1.5 ± 0.40	1.7 ± 0.08	Aldrich
β-Citronellol	—	—	0.6 ± 0.11	0.20 ± 0.13	Sigma
Neral	—	8.0 ± 2.20	28.5 ± 0.75	28.5 ± 0.91	Aldrich
Nerol	—	—	—	0.2 ± 0.01	Sigma
Geraniol	—	9.42 ± 3.93	4.1 ± 0.55	2.35 ± 0.35	Sigma
Geranial	—	11.5 ± 2.95	60 ± 1.38	55.8 ± 0.71	Aldrich
Unknown 1	—	—	Trace ^c	0.62 ± 0.25	—
1-Decanol	—	—	—	0.3 ± 0.18	Sigma
δ-Decalactone ^b	—	Trace ^c	Trace ^c	Trace ^c	MS
Geranylacetone	—	—	0.7 ± 0.21	0.7 ± 0.03	Aldrich
(<i>E</i>)-2,3-Dihydrofarnesol ^b	—	—	—	0.2 ± 0.01	MS
Dihydrolinalool ^b	—	—	—	0.1 ± 0.067	MS
Nerolidol	—	—	—	3.4 ± 0.32	Sigma
Farnesol	—	—	—	0.4 ± 0.04	Sigma
Mean total amount/head (μg)	0.04	0.11	13.81	111.26	

MS = mass spectrum.

^aMean values ± standard error (*N* = 5).

^bIdentification of compound is tentative, based solely on matches with database spectra.

^cAmount detected <0.01% of the total secretion.

different colonies of *A. sexdens sexdens* revealed that the components present were mainly low molecular weight ketones, aldehydes, alcohols, and mono-terpenoids (Tables 1 and 2). Geranial and neral were the major components in the extracts of glands of forager and soldier castes from both of the colonies analyzed, followed by small amounts of 4-methyl-3-heptanone. In contrast, extracts of cephalic capsules of generalists contained lower proportions of

TABLE 2. VOLATILE CONSTITUENTS OF THE MANDIBULAR GLANDS OF DIFFERENT CASTES OF *A. sexdens sexdens* (NEST 2)

Component	Composition (%) ^a				Source
	Gardeners	Generalists	Foragers	Soldiers	
4-Methyl-3-heptanone	100	32.9 ± 2.9	2.3 ± 0.26	3.3 ± 0.16	See Methods and materials
4-Methyl-3-heptanol	–	1.9 ± 0.89	–	0.02 ± 0.02	Aldrich
6-Methyl-5-hepten-2-one	–	–	–	0.1 ± 0.07	Sigma
3-Octanone	–	2.3 ± 1.12	–	–	Aldrich
Decane	–	2.4 ± 1.0	–	–	Sigma
Nonanal	–	4.5 ± 1.9	0.20 ± 0.1	–	Aldrich
Unknown 1	–	1.6 ± 0.74	–	–	–
Limonene isomer ^b	–	–	0.4 ± 0.16	–	MS
<i>trans</i> -2-Caren-4-ol ^b	–	–	–	0.4 ± 0.06	MS
<i>trans-p</i> -Mentha-1[7], 8-dien-2-ol ^b	–	–	0.4 ± 0.17	–	MS
<i>cis</i> -4-Decenal ^b	–	–	–	0.25 ± 0.09	MS
Decanal	–	–	2.3 ± 0.27	1.9 ± 0.28	Aldrich
β-Citronellol	–	–	0.2 ± 0.12	0.4 ± 0.10	Sigma
Neral	–	13.4 ± 1.74	28.3 ± 0.56	27.0 ± 2.10	Aldrich
Geraniol	–	13.3 ± 2.16	3.5 ± 0.33	1.9 ± 0.5	Sigma
Geranial	–	24.3 ± 2.37	59.6 ± 0.52	60.9 ± 1.14	Aldrich
Unknown 2	–	–	0.3 ± 0.26	0.69 ± 0.3	–
1-Decanol	–	–	–	0.1 ± 0.04	Sigma
δ-Decalactone ^b	–	Trace ^c	Trace ^c	Trace ^c	MS
Geranylacetone	–	3.2 ± 1.33	0.8 ± 0.21	0.6 ± 0.07	Aldrich
(<i>E</i>)-Myrtenol ^b	–	–	–	0.4 ± 0.05	MS
Nerolidol	–	–	1.1 ± 0.48	1.2 ± 0.7	Sigma
Farnesol	–	–	0.4 ± 0.01	–	Sigma
Mean total amount/head (μg)	0.05	0.36	11.83	69.68	

MS = mass spectrum.

^a Mean values ± standard error (*N* = 5).

^b Identification of compound is tentative, based solely on matches with database spectra.

^c Amount detected <0.01% of the total secretion.

aldehydes, and the major compound was 4-methyl-3-heptanone. Indeed, this ketone was the only component detected in extracts from cephalic capsules of gardeners. Because extracts from the mandibular glands of soldiers presented the greatest variety, these results were used to assess differences in the composition of secretions among individuals from different colonies of *A. sexdens sexdens*. Accordingly, Table 3 shows that the proportions of 4-methyl-3-heptanone and geranial were different in the mandibular gland secretions of soldiers from different nests.

Identification of Volatile Components in Secretions from Different Castes of A. opaciceps from the Same Colony. Extracts of mandibular glands and cephalic capsules of different worker castes of *A. opaciceps* from the same nest contained mainly low molecular weight ketones and alcohols (Table 4). Again, extracts from soldiers exhibited the greatest variety, the most important compounds were 2-heptanone followed by 2-heptanol. Only trace amounts of the former were found in extracts of glands from foragers, and none at all in extracts from generalists and gardeners. Thus, 2-heptanol was unique to the extract from soldiers. 4-Methyl-3-heptanone was present in the extracts from all castes and was the major component present in the extract from foragers. Whereas only small amounts of this component could be detected in extracts of generalists and gardeners, no other volatile components appeared to be present.

Evaluation of Alarm Response of A. sexdens sexdens and A. opaciceps to Compounds in Mandibular Gland Secretions. Forager workers of *A. sexdens sexdens* and *A. opaciceps* exhibited different levels of response to various mandibular gland components. Workers from both nests of *A. sexdens sexdens* responded most strongly to 4-methyl-3-heptanone. The response of workers from Nest 1 to citral was as strong as the response to 4-methyl-3-heptanone, whereas workers from Nest 2 responded less strongly to citral. Similarly, workers from Nest 1 exhibited a weak but significant response to geraniol,

TABLE 3. COMPARISON OF THE MAJOR COMPONENTS PRESENT IN THE MANDIBULAR GLAND EXCRETIONS OF *A. sexdens sexdens* SOLDIERS FROM NEST 1 AND FROM NEST 2

Component	Nest 1 Amount (%) ^a	Nest 2 Amount (%) ^a
4-Methyl-3-heptanone	4.6 ± 0.5*	3.3 ± 0.1*
Neral	28.5 ± 0.9	27.0 ± 2.1
Geranial	53.8 ± 0.7*	60.9 ± 1.1*
Geraniol	2.3 ± 0.36	1.9 ± 0.5
Geranylacetone	0.7 ± 0.03	0.6 ± 0.07

* $P < 0.05$, one-way ANOVA, *t* test. Values that are on the same line are significantly different.

^aMean values ± standard error ($N = 5$).

TABLE 4. VOLATILE CONSTITUENTS OF THE MANDIBULAR GLANDS OF DIFFERENT CASTES OF *A. opaciceps*

Component	Composition (%) ^a				Source
	Gardeners	Generalists	Foragers	Soldiers	
2-Heptanone	–	–	Trace ^b	68.2 ± 8.04	Aldrich
2-Heptanol	–	–	–	20.3 ± 7.24	Aldrich
4-methyl-3-heptanone	100	100	99.8 ± 0.01	6.1 ± 2.26	See Methods and materials
4-Methyl-3-heptanol	–	–	–	Trace ^b	Aldrich
2-Octanone	–	–	–	Trace ^b	Aldrich
2-Nonanone	–	–	–	4.6 ± 2.86	Aldrich
2-Nonanol	–	–	–	0.6 ± 0.69	Aldrich
Mean total amount/head (ng)	<1.0	5.3	73.3	1961	

^aMean values ± standard error (N = 5).

^bAmount detected <0.01% of the total secretion.

whereas the response of Nest 2 workers to geraniol was no different from the response to the hexane control. Workers from both nests showed no response to nerylacetone. Foragers of *A. opaciceps* responded strongly to 4-methyl-3-heptanone, but showed no response to 2-heptanone (Figures 1 and 2).

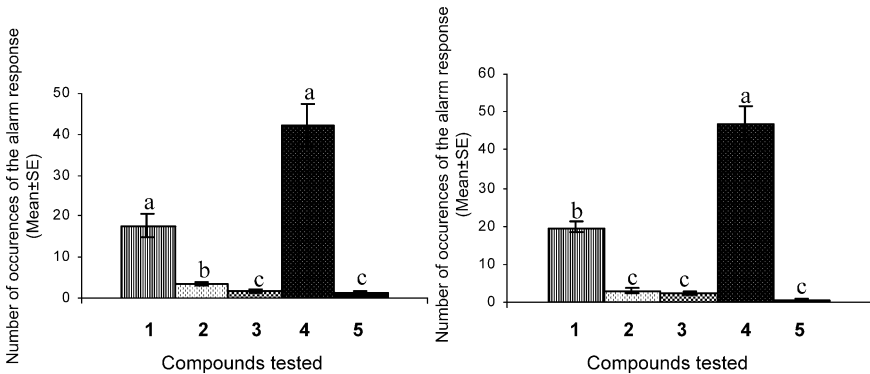


FIG. 1. Alarm response of *A. sexdens sexdens* workers from different nests to 10 µl of a solution of the synthetic mandibular gland compounds from the secretions of generalists. 1, Citral (mixture of neral and geraniol); 2, geraniol; 3, nerylacetone; 4, 4-methyl-3-heptanone; and 5, hexane control. Means were separated by Tukey’s test ($P < 0.05$).

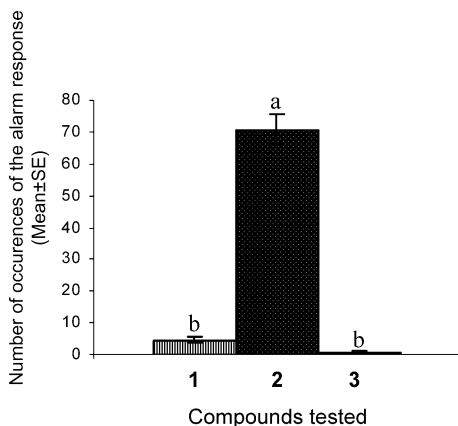


FIG. 2. Response of *A. opaciceps* foragers to two synthetic mandibular gland compounds from the secretions of generalists. 1, 2-heptanone; 2, 4-methyl-3-heptanone; and 3, hexane control. Means were separated by Tukey's test ($P < 0.05$).

DISCUSSION

Chemical compositions of secretions of mandibular glands of a number of species of leaf-cutting ants of the genus *Atta* have been previously reported (Blum et al., 1968; Moser et al., 1968; Riley et al., 1974; Do Nascimento et al., 1993; Hernández et al., 1999; Hughes et al., 2001b). Thus, Blum et al. (1968) demonstrated that geranial and neral accounted for ca. 90% of the mandibular gland secretion in *A. sexdens*, and these aldehydes were also shown to be the most abundant components in the mandibular glands of soldiers and foragers of *A. sexdens rubropilosa* (Do Nascimento et al., 1993). However, geranial and neral did not provoke alarm reactions when tested against a number of species of leaf-cutting ants including *A. sexdens* (Blum, 1969). Della Lucia et al. (2001) suggested that these aldehydes might be used in defense because they are present in the mandibular glands of the soldier castes. On the other hand, 4-methyl-3-heptanone, a major component of the mandibular gland secretions of a number of *Atta* species, has been demonstrated to be an alarm pheromone for *A. texana* (Moser et al., 1968), *A. laevigata* (Hernández et al., 1999), *A. bisphaerica*, and *A. capiguara* (Hughes et al., 2001a).

It had been previously reported that the nature of the alarm pheromone can vary among castes of leaf-cutting ants (Hughes et al., 2001b), and this was confirmed in the present study by the demonstration that the proportion of 4-methyl-3-heptanone in the mandibular gland secretions increased as the size of the cephalic capsule decreased. Such results are also consistent with the

hypothesis that gardeners and generalists of leaf-cutting ants are principally responsible for the production and release of alarm pheromone, a proposition arising from the finding of Do Nascimento et al. (1993) that the mandibular gland secretions of gardeners and generalists of *A. sexdens rubropilosa* consisted almost entirely (89.5%) of 4-methyl-3-heptanone. Such views have received further support from Hernández et al. (1999) who demonstrated that this ketone is the major component of the secretions of lower workers of *A. laevigata*.

The present results reveal that in *A. sexdens sexdens* variations occur in the chemical profiles of the mandibular gland secretions derived from the same castes belonging to different colonies: The differences in the amounts of the two major components (4-methyl-3-heptanone and geranial) were particularly significant. Similar results were obtained by Hughes et al. (2001b) who found quantitative differences in the secretions of different colonies of *A. capiguara* and *A. bisphaerica*, although such variations were smaller than the differences observed between the species.

In some species of ants, for example, *Oecophyla longinoda* (Bradshaw et al., 1979) and *Formica lugubris* (Cherix, 1983), qualitative and quantitative variations in the chemical compositions of the alarm pheromones of the mandibular glands have been observed among members of different colonies in the same location. For leaf-cutting ants, some authors (Bonavita-Cougourdan et al., 1987) have suggested that cuticular compounds are responsible for defense and alarm behavior, whereas other researchers (Jaffe, 1979; Salzman and Jaffe, 1991) have shown that such behaviors are associated with the presence in the environment of the odors of recognition released from the cephalic capsules of ants from different colonies. Furthermore, Hernández et al. (2002) compared cuticular extracts with those from mandibular glands and were able to demonstrate that intraspecific recognition was based on odors from the cephalic capsule and that such odors derived primarily from mandibular gland secretions. These findings confirmed a previous report of Hughes et al. (2001b) in which the mandibular glands were described as the producers of the alarm pheromone to which was attributed the function of intracolony recognition. Therefore, the intercolonial differences in the chemical profiles presented herein support the view that such secretions really are involved in intraspecific colony recognition.

The secretion of the mandibular glands of *Atta opaciceps* is a colorless liquid, whereas that from *Atta sexdens sexdens* is yellow in color. Whereas the mandibular secretions of most *Atta* species, including *A. opaciceps*, are relatively similar in composition, those of *A. sexdens sexdens* are different in that the secretions of the majority of worker castes contain large amounts of citral. In contrast, the secretions of *A. opaciceps* consist of alcohols and ketones, typical of those found in other species of leaf-cutting ants (Table 4), although the amounts present appeared to be much reduced. This is probably because these ants are very aggressive when perturbed, and it is likely that a considerable

proportion of the alarm pheromone was liberated during specimen collection. Nevertheless, 4-methyl-3-heptanone was identified in all worker castes, further supporting the view that the ketone is characteristic of the mandibular gland secretion of all species of the genus *Atta* (Blum et al., 1968; Do Nascimento et al., 1993; Hernández et al., 1999; Hughes et al., 2001a,b). The major component of the mandibular gland secretion of *A. opaciceps* soldier castes was 2-heptanone, a compound that has been detected in other species of the subfamily Myrmicinae, including the genus *Atta*, and is considered a common component of alarm pheromones (Hölldobler and Wilson, 1990).

It has been previously reported (Hughes et al., 2001b) that 2-heptanone and 4-methyl-3-heptanone are the main components of the mandibular gland secretions of *A. capiguara* and *A. bisphaerica*, although the proportions of these ketones vary among castes. In the present study, 2-heptanone was not detected at all in gardeners and generalists of *A. opaciceps*; 4-methyl-3-heptanone was the sole compound found in the secretions of these castes. Behavioral bioassays performed in an earlier study involving *A. opaciceps* (unpublished results) indicated a greater stimulation of alarm behavior by minor castes in comparison with major castes. Because chemical analysis of the mandibular gland secretions of the minor castes suggested that 4-methyl-3-heptanone was the major (or perhaps only) component, we suggest that, as is the case for *A. sexdens sexdens*, it is the gardeners and generalists that are responsible for the production and release of the alarm pheromone in *A. opaciceps*.

4-Methyl-3-heptanone elicited strong alarm response in foragers of *A. sexdens sexdens* and *A. opaciceps*. The number of times a single forager exhibited an alarm response to this compound was higher than to any other tested compound. This result agrees with earlier studies carried out with workers of *A. texana*, *A. sexdens*, *A. cephalotes*, *A. capiguara*, and *A. bisphaerica*, which showed 4-methyl-3-heptanone as the alarm pheromone (Blum et al., 1968; Moser et al., 1968; Riley et al., 1974; Knapp, 1995; Pow, 1996; Hughes et al., 2001a). In addition, this alkanone was also found as the major component in mandibular secretions of *A. colombica* and *A. robusta* (Blum et al., 1968). Thus, 4-methyl-3-heptanone seems to be the alarm pheromone of *Atta* and *Pogonomyrmex* species as suggested in the literature (Wilson, 1965; Blum, 1969; Vick et al., 1969; McGurk et al., 1996; Vander Meer and Alonso, 1998).

The role that volatile compounds from the mandibular gland secretions of *A. sexdens sexdens* and *A. opaciceps* play in intraspecific recognition is not known. Further studies need to be conducted to determine what role these compounds may play in nestmate recognition.

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BEYOND 9-ODA: SEX PHEROMONE COMMUNICATION IN THE EUROPEAN HONEY BEE *Apis mellifera* L.

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Abstract—The major component of the mandibular gland secretion of queen honeybees (*Apis mellifera* L.), 9-ODA ((2*E*)-9-oxodecenoic acid), has been known for more than 40 yr to function as a long-range sex pheromone, attracting drones at congregation areas and drone flyways. Tests of other mandibular gland components failed to demonstrate attraction. It remained unclear whether these components served any function in mating behavior. We performed dual-choice experiments, using a rotating drone carousel, to test the attractiveness of 9-ODA compared to mixtures of 9-ODA with three other most abundant components in virgin queen mandibular gland secretions: (2*E*)-9-hydroxydecenoic acid (9-HDA), (2*E*)-10-hydroxydecenoic acid (10-HDA), and *p*-hydroxybenzoate (HOB). We found no differences in the number of drones attracted to 9-ODA or the respective mixtures over a distance. However, adding 9-HDA and 10-HDA, or 9-HDA, 10-HDA, and HOB to 9-ODA increased the number of drones making contact with the baited dummy. On the basis of these results, we suggest that at least 9-HDA and 10-HDA are additional components of the sex pheromone blend of *A. mellifera*.

Key Words—Sex pheromone, mating behavior, mandibular gland, honeybee, *Apis mellifera*, (2*E*)-9-oxodecenoic acid, (2*E*)-9-hydroxydecenoic acid, (2*E*)-10-hydroxydecenoic acid.

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INTRODUCTION

In insects, the most elaborate chemical communication systems have evolved in the context of mating and social behaviors (Wilson, 1971; Phelan, 1992; Ayasse et al., 2001). In honeybees, these communication systems are closely linked because the virgin queen uses the mandibular gland secretions to attract drones on her mating flights (Gary, 1962; Butler et al., 1967), whereas the mated queen uses mandibular gland secretions to signal her presence to workers in the hive (Velthuis, 1985; Free, 1987; Slessor et al., 1988; Brockmann et al., 1998). The complex and phase-dependent (i.e., virgin vs. mated) chemical compositions of queen mandibular gland secretions (Plettner et al., 1997; Free, 1987) suggest that single components or mixtures of components are addressed differentially to these two receiver groups and may serve different functions in specific behavioral contexts.

In the 1970s, (2*E*)-9-oxodecenoic acid (9-ODA), the major component of the mandibular gland secretions, was shown to attract drones to queen dummies (Butler, 1971; Gary and Marston, 1971; Boch et al., 1975; Free, 1987). Further investigations suggested that other components of the secretion might play a role in the communication between sexes (for a detailed discussion of these experiments, see Free, 1987; Koeniger and Koeniger, 2000). More recent experiments, which tested the attractiveness of the six most abundant mandibular gland components: 9-ODA, (2*E*)-9-hydroxydecenoic acid (9-HDA; 85% (*R*)-(-), 15% (*S*-(+)), (2*E*)-10-hydroxydecenoic acid (10-HDA), *p*-hydroxybenzoate (HOB), and 4-hydroxy-3-methoxyphenylethanol (HVA), demonstrated that 9-ODA—but none of the other components—attracted drones from a distance (Loper et al., 1996). However, the inability of a single component to attract drones does not necessarily exclude a pheromonal function of a particular component as part of a blend (Linn et al., 1986; Christensen, 1997). Thus, it was not clear whether honeybee sex pheromone communication was based on a single substance or a multicomponent blend, and if the latter, which of the other mandibular gland compounds are also sex pheromone components. In contrast, in the communication between queens and workers, it has been shown that a blend of five mandibular gland substances is necessary to elicit worker retinue behavior [9-ODA, 9-HDA (85% (*R*)-(-), 15% (*S*-(+)), HOB, and HVA] (Slessor et al., 1988; Plettner et al., 1996, 1997).

In the experiments reported here, the potential sex pheromonal function of mandibular gland components were assessed in dual choice experiments using a motor-driven drone carousel, by testing attractiveness of 9-ODA vs. different combinations of 9-ODA and minor mandibular gland components.

METHODS AND MATERIALS

Experimental Site. Experiments were conducted in the vicinity of a drone congregation area close to the bee station and apiary of the Beegroup Würzburg

from May to July 2002. The experimental site was identified by presenting queen dummies containing $70.4 \mu\text{g}$ 9-ODA (equals the amount of 9-ODA in virgin queens' glands; Plettner et al., 1997) on the tip of a 3 m long rod at different spots within an area of about 0.3 km^2 between the bee station and the Biocenter of the University of Würzburg, Germany. The drone carousel was placed in an open meadow near a tree line at the edge of an orchard (marked by an "x" in Figure 1). No drones were detectable at the experimental site when no pheromones were presented, but when we offered a pheromone bait, drones

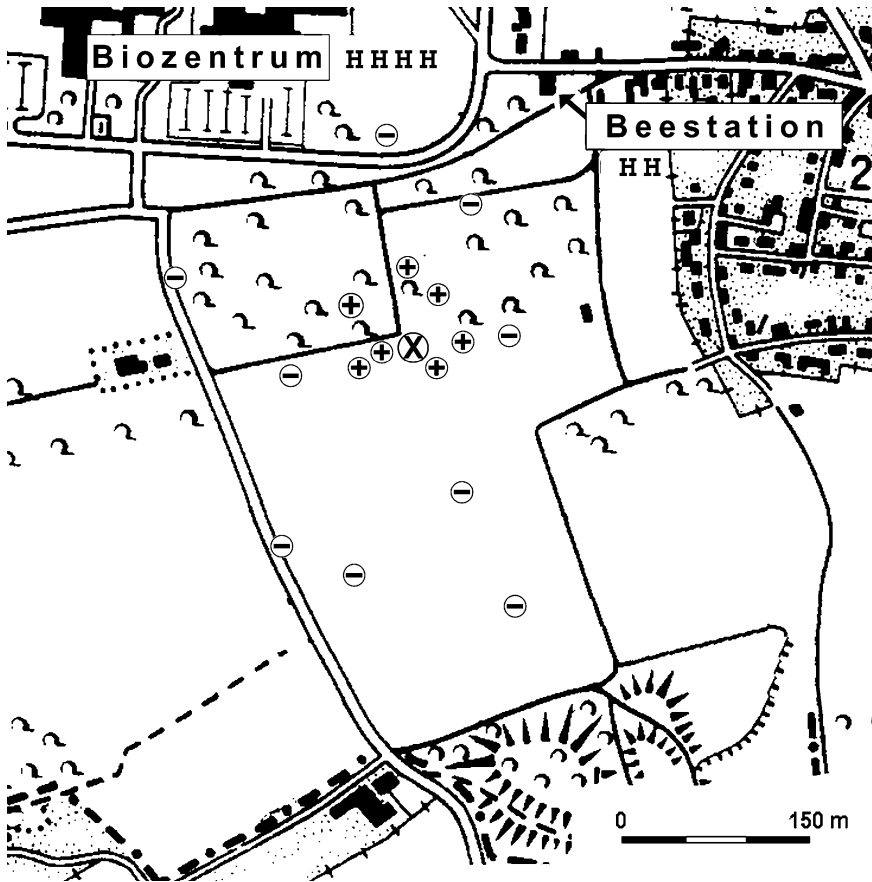


FIG. 1. General map of experimental site. (+) indicates sites at which drones could be attracted; (-) indicates sites at which drones could not be attracted by an artificial pheromone lure attached to a rod (3 m high), containing 1 Qeq of 9-ODA. (x) Position of the drone carousel; (H) position of closest bee hives.

appeared immediately. In some cases, we observed drones coming directly out of the trees lining the field. Thus, we assume that they were either cruising close to or were waiting on the trees. Prior to the experiments, we conducted a dose-response test to determine a pheromone dose that attracted an appropriate number of drones that still could be resolved on videotapes. Despite the use of standardized pheromone doses, drone numbers varied from day to day and between experiments on a single day.

Two-armed Drone Carousel. The drone carousel was a modification of that developed by Renner and Vierling (1977) and further improved by Gries and Koeniger (1996). It was composed of an upright column (adjustable for height from 4 to 6 m) carrying two opposing horizontal aluminum rods (each 2 m in length). The arms could be rotated with an electric motor around the vertical axis with a velocity of 3.1 m/sec at the tips of the arms. The queen dummies were fixed at the distal tips of the arms using a thin nylon thread (10 cm; height of dummies, 4.95 m above ground). A dummy was made of a black aluminum bar (3.5×1.1 cm) with inserted pheromone impregnated glass rods. A video camera was fixed next to the central axis on each arm, facing toward the dummies. Cameras monitored an area of about 1 m² in the plane of a dummy with the dummy in its center.

Pheromone Components and Artificial Blends. 9-ODA, 9-HDA, and 10-HDA were obtained from Phero Tech Inc. (Delta, BC, Canada) and HOB was obtained from Sigma-Aldrich (Taufkirchen, Germany). All compounds were dissolved in absolute ethanol. Test blends were mixed according to published data on the composition of the mandibular secretion in *Apis mellifera* virgin queens (Plettner et al., 1997). The complete artificial virgin queen blend (virgin queen equivalent = $Qe_{q_{\text{virgin}}}$) contained the following: 70.4 μg 9-ODA, 12.6 μg 9-HDA, 76.7 μg 10-HDA, and 0.3 μg HOB. Test blends differed in the number of components. Dose-response tests showed that a dose of 0.1 $Qe_{q_{\text{virgin}}}$ was adequate to attract a sufficient number of drones at our experimental site (see Results). Glass bars (10–12 mm in length) with a small groove in which 10 μl pheromone blends dissolved in ethanol were deposited were used as pheromone dispensers. The solvent was allowed to evaporate for 5 min. Then the glass rods were wrapped in aluminum foil and kept on ice until use. Immediately before the field tests started, the glass rods were fixed to the aluminum dummies.

Two-choice Experiments. On each experimental day, five to nine test runs were conducted with different pairs of pheromone blends in random order, depending on drone abundance. Each test lasted for 15 min, of which the first 5 min were used to attract drones to the carousel and were excluded from the data analysis. Prior to each test run, the video cameras were synchronized to analyze identical periods for both dummies. Altogether, we conducted 86 tests (out of which 63 were included in the analysis, see below) with five different pairs of

pheromone blends: 9-ODA vs. 9-ODA ($N = 11$; control experiment); 9-ODA vs. 9-ODA + 9-HDA ($N = 11$); 9-ODA vs. 9-ODA + 10-HDA ($N = 13$); 9-ODA vs. 9-ODA + 9-HDA + 10-HDA ($N = 13$); and 9-ODA vs. 9-ODA + 9-HDA + 10-HDA + HOB ($N = 15$). The number of drones attracted to each dummy during the 10-min test interval was determined by counting the number of drones every 20 sec, i.e., on 31 video freeze images per test. Because the numbers of drones attracted to the carousel differed strongly among experimental runs (see Figure 3A), we calculated the relative number of drones attracted to each of the two dummies for each test by taking the sum of all drones that were counted on the 62 images from both sides per test as 100%. Only test runs that attracted at least 10 drones to the carousel were included in the analysis. The numbers of drone contacts with the dummies were determined by counting all contacts during each 10-min test interval. Only intense and unambiguous contacts with the dummy, i.e., clinging to the dummy in a clear mounting attempt, were counted. Again, to allow comparisons between tests despite the variation in absolute numbers of drones present in the vicinity of the carousel, we calculated the relative number of contacts to each of the two dummies for each test run. Only test runs in which at least five contacts were scored were included in the final analysis. To test for significant differences of drone numbers or numbers of contacts between the two arms, we applied the Wilcoxon signed-rank test for each of the five pairs of pheromone combina-

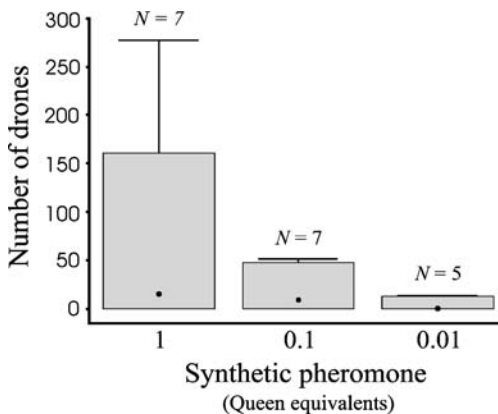


FIG. 2. Effect of different concentrations of synthetic queen pheromone on the attraction of drones to the carousel. One virgin queen equivalent contained 70.4 μg 9-ODA, 12.6 μg 9-HDA, 76.7 μg 10-HDA, and 0.3 μg HOB. Treatments were presented on a dummy at one arm of the carousel. Box and whisker plot indicates median (black dot), first and third quartile (box) and maximum number (whisker) of drones attracted to the dummy per 10-min experimental run.

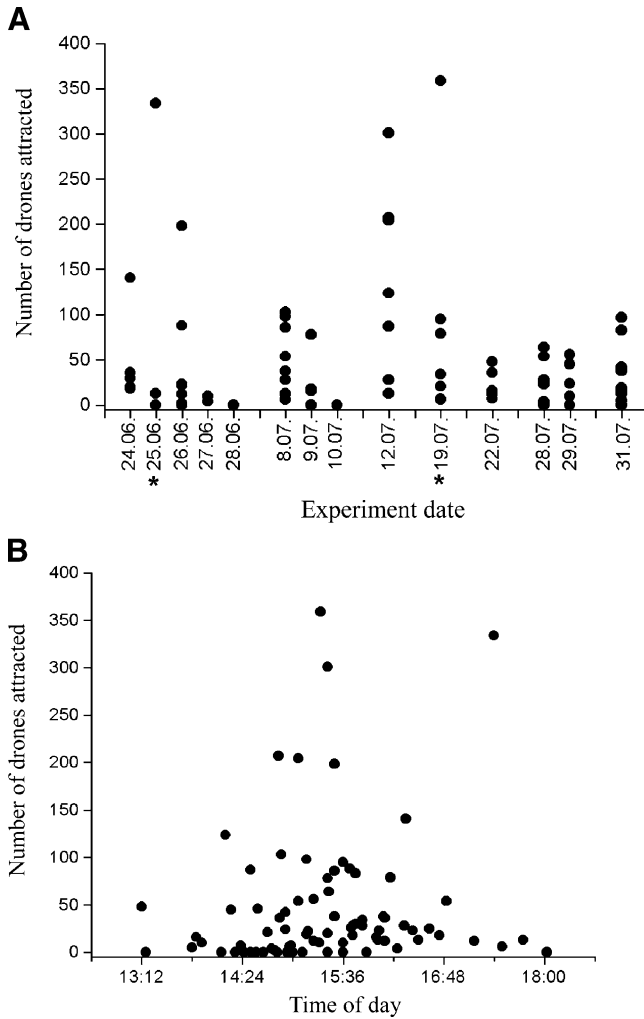


FIG. 3. (A) Number of drones attracted on different days. Each dot indicates number of drones per 10-min experimental run (median = 23, first quartile = 7.3, third quartile = 56, minimum = 0, maximum = 1248). During two experimental runs, a large number of drones were attracted: 528 drones (*) on June 25, and 1248 drones (*) on July 19. These two data points were omitted from both figures. (B) Effect of time of day on the attraction of drones. Dots indicate the number of drones attracted per run for all experimental replicates.

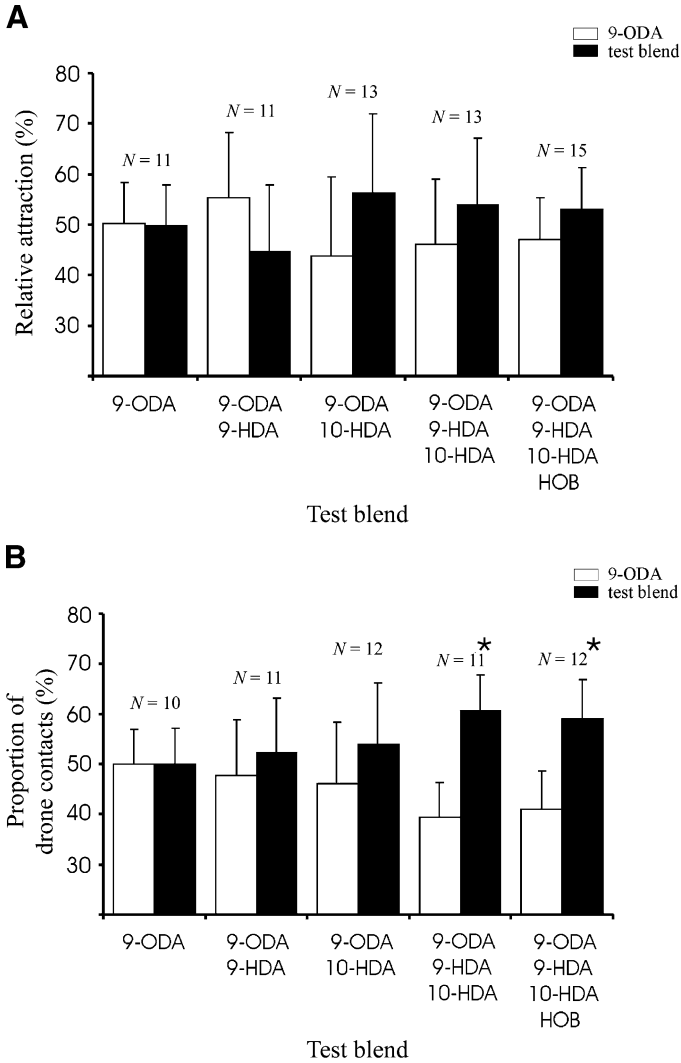


FIG. 4. (A) Proportion of drones attracted (\pm SD) to the vicinity of the treatment and control lures. (B) Proportion of drone contacts with the treated and control dummies. White bars, 9-ODA only; black bars, test blends. *Significant differences between 9-ODA and the blend ($P < 0.05$).

tions. All data were arcsine-transformed prior to analysis. P values exceeding 0.05 were considered as not statistically significant.

RESULTS

Attraction of drones to a synthetic pheromone lure was locally restricted to an area of ~ 100 m in diameter (Figure 1). The distance to the closest honeybee colonies was about 150 to 300 m. The number of drones attracted to the drone carousel was pheromone dose dependent (Figure 2), demonstrating that drones were attracted to treated dummies by olfactory, rather than visual, cues. Numbers of drones attracted to the carousel were stable during each test run but varied among test runs and among test days (Figure 3A,B).

To ensure that there was no bias between the two dummies, we first treated both lures with identical doses of 9-ODA, which resulted in equal numbers of drones being attracted to both dummies (Figure 4A). In subsequent trials, 9-ODA as a single substance was as attractive as any other combination of 9-ODA and minor components (Figure 4A).

We also found no evidence for any asymmetry in drone contacts when equal amounts of 9-ODA were presented on both arms (Figure 4B). Adding 10-HDA or 9-HDA to 9-ODA did not increase the number of contacts compared to 9-ODA alone. However, blends that were more similar to the natural pheromone composition (9-ODA + 9-HDA + 10-HDA or 9-ODA + 9-HDA + 10-HDA + HOB) resulted in significantly higher numbers of contacts with the queen dummy containing the blend compared to the dummy with 9-ODA alone ($Z = -2.81$, $P = 0.005$, and $Z = -2.85$, $P = 0.004$, respectively; Figure 4B).

DISCUSSION

Mating behavior in honeybees is highly competitive between drones, much more than in most other insects. In all honeybee species, drones and virgin queens leave their colonies for mating flights at species-specific times of the day. In our experiments, drones were present at the drone congregation area between 1:00 and 6:00 P.M., with a peak time around 3:30 P.M. Drones gather at the drone congregation areas (estimated sizes range from 50 to 200 m in diameter), waiting for virgin queens to appear (Ruttner, 1985). Upon detection of the queen's sex pheromone, drones initiate their visually driven chasing behavior, all competing for the queen and only a few being successful. To catch a queen, the drones must be fast and precise in detecting and chasing. Although detailed analysis of honeybee mating behavior is still lacking, one may assume that the

chasing behavior of drones is based on a succession of different behavioral steps including detection of the pheromone, searching the sky for small dark flying objects, a rapid pursuit of the queen (Gries and Koeniger, 1996), and finally successful grasping and mounting of the queen.

Our experiment to determine the location of a drone congregation area showed that the effective range of the pheromone was limited to a circular area with a diameter of ~100 m. We assume that the location of this drone congregation area is stable over the years, because of previous reports that one could attract drones with pheromone lures in that area (J. Tautz, pers. comm.). The closest beehives (marked by an “H” in Figure 1) were located about 150–300 m north, with entrances facing south in the direction of the drone congregation area. The most obvious visual feature of the site is the boundary of the orchard and the open meadow, and this may be one of the constitutive visual cues. If drones from the specified colonies join this drone congregation area, the boundary between the orchard and the meadow might function as a visual “stop and stay” stimulus; however, this remains to be investigated.

Our drone carousel experiments demonstrated that in addition to 9-ODA (the so-called queen substance), other components of the queen mandibular gland secretion, particularly 9-HDA and 10-HDA, play a role in pheromone communication between queens and drones. The latter two compounds are not attractive to drones from a distance (Loper et al., 1996), but added to 9-ODA they increased the drone’s contacts with a queen dummy. Whether HOB also mediate drone behavior cannot be inferred from our experiments. The effect of 9-HDA and 10-HDA on the frequency of mounting suggests that 9-HDA and 10-HDA only act over a short range. However, we can not exclude the possibility that these compounds are active at long range (tens to hundreds of meters), because our experimental set-up did not specifically test for those distances. A similar increase in the frequency of mounting behavior in drone carousel experiments was also found when tergite gland extracts were added to 9-ODA (Renner and Vierling, 1977). The conclusion from both experiments is that adding either 9-HDA and 10-HDA or tergite gland secretions to 9-ODA increases the effectiveness of the artificial stimulus, leading to a stronger response and a more complete performance of the mating behavior sequence. Whether the components of the mandibular gland and the tergite gland have distinct functions in the sequence of the mating behavior remains unknown.

It is intriguing to compare our findings on sex pheromone communication with those on queen–worker communication. Slessor et al. (1988) and Keeling et al. (2003) showed that a blend of 9-ODA, 9-HDA, HOB, and HVA similar to that found in mated queens elicited worker retinue behavior. 10-HDA, which is reduced in quantity in mated compared to virgin queen glands, does not play any role in queen–worker communication (Slessor et al., 1988). In contrast to

those experiments, we found clear evidence that 10-HDA, which is produced in large amounts by virgin queens, does have a pheromone function in mating behavior. Taken together, the data suggest that *A. mellifera* queens produce different signals during their adult life stages in relation to different communication contexts. As virgin queens they produce a signal to attract drones on mating flights, and as mated queens they produce a signal for the workers. The signals are produced by the same gland and comprise almost identical substances, but the relative composition changes between virgin and mated queens. This change in mandibular gland composition may be induced by successful mating.

9-ODA is viewed as the major long-distance sex attractant for all honeybee species (Butler et al., 1967; Ruttner and Kaissling, 1968; Shearer et al., 1970; Sanasi et al., 1971; Koeniger and Koeniger, 2000), and it is the main component in the queen mandibular glands of all species except *A. florea* (Plettner et al., 1997), in which 10-HDA is the most abundant mandibular gland component. Our finding that the two other most abundant decenoic acids also have a pheromonal function in *A. mellifera* suggests that they might have sex pheromone functions in other honeybee species as well (Brockmann and Brückner, 2005). Thus, it is likely that 10-HDA is a sex pheromone component for *A. florea*, and it might even be the major functional pheromone component, replacing 9-ODA in this species. In addition, differences in the relative composition of mandibular gland components might be effective in reproductive isolation between sympatric species. GC analyses show that the relative composition of mandibular gland components varies among different *Apis* species (Plettner et al., 1997). Whether drones use these relative differences to discriminate between queens of their own and sympatric species remains to be determined.

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FEMALE SEX PHEROMONE OF A CARPENTER MOTH,
Cossus insularis (LEPIDOPTERA: COSSIDAE)

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Abstract—This study describes the identification of a sex pheromone component of a cossid moth, *Cossus insularis*. Coupled gas chromatographic–electroantennographic detection (GC–EAD) analysis of solid-phase micro-extraction (SPME) collections of volatiles released by live female moths showed that two compounds elicited EAG responses from the antennae of male moths. These compounds were identified as (*E*)-3-tetradecenyl acetate (*E*3-14:Ac) and (*Z*)-3-tetradecenyl acetate (*Z*3-14:Ac) by mass spectral analysis and retention index comparisons with synthetic standards. The ratio of *E*3-14:Ac and *Z*3-14:Ac was 95:5 in the effluvia of a female. In field bioassays, sticky traps baited with blends of *E*3-14:Ac and *Z*3-14:Ac showed that *E*3-14:Ac is an essential component of the pheromone. However, the role of *Z*3-14:Ac is unclear, because *E*3-14:Ac as a single component was as attractive to male moths as blends of *E*3-14:Ac and *Z*3-14:Ac, including the 95:5 blend released by live female moths.

Key Words—Carpenterworm moth, *Cossus insularis*, (*E*)-3-tetradecenyl acetate, (*Z*)-3-tetradecenyl acetate, GC–EAD, SPME, sex pheromone.

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INTRODUCTION

The carpenterworm moth, *Cossus insularis* (Staudinger) (Lepidoptera: Cossidae) is found in Honshu, Kyushu, and Tsushima Islands of Japan (Tadauchi and Inoue, 2000). Although host plants of this species are not well known, Kitajima et al. (1998) reported an aggregation of 236 larvae from a 3.2-cm-diameter branch of a willow tree. Recently, Nakanishi (2005) reported the first incidence of *C. insularis* larvae in Japanese pear, *Pyrus pyrifolia* var. *culta*. On both host trees, aggregations of larvae were observed to bore into woody stems, causing significant damage and frequent mortality. Because burrows are not exposed, insecticides are not a practical measure for control. Isolation and identification of the sex pheromone of *C. insularis* would be useful to help monitor and possibly control populations of this species.

In the Family Cossidae, sex pheromones for *Cossus cossus* L. (Capizzi et al., 1983), *Holcocerus hippophaecolus* Hua (Fang et al., 2005), *Holcocerus insularis* (Zhang et al., 2001), *Prionoxystus robinae* Peck (Solomon et al., 1978), and *Zeuzera pyrina* L. (Tonini et al., 1986) have been identified, and sex attractants for three other species, *Acossus centerensis* Lintner (Doolittle et al., 1976), *Cossus mongolicus* Erschoff (Qi et al., 1985), and *Prionoxystus piger* Grote (Landolt et al., 1985) have been reported. Most of the attractive components for these cossid moths are mono- or diunsaturated 10-, 12-, 14-, or 18-carbon alkenyl acetates. The aim of the present study was to isolate and identify the sex pheromone of *C. insularis*.

METHODS AND MATERIALS

Insects. Late instar larvae and pupae of *C. insularis* were collected from heavily infested logs of willow trees at the Forestry and Forest Products Research Institute, Tsukuba, Japan, in March 2004. Field-collected larvae were reared individually on artificial diet (Insecta LF[®], Nihon Nosan, Yokohama, Japan) under 14L:10D at 25 ± 1°C. Newly emerged female and male moths were used for solid-phase microextraction (SPME) collection of volatiles and coupled gas chromatographic–electroantennographic detection (GC–EAD) analyses, respectively.

Collection of Volatiles from Female Moths. We used SPME for collecting airborne volatiles emitted by the female moths. SPME fibers (100 µm polydimethylsiloxane, Supelco, Bellefonte, PA, USA) were preconditioned for 30 min at 250°C. Newly emerged females were isolated in a 100-ml glass bottle (Sibata, Tokyo, Japan) with a screw cap lined with an acrylic nitril butadiene rubber/Teflon septum (45 mm diameter) with a hole (26 mm ID). Two SPME fibers were inserted into each bottle through a small hole in the septum. The

fibers were exposed to the moths for approximately 16 hr, from 1700 hr until 0900 hr next morning, at $25 \pm 1^\circ\text{C}$. The loaded SPME fiber was then desorbed into the injection port of either a GC–EAD or a GC–MS system. The bottles for volatile collection were ultrasonicated in a detergent solution for 10 min, rinsed, and dried at 100°C before use.

To evaluate the SPME sampling method, we placed 1 mg each of (*E*)-3-tetradecenyl acetate (*E*3-14:Ac) and (*Z*)-3-tetradecenyl acetate (*Z*3-14:Ac) in 100 μl *n*-hexane solution on the bottom surface of a 100-ml glass bottle. An SPME fiber was inserted into the bottle after the *n*-hexane evaporated and kept for one night at $25 \pm 1^\circ\text{C}$. Ten different SPME fibers were used to collect volatiles from the headspace of the bottle and were then desorbed in the injection port of a GC.

Coupled Gas Chromatographic-Electroantennographic Detection (GC–EAD) Analysis. Volatiles collected on the SPME fiber were analyzed by GC–EAD in splitless mode with a Hewlett-Packard 5890 series II GC equipped with a DB-23 column (30 m \times 0.32 mm ID, 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA). Oven temperature was $45^\circ\text{C}/1$ min, $15^\circ\text{C}/\text{min}$ to 150°C , $2^\circ\text{C}/\text{min}$ to 180°C , and then $10^\circ\text{C}/\text{min}$ to 230°C , where it was held for 20 min.

Although the GC effluent splitter followed the general design of Struble and Arn (1984), some parts were replaced with those made from inactivated material. To achieve proper elution of the sample to the flame ionization detector (FID) and the EAD, nitrogen makeup gas (30 ml/min) was introduced into the stainless T-union, in which the end of the analytical capillary column (0.32 mm ID) was inserted into a deactivated fused-silica capillary column (0.53 mm ID, Agilent Technologies, Wilmington, DE, USA). The effluent was split between the FID and the EAD with a press-fit Y splitter (Agilent Technologies). Deactivated fused-silica capillary columns (0.53 mm ID, Agilent Technologies) were also used as transfer lines from the Y splitter to each detector. The GC effluent for EAD emptied into a glass transfer tube (15 mm ID) mounted on the GC and was mixed with humidified air (300 ml/min, 20°C) passing through the tube.

An excised antenna was placed on the antenna holder of an EAG probe (Syntech, Hilversum, the Netherlands). The antenna was connected with the recording and indifferent electrode with electrode gel (Spectra[®] 360, Parker Lab. Inc., Orange, NJ, USA). EAG and FID signals were fed into a computer through an analog-to-digital conversion board (IDAC-232, Syntech). Signal reception and analysis was performed with GC–EAD software (Syntech).

Preparative Gas Chromatography. An HP 5890 Series II gas chromatograph modified for GC–EAD as described above was converted to a preparative GC by exchanging a press-fit Y splitter with a press-fit column connector (Agilent Technologies). The GC–EAD active compounds were trapped in a

glass capillary (5.5 mm ID, 107 mm long) placed on the column outlet after removing the collector nut, an igniter castle, a collector, and collector insulators from the FID port of the GC. The glass capillary was eluted with *n*-hexane, and the eluate was stored at -5°C until analysis. The same column and temperature program as the GC-EAD system was used to trap the compounds of interest except that nitrogen makeup gas flow was adjusted to 15 ml/min.

Chemical Analysis. Samples collected by SPME were analyzed with an Agilent 6890N GC interfaced to an Agilent 5973 mass-selective detector run in electron impact ionization mode at 70 eV. The GC-MS was equipped with a DB-23 column (30 m \times 0.25 mm ID, 0.25 μm film thickness, J & W Scientific), using a temperature program of $120^{\circ}\text{C}/1$ min, $5^{\circ}\text{C}/\text{min}$ to 200°C , and held for 10 min. Helium was used as carrier gas (1 ml/min).

Double bond positions and geometries in unsaturated compounds were determined from dimethyldisulfide (DMDS) adducts of the insect-produced compounds (Buser et al., 1983). Fifty microliters of DMDS and 5 μl of iodine solution (60 mg I_2 in 1 ml diethyl ether) were added to 50 μl of *n*-hexane solution containing EAD-active compounds from female volatiles collected by preparative GC. The mixture was stored at 60°C for 24 hr, and then quenched with 200 μl of 5% $\text{Na}_2\text{S}_2\text{O}_3$ solution in distilled water, and the mixture was extracted with 100 μl *n*-hexane. EI-MS spectra of the derivatives were obtained with the same GC-MS system described above equipped with an HP-5MS capillary column (30 m \times 0.25 mm ID, 0.25 μm film thickness, J & W Scientific), using a temperature program of $100^{\circ}\text{C}/5$ min, $5^{\circ}\text{C}/\text{min}$ to 280°C .

Geometry of the double bond in each monounsaturated compound was determined by comparing the GC retention time (Rt) of DMDS adducts of a compound eliciting an antennal response with those of synthetic standards using the same HP-5MS column described above.

Chemicals. Samples of *E*3-14:Ac (purity > 95%) and *Z*3-14:Ac (purity > 95%) were kindly provided by Prof. Chul-Sa Kim of Kochi University, Japan, and Dr. Huang Yongping of Shanghai Institute of Plant Physiology and Ecology, China, respectively.

For field trapping we synthesized Δ 3-14:Ac from Δ 3-tetradecen-1-ol (Δ 3-14:OH). Thus, the Grignard reagent from 1-dodecyne (Tokyo Kasei Kogyo Co., Ltd., Tokyo) was reacted with ethylene oxide in dry tetrahydrofuran. The product (3-tetradecyn-1-ol) was reduced to Δ 3-14:OH by hydrogenation over Lindlar's catalyst. The *E/Z* ratio of synthesized Δ 3-14:OH was 2:98. To enrich the *E* isomer, isolated Δ 3-14:OH was isomerized by stirring for 3 hr at 80 – 90°C after mixing with 2% (w/w) of nitric acid as a catalyst (Terauchi et al., 2002), which was then acetylated with acetic anhydride by standard techniques. Crude *E*3-14:Ac of 76% isomeric purity was purified by a urea adduct method (Fukumoto et al., 1999). Thus, the crude *E*3-14:Ac was poured into a saturated methyl alcohol solution of urea at 50 – 60°C and stirred for 5 min. To precipitate

the urea adduct, the solution was cooled to room temperature over 2 hr and further to 10°C by immersion in an ice-water bath. The free acetate was removed by extraction of the cooled methyl alcohol solution with *n*-hexane. After removal of the *n*-hexane layer, precipitated *E* isomer-rich urea adduct was dissolved by warming the methyl alcohol to 50–60°C. Two cycles of *Z* isomer-rich free acetate removal from the urea adduct solution gave *E*3-14:Ac of > 99.9% isomeric purity.

To obtain *Z*3-14:Ac, Δ 3-14:OH synthesized as above was acetylated and purified by the urea adduct method (Fukumoto et al., 1999), resulting in *Z*3-14:Ac of > 99.9% isomeric purity.

Field Trapping. Delta traps with removable sticky bottoms (SE trap[®], Sankei Chemical Co. Ltd., Tokyo, Japan) were baited with synthetic *E*3-14:Ac and *Z*3-14:Ac (1 mg in total) mixed in a series of different ratios (Table 1). The pheromone components were impregnated in gray halobutyl isoprene blend elastomer septa (West Co., Singapore). Three replicates of the trap treatments were placed in a randomized block design in a pear orchard in Tokushima, Japan, from June 7 to August 10, 2004. Traps were hung from tree branches at a height of about 2 m. Traps were inspected at 3- to 6-d intervals and shifted at each inspection to reduce positional effects. Sticky panels within the traps were replaced when they became saturated with captured moths.

Statistics. Data from field tests were analyzed using JMP (SAS Institute, Cary, NC, USA). The analyzed variable was the trap catch after $\sqrt{(X + 0.5)}$ transformation followed by ANOVA. Significantly different means were separated by Tukey–Kramer’s HSD test.

TABLE 1. MEAN AND TOTAL CATCHES OF *C. insularis* IN TRAPS BAITED WITH MIXTURES OF (*E*)-3-TETRADECENYL ACETATE AND (*Z*)-3-TETRADECENYL ACETATE

Blend tested % (<i>E</i> 3: <i>Z</i> 3)	Average catch/trap (mean \pm SE)	Total number of moths captured
100:0	173.0 \pm 21.4a	519
95:5	113.7 \pm 28.3a	335
80:20	124.3 \pm 20.7a	370
60:40	145.7 \pm 23.1a	429
20:80	74.3 \pm 6.8b	223
0:100	10.3 \pm 4.5c	31
Control	0	0

Means followed by the same letter are not significantly different ($P = 0.05$, Tukey–Kramer’s HSD test). Control was omitted from ANOVA to avoid heterogeneity among variances (Levene test).

RESULTS

Injections of SPME-collected volatiles released by an unmated female of *C. insularis* into the DB-23 column gave two consistent EAD responses with retention times of 13.72 (peak I) and 13.89 (peak II) min, respectively (Figure 1a). Although some EAD responses different from those to peak I and II were observed (Figure 1a), they were not consistent, suggesting that they were artifacts and, therefore, not subjected to further analysis.

Although it did not show a molecular ion, the EI-MS spectrum for GC peak I (Figure 2) showed m/z 61 ($\text{CH}_3\text{COOH}_2^+$), a diagnostic fragment ion for an acetate ester, and a characteristic fragment ion at m/z 194 ($\text{M}^+ - 60$). This suggested that the compound was a 14-carbon monounsaturated acetate.

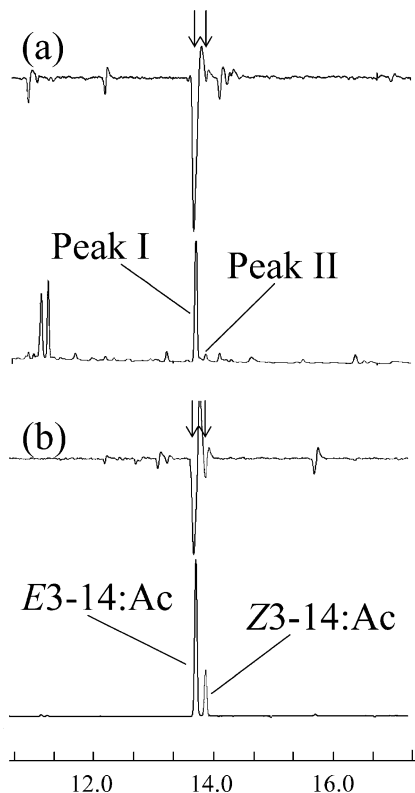


FIG. 1. Coupled GC-EAD responses from an antenna of a male *C. insularis* to female volatiles collected by SPME from an unmated female moth (a) and to synthetic *E3-14:Ac* and *Z3-14:Ac* (b).

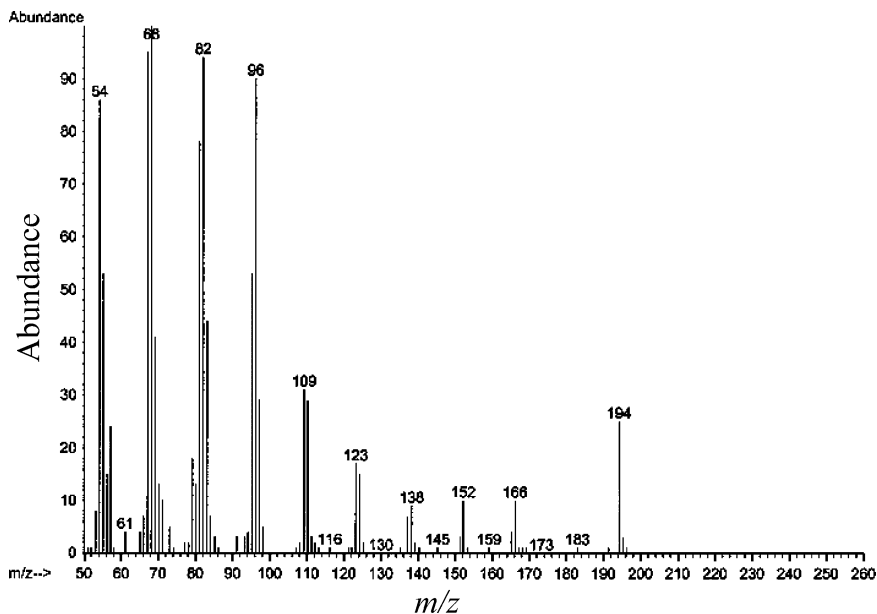


FIG. 2. Mass spectrum of peak I in Figure 1a.

The mass spectrum of the DMDS adduct of GC peak I showed diagnostic fragments at m/z 87 ($\text{CH}_3\text{COOC}_2\text{H}_4^+$, 31%), 147 ($\text{CH}_3\text{COOC}_2\text{H}_4\text{CH}=\text{SCH}_3^+$, 3%), 201 ($\text{H}_3\text{CS}^+=\text{CH}-\text{C}_{10}\text{H}_{21}$, 100%), and 348 (M^+ , 11%), demonstrating that the peak I was a $\Delta 3$ -14:Ac isomer.

Retention times of the DMDS adducts of GC peak I and peak II on the HP-5MS column were 34.39 and 34.29 min, respectively, which were the same as those of the DMDS adducts of the synthetic *E*3-14:Ac (34.38 min) and *Z*3-14:Ac (34.29 min). The GC retention times of the synthetic *E*3-14:Ac and *Z*3-14:Ac on a DB-23 column matched those of peak I and II compounds from female moths. Each of the synthetic compounds elicited as strong an EAG response as elicited by the natural compounds (Figure 1b).

The mixture ratio of the *E* and *Z* isomers of $\Delta 3$ -14:Ac was estimated from the area of each peak obtained from the GC analysis of SPME-collected samples. The *E*:*Z* ratio was $95 \pm 0.9\%$ to $5 \pm 0.9\%$ ($N = 12$), indicating that individual variation in pheromone ratio among females was low. GC analysis of the samples collected by SPME from a 1:1 mixture of synthetic *E*3-14:Ac and *Z*3-14:Ac gave an *E*/*Z* ratio of $48.5 \pm 2.0\%$ *E* and $51.5 \pm 2.4\%$ *Z* isomer, which was not different from 1:1 ($P > 0.05$, Binomial test, $N = 10$). This demonstrated that the ratio of $\Delta 3$ -14:Ac isomers collected by SPME reflected the actual ratio released by the female moths.

Traps baited with lures of *E3-14:Ac* and *Z3-14:Ac* mixed in different ratios captured 1907 male moths from June 7 to August 10. All the lures tested attracted *C. insularis* (Table 1). Numbers of males attracted to the 100:0 to 60:40 blends of *E3-14:Ac* and *Z3-14:Ac* were equivalent (Table 1), whereas the 20:80 and 0:100 blends of *E3-14:Ac* and *Z3-14:Ac* attracted fewer males. No moths were captured in control traps.

DISCUSSION

Our data indicate that *E3-14:Ac* is the main and possibly only component of the sex pheromone of *C. insularis*. Although the *Z* isomer itself was weakly attractive (Table 1), it does not appear to be a critical component of the pheromone because 100% *E3-14:Ac* was as attractive as the 95:5 naturally produced ratio. Alcohols, aldehydes, or acetates of 14-carbon compounds are known sex pheromones and attractants for many lepidopteran species (Mayer and McLaughlin, 1991). However, 14-carbon compounds with the double bond in position 3 are rare. *E3-14:Ac* has been identified only from *Symmetrischema tangolias* (Gelechiidae) (Griepink et al., 1995) and *H. hippophaecolus* (Cossidae) (Fang et al., 2005) as a sex pheromone component, and also has been reported as a sex attractant for two gelechiid moths (Priesner, 1987; Tóth and Doolittle, 1992) and a cossid moth (Doolittle and Solomon, 1986). *Z3-14:Ac* has been identified only from two cossid moths, *H. insularis* (Zhang et al., 2001) and *H. hippophaecolus* (Fang et al., 2005), and has been reported as a sex attractant for several gelechiid species (Priesner, 1987; Willemse et al., 1987).

A cossid moth, *H. insularis* Staudinger, occurs in China where the larvae inflict serious damage to broad-leaved trees (Wang and Zhang, 1993). The genus *Holcocerus* was established as a subgenus of *Cossus* Fabricius, 1793 (Fletcher and Nye, 1982). However, Inoue (1987) considered it doubtful whether to separate *Holcocerus* Staudinger, 1884 from *Cossus* Fabricius, 1793 as a different genus. He did not conclude that *H. insularis* was a synonym of *C. insularis* because he had not checked the type species, *Cossus nobilis* Staudinger, 1884. Because the male genital structures of *C. insularis* from Japan and *H. insularis* from China are nearly identical (Yoshimatsu, personal communication), these moths are probably the same species, but this remains to be conclusively demonstrated. At the present time, therefore, we take the position that *C. insularis* and *H. insularis* are different species, meaning that the sex pheromone of *C. insularis* has been identified for the first time in the present study.

Furthermore, the sex pheromone identified from *H. insularis* was a 51:39:10 blend of *Z3-14:Ac*, (*E*)-3-tetradecen-1-ol, and (*Z*)-3-tetradecen-1-ol, but *E3-*

14:Ac, the main pheromone component of *C. insularis*, was not positively identified (Zhang et al., 2001). *E*3-14:Ac has been identified as a sex pheromone component from another cossid moth, *H. hippophaecolus*, but it attracted male moths only when it was combined with (*Z*)-7-tetradecenyl acetate in a 1:1 ratio (Fang et al., 2005). Thus, our study is the first to demonstrate *E*3-14:Ac as an attractive pheromone component by itself. The Δ 3-14:Ac motif has appeared in several closely related species in *Cossus* and *Holcocerus*, but the role of the isomers differed between species, as described above. It will be intriguing to study sex pheromones in other *Cossus* or *Holcocerus* species to understand the phylogenetic relationships among these genera.

Because the larvae of many forest insect species, especially wood borers, have long life cycles and low emergence rates even when reared on artificial diet (e.g., Ohya and Ogura, 1993), it is not easy to obtain sufficient numbers of adults for pheromone research. *C. insularis* takes about 6 months to complete its life cycle when reared on artificial diet, and the survival rate is not high (Chen, unpublished), resulting in a limited number of adult moths emerging. Thus, conventional extraction methods used with other lepidopteran species were not practical. In the present study, female sex pheromone components could be collected repeatedly from the same individual by SPME, demonstrating the value of SPME for collecting volatiles repeatedly from organisms that are available only in limited quantities.

Agelopoulos and Pickett (1998) mentioned that the ratio of compounds such as pentan-3-ol, 4-penten-1-ol, hexan-1-ol, 6-methyl-5-hepten-2-one, hexyl acetate, and limonene in samples collected by SPME can deviate markedly from samples collected by syringe or porous polymers, and that for estimation of proportions, SPME should be used in conjunction with another sampling method. However, the results presented here from the live females and the standard synthetic compounds show that SPME sampling can be used in the determination of the relative mixture ratios of geometric isomers because the vapor pressures of the isomers and their absorption to polydimethylsiloxane do not differ.

Three species of *Cossus* (*C. cossus orientalis*, *C. ezoensis*, and *C. insularis*) occur in Japan (Tadauchi and Inoue). The larvae of these species feed on broad-leaved trees including poplar and willows. However, the previous reports on the genus *Cossus* in Japan may have confused these three species because of a poor understanding of their life histories, distributions, and host plants (Enda, 1994). It is now possible to document the distribution, phenology, and abundance of *C. insularis* using the sex pheromone identified in the present study.

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ATTRACTION OF *Piezodorus hybneri* TO THE
AGGREGATION PHEROMONE COMPONENTS
OF *Riptortus clavatus*

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Abstract—The aggregation pheromone of *Riptortus clavatus* (Heteroptera: Alydidae) consists of (*E*)-2-hexenyl (*Z*)-3-hexenoate (E2HZ3H), (*E*)-2-hexenyl (*E*)-2-hexenoate (E2HE2H), and myristyl isobutyrate (MI). Experiments were conducted to examine the attraction of *Piezodorus hybneri* (Heteroptera: Pentatomidae) to the pheromone components of *R. clavatus*, individually and in combination, and to determine if *P. hybneri* produces these compounds. Electroantennogram (EAG) responses of *P. hybneri* to E2HE2H were significant in a dose dependent manner, while those to E2HZ3H were not significantly different from those to hexane and air. Field trapping tests using E2HZ3H, E2HE2H, and MI, individually or in combination, showed that only E2HE2H was attractive to *P. hybneri*. E2HE2H was not detected in whole-body extracts or volatile collections from either sex of *P. hybneri* adults. We propose that the response of *P. hybneri* adults to the *R. clavatus* aggregation pheromone may be related to finding suitable host plants.

Key Words—Pentatomidae, Alydidae, bean bug, (*E*)-2-hexenyl (*Z*)-3-hexenoate, (*E*)-2-hexenyl (*E*)-2-hexenoate, myristyl isobutyrate, aggregation pheromone, soybean.

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INTRODUCTION

A number of stink bug species are important pests of soybean, sweet persimmon, citrus, grapevine, and rice production in Korea. *Riptortus clavatus* (Thunberg) (Heteroptera: Alydidae) and *Piezodorus hybneri* (Gmelin) (Heteroptera: Pentatomidae) are the most important pests of soybean in Korea (Son et al., 2000), while *P. hybneri* is a key pest in Japan (Higuchi, 1999; Osakabe and Honda, 2002; Endo et al., 2003) and Australia (Brier, 1993).

Adult *R. clavatus* males release a pheromone consisting of (*E*)-2-hexenyl (*Z*)-3-hexenoate (E2HZ3H), (*E*)-2-hexenyl (*E*)-2-hexenoate (E2HE2H), and myristyl isobutyrate (MI) in a ratio of 1:5:1 (Leal et al., 1995). This blend attracts conspecific nymphs and adults of both sexes, as well as the egg parasitoid, *Ooencyrtus nezarae* Ishii (Numata et al., 1990; Leal et al., 1995; Mizutani et al., 1997, 1999; Masuta et al., 2001; Huh and Park, 2005).

We caught a large number of *P. hybneri* in traps when evaluating the response of *R. clavatus* to different blends of its synthetic aggregation pheromone components in the field. Endo et al. (2003) reported similar findings in Japan and suggested that E2HE2H was responsible for the attraction. Higuchi (1999) observed that live *P. hybneri* males attracted both males and females, suggesting the presence of an aggregation pheromone based on the attraction. However, the male produced pheromone in *P. hybneri* is a sex pheromone consisting of β -sesquiphellandrene, (*R*)-15-hexadecanolide, and methyl 8-(*Z*)-hexadecenoate (ratio: 10:4:1) (Leal et al., 1998). Therefore, we conducted detailed experiments to gain insights into this intriguing phenomenon of cross attraction by examining the response of *P. hybneri* to the three components of the *R. clavatus* pheromone, individually and in combination, as well as determining if *P. hybneri* actually produces them.

METHODS AND MATERIALS

Insects Used for GC Analyses and EAG Recordings. A colony of *P. hybneri* was started from individuals captured in traps baited with aggregation pheromone of *R. clavatus* in soybean fields in Sacheon, Korea in the autumn of 2002. The colony was maintained at $25 \pm 2^\circ\text{C}$, $50 \sim 70 \pm 10\%$ R.H., under a 16:8 L-D photoperiod. Insects were regularly provided with fresh water, soybeans, and peanuts.

Syntheses of Pheromone Components. E2HZ3H and E2HE2H were easily prepared from commercially available (*Z*)-3-hexenoic acid and (*E*)-2-hexenoic acid, respectively. They were chlorinated with oxalyl chloride, then esterified with (*E*)-2-hexenol to give 75% and 96% overall yield, respectively. The MI

was obtained through esterification of isobutyryl chloride with myristol with 80% yield. The reactions were carried out in oven-dried glassware under nitrogen and anhydrous solvents. ^1H and ^{13}C -NMR experiments were conducted on Bruker AW-500 spectrometer. HREIMS was obtained on a JEOLJMS-700 mass spectrometer. IR spectral data were obtained on Bruker IFS66 spectrometer. Gas chromatography (GC) analysis was performed on a GC-14A (Shimadzu, Japan) using a Supelco wax 10 column (60 m \times 0.32 mm; 0.25 μm). The oven temperature was programmed at 80°C for 5 min, increased to 120°C at a rate of 20°C/min, held at this temperature for 30 min, increased to 260°C at a rate of 4°C/min, held for 50 min. Details of NMR, IR, and HREIMS spectroscopic assignments for the three compounds are described in a previous paper (Huh et al., 2005).

Preparation of Pheromone Lures. The synthetic pheromone components were diluted in hexane (97%, Merck, Germany) at the required ratios for each experiment. Rubber septa (13 mm O.D., Sigma-Aldrich, Germany) were filled with 2 mg of the test solutions, placed in a fume hood for around 3 hr for absorption of the chemical(s) and evaporation of hexane, then packed in polyethylene lined foil pouchs (6.5 \times 10 cm, Green Agro-Tech, Korea), and stored at 4°C until used. Rubber septa impregnated with hexane only served as controls.

Field Bioassays. Two field experiments were carried out in 3 ha of soybean field in Sacheon city, Korea. Traps (17 cm O.D., 33 cm long), similar to gee minnow trap (BioQuip, USA) with netted funnel inlets at both sides, were mounted on poles 1 m above ground, and set 12 m apart. Three traps were employed in each treatment.

*Effect of MI on Attractiveness of *P. hybneri*.* This trial was done from September 7 to October 4, 2002, testing MI alone at 1, 10, 50, and 100 mg per septum or together with E2HZ3H and E2HE2H (at 7 and 36 mg, respectively) at 0.1, 1, 7, and 50 mg. The ratio of E2HZ3H and E2HE2H (1:5) was based on the results of Leal et al. (1995). This experiment was repeated 7 times and the number of *P. hybneri* captured were counted and sexed every three days. At the same time, the placement of traps was interchanged randomly.

*Attractiveness of E2HZ3H and E2HE2H to *P. hybneri* Adults.* The attractiveness of E2HZ3H + E2HE2H (mg) at ratios of 10 + 0, 0 + 50, and 10 + 50 were tested from August 2 to August 29, while 10 + 0, 0 + 50, and 50 + 50 mixtures were tested from August 30 to September 19, 2004. Hexane and ten live males of *R. clavatus* were used as blank and positive control, respectively. Trials were repeated 4 and 3 times, respectively, with counts and trap rotation being carried out once a week.

*EAG Responses of *P. hybneri* to E2HZ3H and E2HE2H.* The antennae of 3–4 wk old males and females reared in a group were used. The recording and gold reference electrodes were connected with electrode gel (Spectra 360,

PARKER, USA) to the distal antennal segment and the base of the antenna, respectively. Based on the results of preliminary experiments we tested 0 + 2, 0 + 5, 2 + 0, 2 + 2, 2 + 5, 5 + 0, 5 + 2, and 5 + 5 mg of E2HZ3H and E2HE2H applied to a piece (2 mm×50 mm) of filter paper (Toyo No. 2), with a filter paper strip alone and another strip treated with 20 µl hexane as blank controls. One strip of the test filter paper was inserted into a glass Pasteur pipette (15 cm long) that was inserted into a glass tube (10 mm O.D., 18 cm long) positioned 10 mm from the antennal preparation. An air stimulus controller (CS-05, Syntech, the Netherlands) was used for air and odor delivery at a constant flow of 2 l/min. Stimulations, from the lowest to the highest dose, each lasted for 1 sec at 20 sec intervals. EAGs to air and hexane were recorded before the test compounds and then after every 8 recordings. Pasteur pipettes, filter paper strips, and antenna were changed after 3 sets of 10 stimuli.

GC Analyses. *P. hybneri* whole body extracts were obtained from either 4 males or females (12–16-d old virgins) by immersing them in 1 ml hexane for 3 min. One µl of the extract, was submitted to GC analysis and compared with those of 1 µl of laboratory-synthesized E2HZ3H, E2HE2H, and MI (each at 0.05 µl per ml hexane). In all cases, the test materials contained 0.0001 µl *trans*-2-hexenyl acetate as an internal standard (IS). This analysis was triplicated.

Volatile collections were taken from either 4 virgin males or females (all 12–16-d old), using solid phase microextraction (SPME). The bugs were anesthetized by CO₂, placed in a 100 ml glass vial with five soybeans. After they had recovered, the vial was sealed with parafilm. All volatile collections were done from 10–13 hr after the photophase by inserting the SPME fiber [Supelco, Bellefonte, PA, USA: blue type polydimethylsiloxane/divinylbenzene (PDMS/DVB) 65-µm film thickness] into the vial for 10 min. The sample was immediately analyzed by introducing the fiber into the heated injector for 2 min. The volatiles from 20 µl of synthetic E2HZ3H, E2HE2H, and MI (0.5 µl each per ml hexane) on filter paper were collected using the same protocol. Twenty µl of IS solution were dropped on a separate strip of filter paper in the vial which contained *P. hybneri* adults or pheromone compounds.

Samples were analyzed by gas chromatography (GC: Shimadzu 17-A, Japan) equipped with DB-wax capillary column (30 m×0.25 mm; 0.25 µm) and RTx-225 capillary column (60 m×0.25 mm; 0.25 µm) for whole body extract analyses, and DB-wax capillary column for volatile collection analyses. The GC was operated in splitless mode. The oven temperature was programmed at 50(1)-210(10)/10 for DB-wax column, and at 80(2)-150(1)/15-200(3)/10-210(10)/5 for RTx-225 column.

Statistical Analyses. Data for EAG response and the transformed [$\log(x + 1)$] trap catches were analyzed by ANOVA followed by Duncan's multiple range test (DMRT) (SAS Institute, 1998).

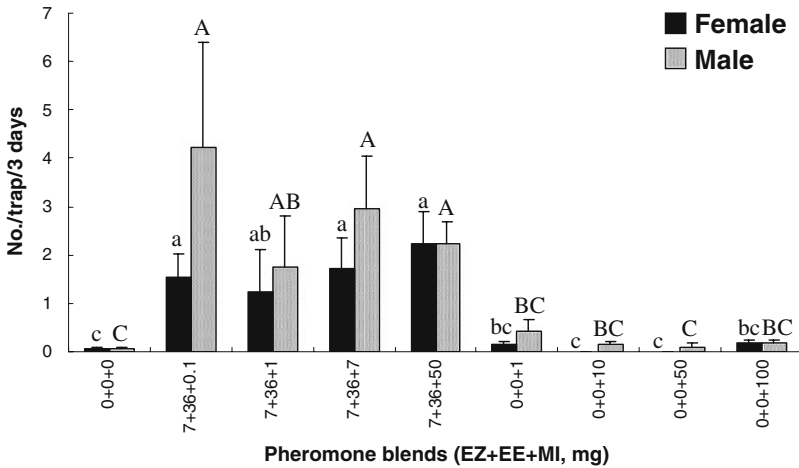


FIG. 1. Number (mean ± SE) of *Piezodorus hybneri* adults captured in pheromone traps baited with different concentrations of MI in the presence and absence of E2HZ3H and E2HE2H. Bars with the same upper or lower cases are not significantly different (Duncan’s multiple range test at $\alpha = 0.05$).

RESULTS

Effect of MI on Attractiveness of P. hybneri in the Field. A total of 147 females and 254 males were captured during the entire experimental period. Interestingly, all the females were non-reproductive (i.e., they did not have any developed eggs in their ovaries). Generally, the tertiary blends of E2HZ3H,

TABLE 1. NUMBER (MEAN ± SE/WEEK) OF MALE AND FEMALE *Piezodorus hybneri* CAPTURED IN PHEROMONE TRAPS BAITED WITH SINGLE COMPONENTS OR BINARY BLEND OF E2HZ3H AND E2HE2H

E2HZ3H + E2HE2H (mg)	1st trial		2nd trial	
	Female	Male	Female	Male
Hexane	0.0 ± 0.0 d ^a	0.0 ± 0.0 c	0.3 ± 0.3 b	0.5 ± 0.3 b
10 + 0	0.0 ± 0.0 d	1.0 ± 0.6 bc	0.3 ± 0.3 b	0.5 ± 0.5 b
0 + 50	9.3 ± 3.0 a	10.7 ± 3.7 a	12.5 ± 6.6 a	8.3 ± 3.7 a
10 + 50	4.0 ± 0.6 b	3.0 ± 1.2 b	—	—
50 + 50	— ^b	—	4.0 ± 1.6 a	5.3 ± 1.1 a
Ten live <i>R. clavatus</i> males	1.0 ± 0.0 c	0.0 ± 0.0 c	0.5 ± 0.5 b	1.0 ± 0.7 b

^a Means followed by the same letters within a column are not significantly different (Duncan’s multiple range test at $\alpha = 0.05$).

^b No treatments.

E2HE2H, and MI attracted higher number of females ($df = 16,8$; $F = 6.11$; $P = 0.001$) and males ($df = 16,8$; $F = 6.74$; $P < 0.001$) than those baited with MI alone or the control (Figure 1). Increasing the concentration of MI did not affect the attractiveness of the blends (Figure 1).

Attractiveness of E2HZ3H and E2HE2H to P. hybneri Adults in the Field. There was a significant effect of treatment on the two sexes in both trials (females, $df = 12,4$; $F = 8.92$; $P = 0.001$ and $df = 8,4$; $F = 57.17$; $P < 0.001$; males, $df = 12,4$; $F = 7.39$; $P = 0.003$ and $df = 8,4$; $F = 15.74$; $P < 0.001$) (Table 1). E2HE2H alone always attracted the highest number of females while

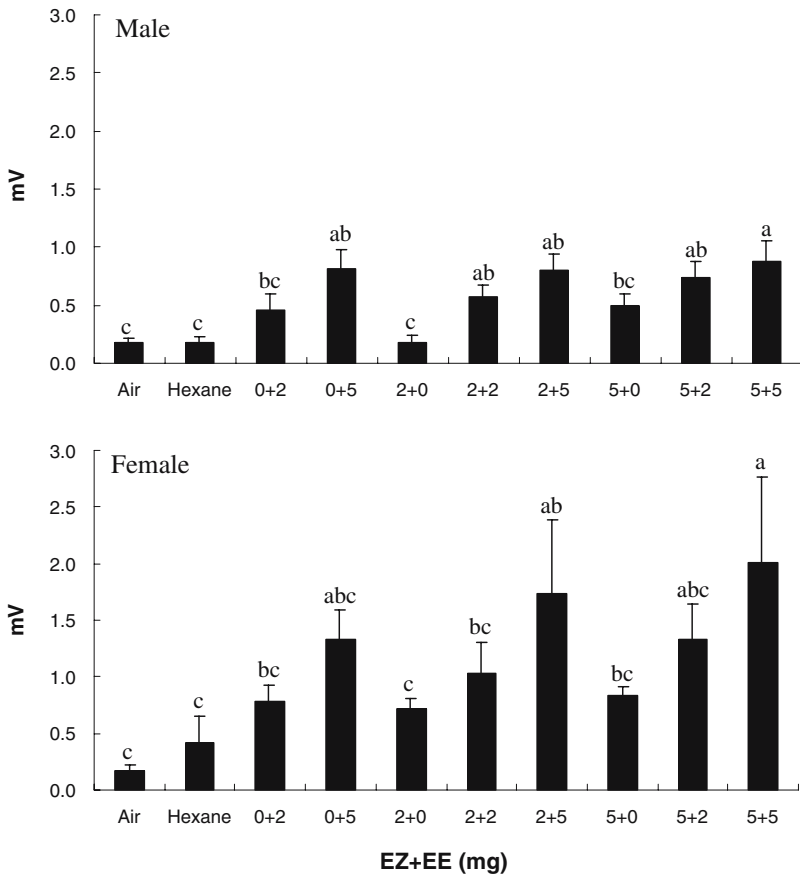


FIG. 2. EAG responses (mean \pm SE) of antennae from male and female *Piezodorus hybneri* adults to blends of E2HZ3H and E2HE2H. Bars with the same letters are not significantly different (Duncan's multiple range test at $\alpha = 0.05$).

E2HZ3H alone was no more attractive than the controls. The addition of 10 or 50 mg of E2HZ3H to E2HE2H did not increase trap catches, and resulted in a decrease in the number of bugs caught in trail 1 (Table 1).

EAG Responses of P. hybneri to E2HZ3H and E2HE2H. There were effects of increasing amount of E2HE2H on both males ($df = 30,9; F = 5.41; P < 0.001$) and females ($df = 30,9; F = 2.48; P < 0.03$), with the latter showing somewhat stronger responses ($N = 40, t = -4.545, P < 0.001$). In the case of males 5 mg of E2HE2H alone and all binary combinations gave responses significantly higher than the controls, while in females only the 2 + 5 and 5 + 5 blends of E2HZ3H and E2HE2H did so (Figure 2).

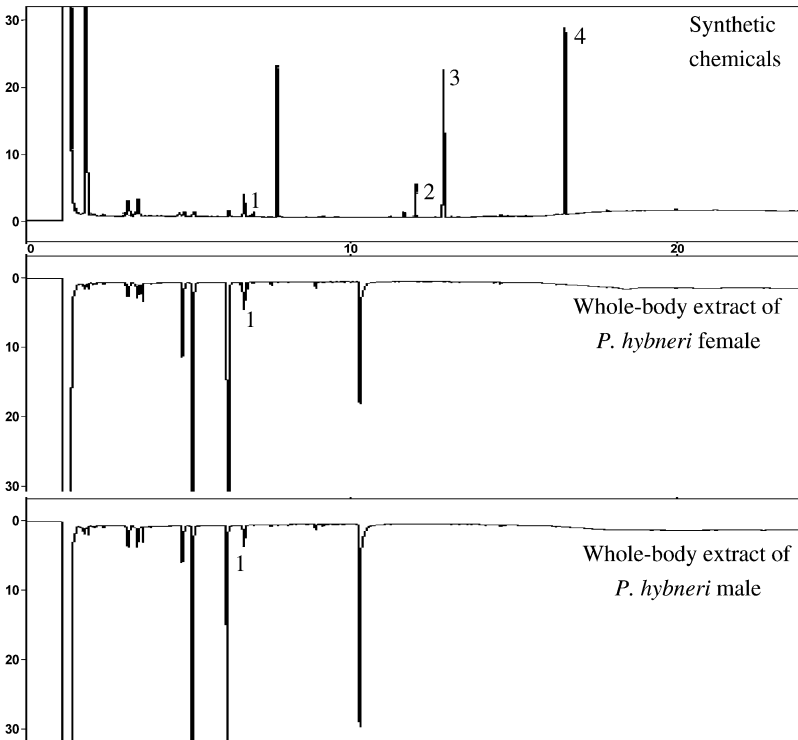


FIG. 3. Chromatograms of synthetic E2HZ3H, E2HE2H, MI, and whole body extracts of *Piezodorus hybneri* adult females and males. The retention times of internal standard (no. 1), *trans*-2-hexenyl acetate, were 6.645, 6.647, and 6.664 min in upper, middle, and lower graphs, respectively. The retention times of E2HZ3H, E2HE2H, and MI (no. 2, 3, and 4) were 11.922, 12.769 and 16.505 min, respectively.

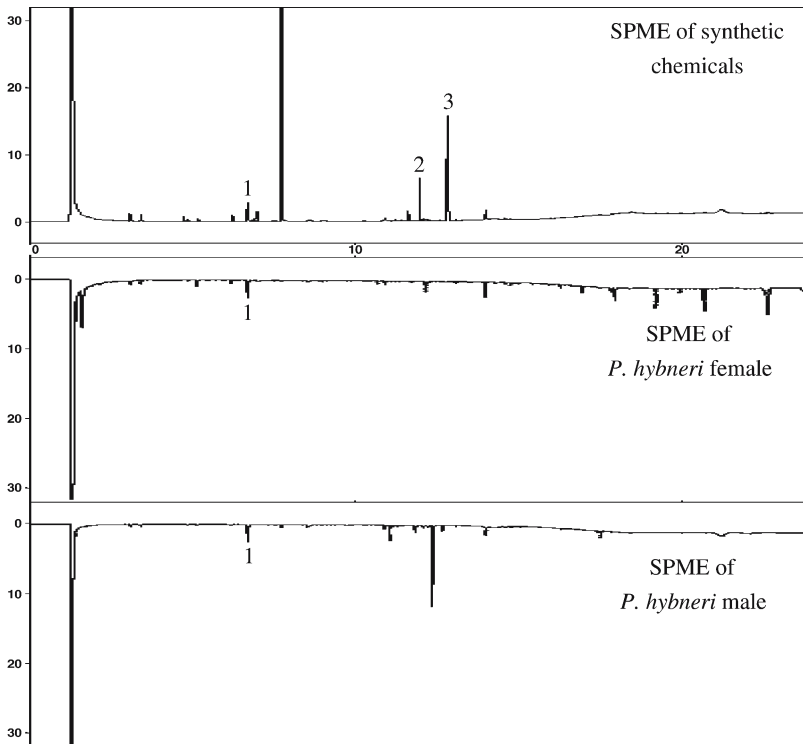


FIG. 4. Chromatograms of volatiles from synthetic E2HZ3H and E2HE2H, and adult *Piezodorus hybneri* females and males, using SPME. The retention times of internal standard (no. 1), *trans*-2-hexenyl acetate, were 6.630, 6.629, and 6.635 min in upper, middle, and lower graphs, respectively. The retention times of E2HZ3H and E2HE2H (no. 2 and 3) were 11.916 and 12.763 min, respectively.

GC Analyses of P. hybneri Adults. None of the three compounds of the *R. clavatus* aggregation pheromone were detected in either the whole body extracts (Figure 3) or the volatiles (Figure 4) collected from *P. hybneri* females and males.

DISCUSSION

The results of the field trials show that *P. hybneri* adults are attracted to E2HE2H, but not to either MI or E2HZ3H. Furthermore, adding MI or E2HZ3H to E2HE2H did not increase the number of insect captured. Endo et al. (2003)

also reported that neither MI nor E2HZ3H were attractive to *P. hybneri*, even though MI increased the attractiveness of E2HZ3H and E2HE2H to *R. clavatus* (Mizutani et al., 1997; Endo et al., 2005; Huh et al., 2005).

The attraction of *P. hybneri* to E2HE2H, a component of the *R. clavatus* aggregation pheromone, is intriguing. There are a number of examples of different hemipteran species having some of the same components in their pheromone blends (Kitamura et al., 1984; Aldrich et al., 1993; Borges et al., 1999), so lures containing a single common component could attract adults of several species. However, this is not the case for *P. hybneri* and *R. clavatus*, as neither sex of *P. hybneri* produce E2HE2H. One alternate explanation is that E2HE2H could be a component of the volatiles produced by host plants. However, this seems somewhat unlikely as soybean and horse bean seeds, common food sources for these two bug species, do not produce this compound (Leal et al., 1995).

As both *P. hybneri* and *R. clavatus* share a number of host plants, we postulate that the response of *P. hybneri* to one component of the *R. clavatus* aggregation pheromone is an adaptation that increases the probability of finding suitable host plants. If this is true, then one can expect that traps baited with *R. clavatus* males will capture *P. hybneri* adults, but this was not the case (Table 1). However, in another experiment run August 2 to November 22, 2004, traps baited with *R. clavatus* caught 19 female and 14 male *P. hybneri* adults, while the control traps captured none. Why there is such variability in the attractiveness of *R. clavatus* for *P. hybneri* is unknown, and further research is required, examining factors such as the physiological state of males in traps and the actual densities of *P. hybneri* at the sites. It does not seem likely that the differences are related to the physiological status of *P. hybneri*. The majority of females we captured in traps baited with E2HE2H from September 7 to October 4 in Jinju (latitude, about 35° 2') were sexually immature, suggesting they were in reproductive diapause. In their study from July 29 to August 12 in Kumamoto (latitude, about 32° 8'), Endo et al. (2003) captured both of *P. hybneri* adults and nymphs, and it is likely that the adults captured were sexually mature, because diapausing individuals usually appear around mid September at this location (Higuchi, 1994).

There are a number of studies showing that hemipteran parasitoids (Leal et al., 1995; Masuta et al., 2001; Huh and Park, 2005) or predators (Zegelman et al., 1993; Mendel et al., 1995) use host pheromones as kairomones to locate suitable prey/hosts. However, we believe this is the first suggestion that the aggregation pheromone of one herbivorous bug may serve as a kairomone for a second herbivorous species. It has been shown that *Halyomorpha mista* (=halys) was attracted to traps baited with aggregation pheromone [methyl (E,E,Z)-2,4,6-decatrienoate] of *Plautia stali* in Japan (Adachi, 1998) and in Korea (Lee et al., 2002) but this may result from shared pheromone components

(the chemical communication system of *H. mista* has not been studied to date) rather than *H. mista* using the aggregation pheromone of *P. stali* as a kairomone. The possibility of one herbivorous species using the aggregation pheromone of another, sympatric one, to locate suitable host plants merits further attention for truth. The multi-species management systems could be developed, such as using E2HE2H to control both *R. clavatus* and *P. hybneri*.

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DIMETHYLFURAN-LACTONE PHEROMONE FROM MALES OF *Galerucella californiensis* AND *Galerucella pusilla*[†]

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Abstract—Male *Galerucella californiensis* and *Galerucella pusilla* (Coleoptera: Chrysomelidae) emit an aggregation pheromone while feeding on host foliage. Isolation of the compound from collected volatiles was guided by comparisons of gas chromatograms of extracts from males and females and by gas chromatography–electroantennographic detection. The compound was identified by a combination of spectrometric methods and microchemical tests as the novel dimethylfuran lactone, 12,13-dimethyl-5,14-dioxabicyclo[9.2.1]-tetradeca-1(13),11-dien-4-one. The structure was confirmed by synthesis, and the synthetic compound attracted males and females of both species in field bioassays. These beetles were previously introduced into North America as biological control agents for the invasive wetland weed, purple loosestrife *Lythrum salicaria*, and the pheromone could become a tool for monitoring populations. A new method is described for distinguishing the two species based on the tibial spurs of the males.

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[†] Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Key Words—*Galerucella californiensis*, *Galerucella pusilla*, Coleoptera, Chrysomelidae, purple loosestrife, *Lythrum salicaria*, biological control, lactone, synthesis, attraction, aggregation pheromone.

INTRODUCTION

Galerucella californiensis (L.) and *Galerucella pusilla* (Duftschmidt) are leaf beetles (Coleoptera: Chrysomelidae) that were introduced into the United States from Europe as biological control agents for the invasive wetland weed, purple loosestrife *Lythrum salicaria* L. (Hight et al., 1995). The adults are small (3.5–5.5 mm) and brownish with dark markings; the species and sexes are nearly identical in appearance. The insects consume the foliage of purple loosestrife, both as adults and as larvae. For biocontrol purposes, methods of monitoring release sites for beetle establishment, abundance, seasonal timing, dispersal rates, and other population properties are required. Pheromone-baited traps could be sensitive and selective tools for acquiring such information. Observations of aggregation behavior in these beetles suggested that a pheromone exists (Grevstad and Herzig, 1997), but none had been chemically identified.

Existing knowledge about chrysomelid pheromones gave little guidance on *Galerucella* spp. because of the considerable chemical and biological diversity within the family. Pheromones of the rootworm beetles (*Diabrotica* spp.) are female produced and attract only males; they consist of esters of methyl-branched secondary alcohols and structurally related ketones (reviewed in Krysan et al., 1989). The more recently identified chrysomelid pheromones are all male produced and attract both sexes. These include a hydroxyketone from the cereal leaf beetle *Oulema melanopus* L. (Cossé et al., 2002; Rao et al., 2003), a dihydroxyketone from the Colorado potato beetle *Leptinotarsa decemlineata* Say (Dickens et al., 2002), a series of himachalene and cadinene sesquiterpenes from *Aphthona* and *Phyllotreta* flea beetles (Bartelt et al., 2001, 2003; Muto et al., 2004; Soroka et al., 2005), and a 7-carbon diene aldehyde and alcohol from *Diorhabda elongata* Brullé (Cossé et al., 2005).

An approach was chosen that would detect pheromone emission by either sex. Volatiles were collected from male and female beetles separately and analyzed for sex-specific compounds that elicited strong antennal responses in gas chromatography–electroantennographic detection (GC–EAD) studies as possible pheromone components. Only one such compound was found from *G. californiensis* (from males), and it was subsequently found from *G. pusilla* males also. We report here the identification, synthesis, and field testing of the pheromone, which was attractive to both sexes of each species. Structurally, the compound is unlike any of the previously identified chrysomelid pheromones.

METHODS AND MATERIALS

Insects. *G. californiensis* were reared on potted purple loosestrife plants in a greenhouse at the Illinois Natural History Survey. Adults for volatile-collection efforts in early spring were obtained from a stock of beetles that had diapaused in cold storage over the winter, and in late spring or summer, adults were collected as they emerged from pupation in the soil. The sexes of *G. californiensis* were separated based on the characteristic tibial spurs on the meso- and metathoracic legs of males (Cossé, 2004). Early in the project, when it was unknown which sex emitted a long-range pheromone, adults were placed individually into vials as they emerged from overwintering diapause or from pupation to increase the likelihood that they were unmated when used for collection of volatiles. This precaution was taken because in another chrysomelid, *Diabrotica virgifera*, emission of the female-produced sex pheromone ceased upon mating (Bartelt and Chiang, 1977). Once evidence emerged for a male-produced pheromone in *G. californiensis*, the time-consuming procedure of isolating beetles as they emerged was discontinued. In chrysomelids with male-produced pheromones (e.g., *Phyllotreta cruciferae*, *Aphthona* spp., and *Diorhabda elongata*), pheromones were collected from freely mating, field-collected males (Bartelt et al., 2001; Cossé et al., 2005).

During summer of 2003, *G. pusilla* became available from a population in Minnesota, allowing collection of volatiles and electrophysiology to be conducted for this species also. *G. pusilla* were also studied under the microscope for sexually dimorphic characters and differences from *G. californiensis* that would allow identifications to be made without injuring the beetles. Additional field-collected *G. pusilla* were obtained during summer of 2005 for volatile collections.

Collection of Volatiles. Volatiles were collected from *G. californiensis* during 2002–2004 at the National Center for Agricultural Utilization Research (NCAUR). Initial collections were made from both males and females feeding on purple loosestrife foliage and from foliage alone. Once it became clear that a male-produced pheromone was probable, subsequent collections were made almost entirely from males, accumulating material for identification. In 2002, 122 1- to 3-d collections were made from feeding males, 60 were made from feeding females, and 8 were made from plants only. In 2003, 567 1-d collections were made from males, and 23 from females. In 2004, 117 1-d collections were made, all from males. For *G. pusilla*, 50 1-d collections from males and 21 from females were made during 2003, and 100 1-d collections from males and 40 from females were made during 2005.

The conditions and apparatus for collecting beetle-produced volatiles evolved during the course of this study. Details for the most successful procedure (used in 2004 for *G. californiensis*) are described here: The body of

the collector apparatus was a 45-cm-long by 3-cm-diameter straight glass tube with a female 24/40 ground glass joint on each end. A glass adapter was fitted into each end. (These 9-cm-long adaptors, suitable for holding thermometers or objects of similar diameter, have a male 24/40 joint on one end and a threaded fitting with an airtight O-ring seal on the other, Ace Glass, Vineland, NJ, USA #5028-30). Glass filter tubes (6 mm O.D. by ca. 8 cm length) containing a 1-cm plug of Super-Q were prepared as described previously (Cossé et al., 2002) and inserted into each adapter.

Each aeration chamber contained a shoot of purple loosestrife foliage about 30 cm in length after the growing tip was removed, obtained from potted plants maintained in the NCAUR greenhouse. Leaves were detached from the shoot until only three or four remained, each about 5–8 cm in length, well spaced along the stem. The base of the stem was placed in a 5-ml glass vial containing water. A Teflon seal in the ring-shaped cap of the vial kept the water from spilling. Beetles (usually 5–10) were added to the collector, which was then oriented horizontally. This configuration provided ample, but not excessive, food for the beetles for 1 d; using only this amount of foliage helped minimize the background levels of plant-related chemicals in the volatile collections. The beetles were not overcrowded and were able to move about on the plants in a natural manner.

Clean air was pulled through the aeration chamber and volatile trap at 300 ml/min. This rate was rapid enough that transpired water from the leaves did not condense on the walls of the tubes (the target compound was almost never detected when water droplets were visible). An inlet filter cleaned the entering air, and the outlet filter captured the volatiles emitted by the insects and plants. The collectors were located in an environmental chamber at 27°C, ~50% RH, and with a 17:7 (L:D) photoperiod, chosen to maintain the beetles physiologically in a reproductive state (Velarde et al., 2002). Light was provided by four 40-W fluorescent tubes about 1 m above the collectors.

Traps were changed each morning, eluting the traps with 400 µl methylene chloride. The beetles were transferred to clean collectors with fresh foliage, and the collection process was restarted. Maintenance was done daily because amounts of the target compound decreased if foliage was not fresh, and the chemical “background” in the samples increased when the glassware was not cleaned frequently. This procedure was repeated as long as the beetles survived (usually > 30 d). Each collection was analyzed by gas chromatography–mass spectrometry (GC–MS) and then stored in a freezer (–70°C) until it was processed further.

For *G. pusilla*, the procedure in place during 2003 (when the initial collections were made from this species) employed groups of up to 50 males or females held in glass tubes of ca. 8 cm diam and 100 cm length, and the foliage amounts were greater. However, the 2005 collections from *G. pusilla* used the optimum procedure described above for *G. californiensis*.

Electrophysiology. Samples from feeding male or female beetles of both species were analyzed by GC–EAD. The effluent from the GC column was split to a flame ionization detector (FID) and to the antenna of a male or female beetle. A glass pipette Ag/AgCl grounding electrode was inserted into the back of an excised beetle head. A second pipette serving as the recording electrode was placed in contact with the distal end of one antenna. Both pipettes were filled with physiological saline. GC and EAD data were analyzed by Syntech (Hilversum, the Netherlands) software, using previously described methods and equipment (Cossé and Bartelt, 2000).

Gas Chromatography–Mass Spectrometry. Coupled GC–MS was used to analyze all extracts (EI mode, 70 eV), using a Hewlett Packard 5973 mass selective detector, interfaced to an HP 6890 GC, controlled with Agilent ChemStation software (Release C.00.00), and with the Wiley spectral library with 275,000 spectra. A DB-1 column (30 m length, 0.25 mm ID, 0.25 μ m film thickness, J&W Scientific, Folsom, CA, USA) was used, programmed from 50°C/1 min, 10°C/min to 280°C, hold 5 min at 280°C. Other column types were also used. Injections were made in splitless mode, with He carrier gas and inlet and transfer line temperatures of 280°C. Positive chemical ionization (CI) mass spectra were acquired on the same instrument, with either methane or isobutane reagent gas. The high-resolution EI mass spectrum was obtained at the University of Illinois on a VG 70SE instrument with a GC inlet.

Gas Chromatography. Selected samples were analyzed by GC to estimate amounts of material present, using an HP 5890 Series II instrument with FID and column and parameters similar to those used for GC–MS. Quantitation was by the external standard method, relative to alkanes.

Preparation of NMR Sample by HPLC. The *G. californiensis* compound was purified in one step by HPLC, using a Waters 515 pump with a Supelcosil LC-SI silica column (25 cm by 0.46 cm ID, 5 μ m particle size), eluting with 5% ethyl ether (redistilled) in hexane (1 ml/min). The LDC/Milton Roy Spectro-Monitor D variable-wavelength UV detector was operated at 229 nm and was interfaced to an HP 3396 Series III integrator. Volatile collections were combined and concentrated under a stream of argon (three batches, each concentrated to about 500 μ l). HPLC injections were 100 μ l. The target compound eluted between 10 and 11 min and was essentially free of impurities, by GC–MS. Finally, the combined fractions were taken to dryness under a stream of nitrogen, and the compound was quickly taken up in 200 μ l deuterobenzene. The sample for NMR contained 17 μ g.

NMR Spectroscopy. NMR spectra were acquired at NCAUR on a Bruker Avance 500-MHz instrument with a 5-mm inverse broadband probe with a Z-gradient. Samples were run in a Bruker Microbore capillary-end tube (Wilma-Labglass, Buena, NJ, USA #520-1A). The experiments conducted included 1D 1 H NMR and 2D 1 H COSY and HMBC.

Additional NMR spectra were acquired on this sample at Pfizer on a Bruker 500-MHz instrument equipped with a Bruker Dual CryoProbe™. The sample was transferred to a Shigemi tube, and 1D ¹H NMR, ¹³C proton decoupled, DEPT135, and DEPT90 experiments and the 2D HMBC, HSQC, and HSQC-TOCSY experiments were conducted.

UV Spectroscopy. The UV spectrum was obtained on an HP 1040A diode array detector, using the Waters HPLC system described above. A quantitated sample was also run in a 1-cm cuvette on a Shimadzu UV-1601PC spectrophotometer to measure the extinction coefficient.

Microchemical Tests. Hydrogenation was conducted over Adam's catalyst (PtO₂) and over 10% palladium on carbon. Samples were dissolved in methylene chloride (ca. 1 μg in 100 μl) in a conical vial, and a small amount of catalyst was added (1 mg or less, barely visible in the vial). Hydrogen was bubbled through the solution from a fine needle for 5 min at room temperature, and then the sample was analyzed by GC-MS.

An aliquot was also reduced with LiAlH₄. The reagent (1 M in ether, Aldrich Chemical Co., Milwaukee, WI, USA) was diluted further with dry ether, and a drop was added to a sample of the beetle compound (ca. 500 ng) in ether. After several minutes, the reaction was quenched with a minimal amount of water and analyzed by GC-MS. Treatment with diazomethane was done essentially as described by Levitt (1960) on the beetle compound and on the standards, heptanoic acid and γ-decalactone.

Synthesis. The synthetic scheme is summarized in Figure 1. Briefly, the strategy was to prepare 3,4-dimethylfuran (**7c**), construct hydroxyl and acyl side chains at the 2 and 5 positions, and then close the lactone ring.

To make 3,4-dimethylfuran, 3,4-bis-(acetyloxymethyl)furan **6** was first prepared by a Diels-Alder reaction between butynediol diacetate **3** (obtained by acetylating diol **2**) and 4-phenyloxazole **5** (made from phenacyl bromide **4** and ammonium formate). The Diels-Alder adduct decomposed spontaneously to **6** and benzonitrile. All of the above reactions were done as described by Hutton et al. (1979). Diacetate **6** was converted to diol **7a** by transesterification with MeOH/MeONa (Christie, 1993). Diol **7a** was converted to dichloride **7b** with thionyl chloride, which was reduced to 3,4-dimethylfuran **7c** with LiAlH₄ (see Rawson et al., 1979, for both of these reactions).

The precursor for the 5-carbon side chain was prepared by converting 1,5-pentanediol **8** to iodohydrin **9a** by refluxing with HI in a two-phase system (Kang et al., 1985). The hydroxyl group of **9a** was protected as the tetrahydropyranyl (THP) ether **9b** (Miyashita et al., 1977). Furan **7c** was smoothly alkylated with **9b** after the 2 position was lithiated with butyllithium, giving trisubstituted furan **10** (Nolan and Cohen, 1981). To prepare the 3-carbon acyl side chain, **10** was first carbonylated to **11** by lithiation of the 5 position, followed by reaction with *N,N*-dimethylformamide (Koenig, 2002). Aldehyde

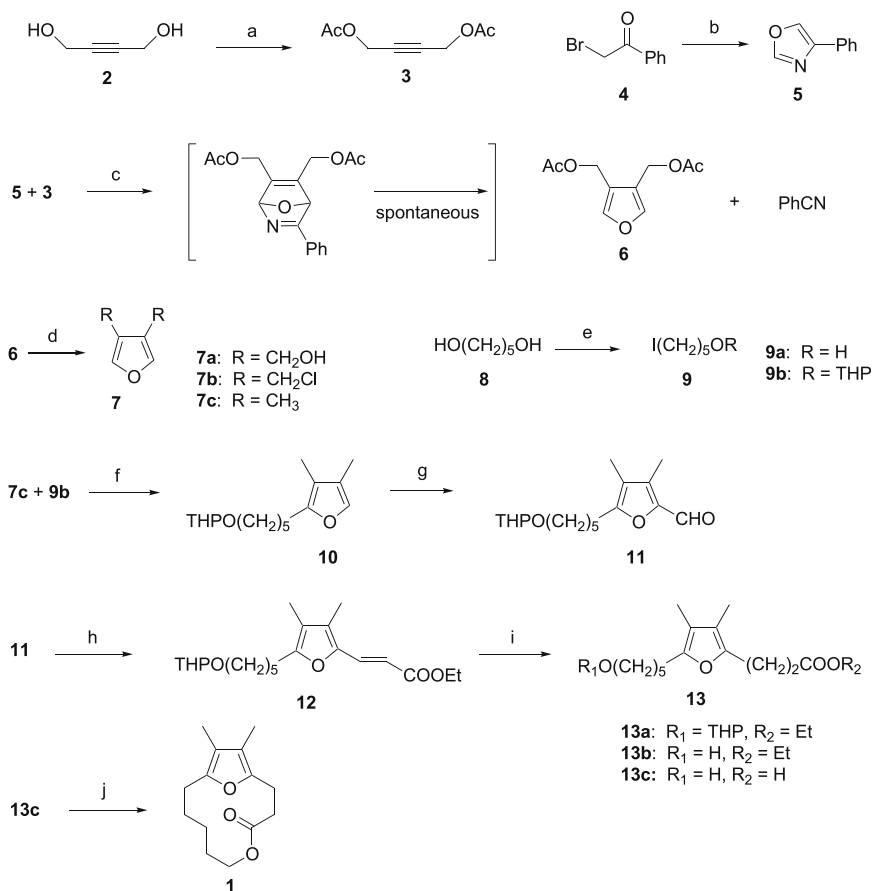


FIG. 1. Synthesis of *Galerucella* furan lactone (**1**). (a) Ac₂O, PTSA, 110–115°C, 3 hr; quant.; (b) ammonium formate, isobutyl formate, formic acid, azeotropic removal of water at 92–94°C, 7 hr; 51%; (c) Na₂CO₃, 205–206°C, 24 hr; 64%; (d) (i) NaOMe (0.2 equiv.), MeOH, rt, 1 hr; quant.; (ii) SOCl₂, pyridine, CH₂Cl₂, –5°C, 15 min; 60%; (iii) LiAlH₄, ether, addition at 0°C, then reflux, 4 hr; 70%; (e) (i) concentrated HI, hexane (two phases), reflux, 4 hr; 35%; (ii) dihydropyran, pyridinium *p*-toluenesulfonate (PPTS), CH₂Cl₂, rt, 4 hr; 86%; (f) addition of BuLi to **7c**, THF, –15°C, 2 hr, followed by addition of **9b**, –15°C, 1 hr, then rt, 1 hr; 62%; (g) BuLi, THF, 0°C, 2 hr, then dimethylformamide, 0°C, 1 hr; 40%; (h) triethyl 2-phosphonoacetate, BuLi, THF, 0°C, 45 min; 40%; (i) (i) 10% Pd on carbon, hexane, H₂ (1 atm), rt, 5 min; 95%; (ii) PPTS, EtOH, 55°C, 2.5 hr; quant.; (iii) KOH, 1:1 MeOH/H₂O, 45°C, 2 hr, by GC reaction complete; (j) 2,4,6-trichlorobenzoyl chloride, Et₃N, THF, rt, 2 hr, then 4-(*N,N*-dimethylamino)pyridine, toluene, reflux, addition over 3.5 hr; 75%.

11 was elaborated to unsaturated ester **12** by a Wittig–Horner condensation with triethyl 2-phosphonoacetate (Boutagy and Thomas, 1974). The side-chain double bond was selectively reduced by hydrogenation over 10% Pd on carbon in hexane to give **13a** (Lie Ken Jie, 1981).

Compound **1** was completed by deprotecting the hydroxyl (Miyashita et al., 1977, giving **13b**) and acyl functions (KOH in aqueous MeOH) and closing the ring. Hydroxy acid **13c** was activated by conversion to the mixed anhydride with 2,4,6-trichlorobenzoyl chloride, followed by cyclization under high-dilution conditions using 4-(*N,N*-dimethylamino)pyridine as the catalyst (Sinha et al., 1993). Purification on silica gel (10% ether in hexane, after 5% ether in hexane) yielded 30 mg of **1** (91% pure).

The synthesis is described in detail in the online supplementary information available for this article, at <http://dx.doi.org/10.1007/s10886-005-9026-3> and is accessible for authorized users.

Field Lures. Red rubber septa (Aldrich) were cleaned by Soxhlet extraction with CH₂Cl₂, and loaded with synthetic **1** (500 µg in 100 µl of hexane). Loaded septa were stored at –20°C until used. One freshly prepared septum was placed into a laboratory volatiles-collection apparatus to determine the emission rate of **1**. Volatiles were collected continuously for 31 d and were quantitated every 1–4 d by GC, using heptadecane as internal standard.

Field Tests. Field tests of synthetic **1** were conducted on May 10, 17, and 18, 2005, in two wetlands in northeastern Illinois (Cook County) known to have infestations of purple loosestrife and populations of *Galerucella* spp. The field sites were at Hyde Lake (41°39.9'N, 87°33.1'W) and Powderhorn Lake (41°38.6'N, 87°32.0'W). The three test days were mostly sunny, with maximum temperatures of ca. 20°C. Beetles were observed on plants at both locations, but the season was slightly more advanced at Hyde Lake: Eggs were easily found at Hyde Lake on May 17, but none were apparent at Powderhorn Lake on that date.

Traps were 15 × 15-cm yellow sticky cards with adhesive on both sides (“Sticky Strips,” Olson Products, Medina, OH, USA, cut in half). These were attached near the tops of bamboo stakes (1.2 m in length). Lures were fastened to the tops of the sticky cards with wire.

Experiments were organized as paired *t* tests, with a treated and a control trap in each pair. Traps were placed near purple loosestrife, but not necessarily in dense stands. Trap positions of a pair were separated by 3–4 m and were chosen to be as similar as possible with respect to height, density, and type of vegetation. Assignment of treatments within trap pairs was randomized. Spacing between pairs was at least 10 m. Overall, 15 pairs of traps were run at Hyde Lake (on May 10 and 17), and 20 pairs were run at Powderhorn Lake (on May 17 and 18).

Traps were deployed in midmorning, before beetles became active, and the experiment was terminated between 3:00 PM and 5:00 PM. Beetle numbers were recorded at the end of the experiment, and all specimens were examined under the microscope for tibial spurs. Samples of males (five of each species from each field site) were dissected to examine genitalia (Manguin et al., 1993).

RESULTS AND DISCUSSION

Identification and Sexing of Beetles. As was found previously with *G. californiensis* (Cossé, 2004), the males of *G. pusilla*, but not the females, have spurs on their mesotibiae. However, analogous spurs were not found on the metatibiae of male *G. pusilla*, as they had been on males of *G. californiensis*. These characters allowed males of the two species to be distinguished from each other and from females. Moreover, these determinations could be done without causing injury (compare to Manguin et al., 1993). Species determinations made using the spur characters were subsequently verified by examination of genitalia at NCAUR and by submission to experts at other institutions. We were unable to confidently separate females of *G. californiensis* and *G. pusilla* from mixed populations using the criteria of Manguin et al. (1993).

Male-Specific Compound. GC–MS comparison of volatiles from feeding male and female *G. californiensis*, supplemented by GC–EAD analysis, revealed a likely pheromone. Figure 2 (top) shows example GC traces from feeding males and females. Most of the peaks in these samples were from the plant material. However, one compound was found only from males, and it elicited an intense response from the antennae of both sexes (lower part of Figure 2). The amount of the male-specific compound depended on the conditions during collections and tended to be low early in the project when techniques were still evolving. Some early samples that elicited the characteristic EAD response had such a small amount of the male-specific compound that it was not detected by GC–FID. As the beetle-handling and volatile-collecting procedures were improved, the compound became more prominent in chromatograms (e.g., Figure 2), and it amounted to as much as 5–10% of the total GC peak area in some samples. Males produced a maximum 30–50 ng per male per day. Production typically began within a week of setting up collectors and then continued for a month or more. In the final collection effort during 2004, the compound was detected in 69 of the 81 samples, and all of those not showing it were from the initial 9 days. There was no evidence for other sex-specific compounds that elicited antennal responses.

For *G. pusilla*, 36 of the 50 collections from males in 2003 had detectable amounts of the same compound, but none was found in 21 collections from

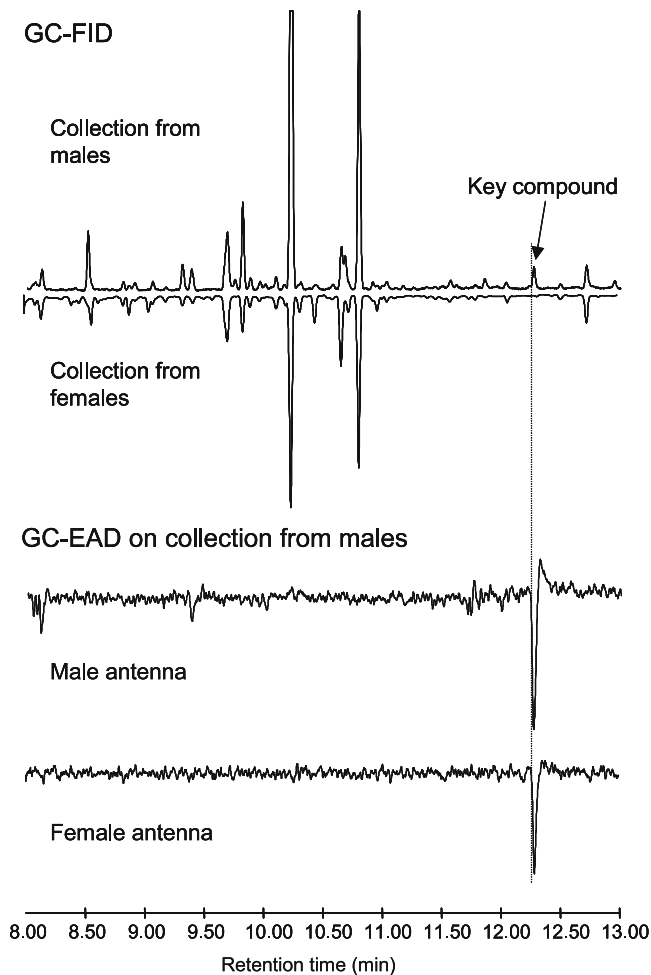


FIG. 2. GC comparison of volatiles from male and female *G. californiensis* feeding on purple loosestrife foliage, showing key male-specific compound (top), and GC-EAD responses of antennae of males and females to the volatile collection from males.

females. In 2005, the compound was present in 94 of the 100 collections from males but not in any of the 40 collections from females. The antennae of both sexes responded to the compound (GC-EAD) in the same way as did *G. californiensis*. For *G. pusilla* also, there was no evidence for other sex-specific compounds that elicited antennal responses.

Mass Spectrometry, UV Spectroscopy, and Microchemical Tests. The EI mass spectrum of the male-specific compound (Figure 3, top) had an “aromatic”

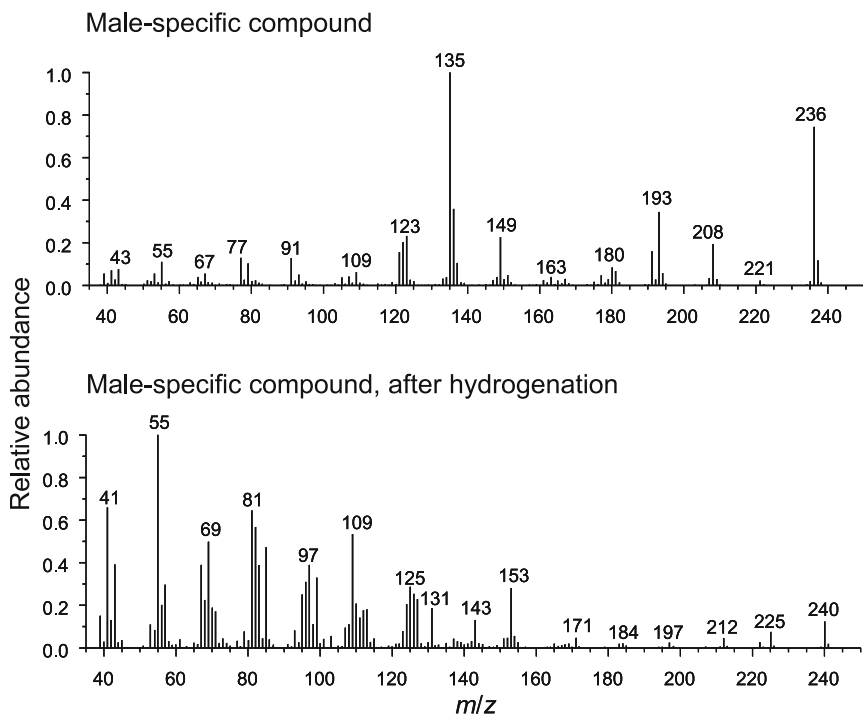
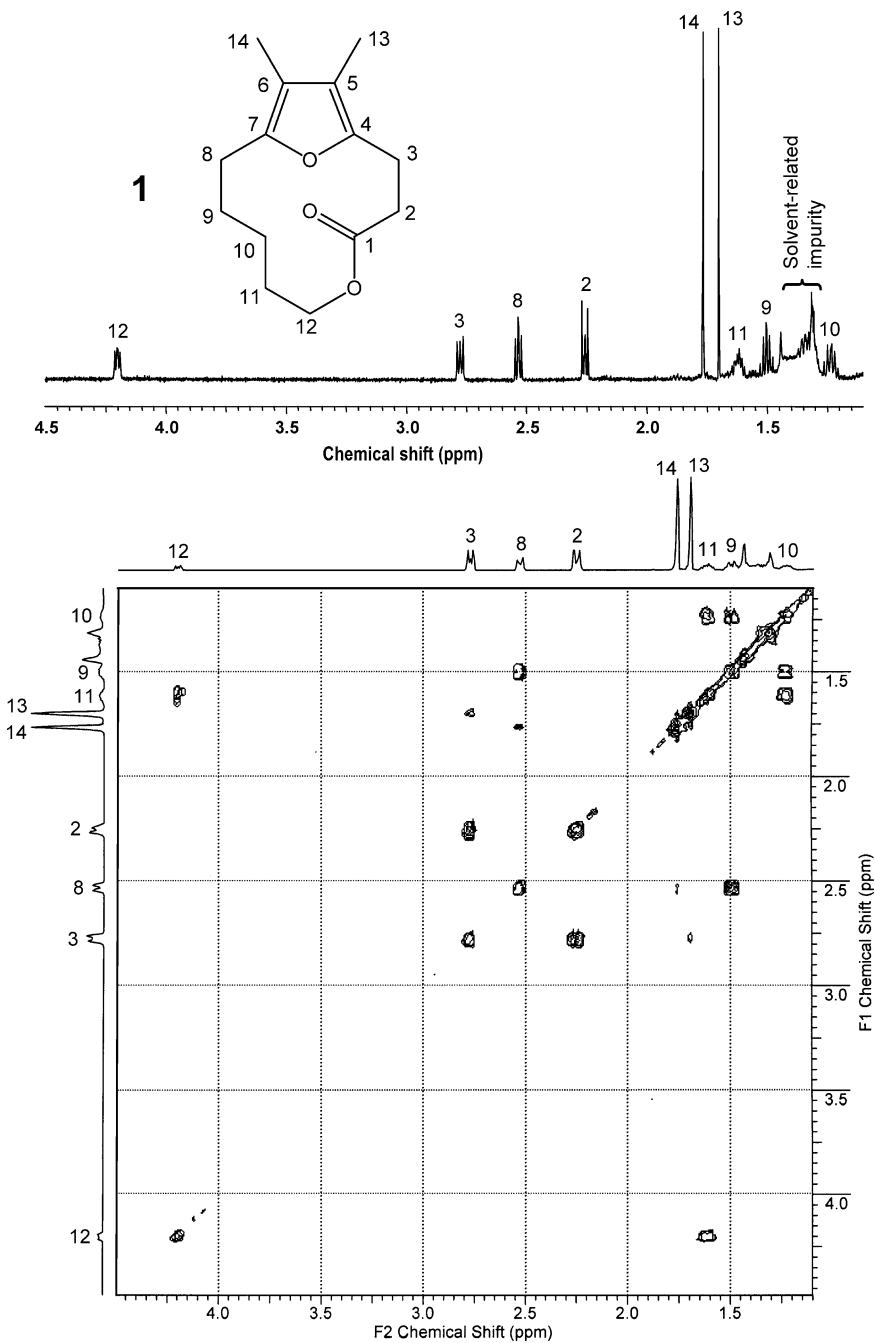


FIG. 3. EI mass spectra of the male-specific compound before and after hydrogenation.

appearance but did not match any spectra in the data system library. The apparent molecular weight of 236 was supported by positive ion CI mass spectra. With methane reagent gas, the base peak was m/z 237 $[(M + H)^+]$, and typical adduct peaks were seen at m/z 265 (15% of the base peak, $M + 29$) and m/z 277 (2%, $M + 41$). With isobutane reagent gas, the base peak was again m/z 237, and adduct peaks were seen at m/z 275 (3%, $M + 39$) and m/z 293 (2%, $M + 57$). The high-resolution EI mass spectrum was consistent with a molecular formula of $C_{14}H_{20}O_3$ (observed m/z : 236.1424; calculated m/z for $C_{14}H_{20}O_3$: 236.1412). This molecular formula indicates five degrees of unsaturation. Hydrogenation over palladium and platinum catalysts suggested that two of the degrees of unsaturation were due to carbon-carbon double bonds. With both catalysts, GC-MS revealed two products (probably diastereomers) with nearly identical spectra and apparent molecular weight of 240 (uptake of four hydrogen atoms). The mass spectrum of the more abundant, later-eluting one is shown in Figure 3, bottom. Thus, three degrees of unsaturation remained to be accounted for with rings, carbonyls, or other features.



The beetle-derived compound had a UV maximum at 229 nm (extinction coefficient 7000), suggesting that there were two double bonds in conjugation but not three.

Other microchemical reactions and liquid chromatography results gave useful structural information. The beetle compound reacted with LiAlH_4 , indicating that a carbonyl or other reducible group was present, although the product from this reaction was not recovered. The compound did not react with diazomethane, indicating no acidic protons. The polarity of the beetle compound on silica (HPLC) was lower than would be expected if a free hydroxyl group were present; the compound eluted with 5% ether in hexane, rather than requiring 25–50% ether in hexane, typically needed to elute an alcohol.

NMR Spectroscopy. NMR analysis was conducted on the purified compound (17 μg). The 1D proton spectrum is shown in Figure 4 (top), and the proton shifts and coupling information are summarized in Table 1. The proton spectrum accounted for all 20 of the protons expected from the high-resolution mass spectrum. The most downfield resonance (signal 12, two protons) suggested a methylene attached to an oxygen, and three midfield signals (2, 3, and 8, all with two protons) suggested methylenes adjacent to unsaturated carbons. The methyl groups (13 and 14) had shifts suggesting attachment to unsaturated carbons. The three remaining resonances (9, 10, and 11, each with two protons) were consistent with methylenes attached only to saturated carbons. Importantly, there was no evidence for olefinic protons.

The COSY spectrum (Figure 4, bottom) indicated two spin systems that were isolated from each other, consisting of two and five methylene units (signals 2 and 3 and 8–12, respectively). In addition, there was evidence for long-range, homoallylic coupling of the methyl groups into the main spin systems (specifically, between methyl 13 and methylene 3 and between methyl 14 and methylene 8). The methyl signals were broadened by this coupling but were not obviously split.

There were two other interesting features of the ^1H data. First, both protons of each methylene pair apparently had the same chemical shift, which would be extremely unlikely if one or more asymmetric centers were present in the molecule. Second, four of the methylenes (2, 3, 8, and 12) were nominally triplets but had second-order character (appearing as $aa'bb'$ multiplets). This was probably due to restricted rotation, perhaps because of a ring structure.

FIG. 4 Proton NMR spectrum of the male-specific compound from *G. californiensis* and assigned structure (top) and the proton COSY spectrum for the compound (bottom). A solvent-related impurity is evident in the proton spectrum; it does not correlate to any of the numbered signals in the COSY spectrum, indicating that it is unrelated to the beetle compound.

TABLE 1. SUMMARY OF ^1H AND ^{13}C NMR DATA FOR THE MALE-SPECIFIC *Galerucella* COMPOUND

Carbon position	^1H : shift (multiplicity), J	^{13}C : shift, ^a (multiplicity)	2D $^{13}\text{C}/^1\text{H}$ (protons observed)	
			HMBC	HSQC- TOCSY ^b
1	–	172.0, ^c (C)	2, 3, 12	
2	2.26 (2nd order) ^d	34.36 (CH ₂)	3	2, 3
3	2.78 (2nd order) ^d	22.38 (CH ₂)	2	2, 3
4	–	146.1, ^c (C)	2, 3, 13	
5	–	114.4, ^c (C)	13, 14	
6	–	114.4, ^c (C)	8, ^e 13, 14	
7	–	147.6, ^c (C)	8, 9, 14	
8	2.53 (t with 2nd order character), $J_{8,9} \sim 6.5$ Hz	22.98 (CH ₂)	9	8, 9
9	1.50 (p), $J_{8,9} \sim J_{9,10} \sim 6.5$ Hz	24.73 (CH ₂)	8	8, 9
10	1.24 (p), $J_{9,10} \sim J_{10,11} \sim 6.5$ Hz	21.86 (CH ₂)	8, 9, 12	9, 10, 11
11	1.62 (m)	24.95 (CH ₂)	9	10, 11, 12
12	4.20 (2nd order) ^d	62.35 (CH ₂)		11, 12
13	1.70 (s), $J_{3,13} < 1$ Hz	7.50 (CH ₃)		13
14	1.77 (s), $J_{8,14} < 1$ Hz	7.40 (CH ₃)		14

^aUnless otherwise indicated, carbon shifts were observed directly and numbers of attached protons were confirmed by DEPT135 experiment; associations between particular carbons and protons based on HSQC experiment.

^bMixing time was 17 msec; the protonated carbons were correlated to their own protons and also to protons on immediately adjacent carbons.

^cShifts and assignments determined from HMBC experiment.

^dApparent aa'bb' systems; coupling constants are not directly observable.

^eRedundant carbon shifts (δ 114.4) make assignment of cross peak ambiguous.

At this point, the chains of two and five methylenes and the two relatively isolated methyls accounted for 9 of the 14 carbons indicated by high-resolution mass spectrometry (HRMS), but there was little information as to how these protonated groups were connected through the five remaining, unprotonated carbons. The 2D heteronuclear multiple bond correlation (HMBC) experiment (for observing 2- and 3-bond proton/carbon correlations) gave the needed information.

Because of the small sample size, the NCAUR instrument showed only the most intense HMBC cross peaks, those related to the methyl group protons, but these were informative. Methyl protons 13 (δ 1.70) were correlated to carbon resonances at δ 114.4 and 146.1, and methyl protons 14 (δ 1.77), to carbon resonances at δ 114.4 and 147.6. Because three cross peaks were anticipated for each methyl, it was postulated that there were really two carbons at δ 114.4 and that both of these were coupled to both of the methyl

groups. The furan ring in **1** would meet these criteria, and the observed carbon shifts were consistent with a furan (Glass et al., 1975; Levy et al., 1980). The furan structure also accounted for the two double bonds revealed by hydrogenation. Tetrasubstituted furans have been shown to be reactive to catalytic hydrogenation (Glass et al., 1975). Furthermore, a substituted furan could explain the observed UV maximum at 229 nm (Scott, 1964; Glass et al., 1975). The ring of the furan would also account for one of the three remaining, unexplained degrees of unsaturation.

The initial HMBC result was confirmed on the more sensitive instrument at Pfizer, and additional information was obtained as well. Nearly all of the possible HMBC correlations for **1** involving the unprotonated carbons were observed, as were many of those involving the protonated carbons (summarized in Table 1). The experiment showed the attachment of the methylene chains to the furan ring and also gave evidence for the lactone function. In particular, carbon 1 was attached to methylene 2 and, through an oxygen, to methylene 12, and the shift of carbon 1 (δ 172.0) was consistent with a lactone carbonyl (Levy et al., 1980). The lactone group accounted for the two remaining degrees of unsaturation. Other evidence supporting the lactone was the reactivity with LiAlH_4 (ester reduction), failure to react with diazomethane (no free carboxyl), and the relatively low polarity by silica gel chromatography.

The ^{13}C spectrum acquired at Pfizer (30,000 scans) showed peaks for all of the protonated carbons (shifts summarized in Table 1). The DEPT135 experiment confirmed which carbons were methyls and which were methylenes. No peaks were observed in the DEPT90 experiment, consistent with no methine protons. The HSQC experiment showed all of the possible one-bond proton/carbon correlations, and these were used to assign the carbon shifts to the particular positions listed in Table 1. The HSQC–TOCSY experiment (Table 1) supported the proton/carbon and proton/proton correlations obtained from the HSQC and COSY experiments, respectively. Taken together, the data fully determined the structure of the beetle compound **1**, 12,13-dimethyl-5,14-dioxabicyclo[9.2.1]tetradeca-1(13),11-dien-4-one. The beetle-derived and synthetic **1** were identical with respect to GC retention, mass spectrum, proton NMR spectrum, and GC–EAD analysis (males and females of both species).

Septum Emissions. Over a period of 31 d, the mean emission rate of **1** from a septum treated with 500 μg of this compound was 30 (\pm 7 SD) ng/hr (N = 17). The rate remained stable over the 31-d period (linear regression of release rate over time not significant, t = -1.67 , P = 0.12). Calculated from collected amounts and septum load, less than 5% of the applied compound actually volatilized during the test. The release rate was approximately 15 times the maximum observed daily average per live male in the laboratory. However, if the beetles only produce pheromone during certain periods of the day (rather than throughout the day), or if not all of the males in the aeration tubes were

actually emitting, or both, then the peak emission rate from males could approach or exceed the release rate from septa.

Field Tests. Traps baited with synthetic **1** caught more beetles (10.3 ± 9.9 SD) than controls (1.8 ± 2.6 ; $N = 35$, $t = 10.3$, $df = 34$, $P < 0.001$; paired t test after $\log(X + 1)$ transformation). The control catch never exceeded that of the treated trap for any pair, although no beetles were caught in 2 of the 35 blocks.

Examination of the trapped beetles gave the results summarized in Table 2. Species determinations based on tibial spurs were fully supported by dissections of genitalia. By a chi-square test, the overall difference between the pheromone and the control was again highly significant ($P < 0.001$), but the treatment by beetle-category interaction was not significant ($P = 0.16$). Thus, the effect of the pheromone, expressed as the percentage of the catch found in pheromone traps, was reasonably consistent over all six beetle categories (involving species, sexes, and locations).

The overall captures of males (Table 2, totals for pheromone plus control) suggested that *G. californiensis* is the minor species at the Hyde Lake site (24% of the captured males) but is the major species at Powderhorn Lake (88% of the captured males). Based on a 2 by 2 contingency table with the trap totals for males, these percentages of *G. californiensis* at the two locations were

TABLE 2. SUMMARY OF *Galerucella* TRAPPING RESULTS, CHICAGO, IL, MAY 2005 (DATA ARE TOTALS OVER REPLICATIONS FOR INDIVIDUAL TREATMENTS, BEETLE CATEGORIES, AND LOCATIONS)

Beetle category	Trap catch			Percent on pheromone
	Pheromone	Control	Total (pheromone + control)	
Hyde Lake (15 replications)				
Male <i>G. californiensis</i>	25	8	33	76
Male <i>G. pusilla</i>	86	21	107	80
Female <i>Galerucella</i> spp.	150	25	175	86
Powderhorn Lake (20 replications)				
Male <i>G. californiensis</i>	51	8	59	86
Male <i>G. pusilla</i>	7	1	8	88
Female <i>Galerucella</i> spp.	33	1	34	97
Overall totals (35 replications)	352	64	416	85

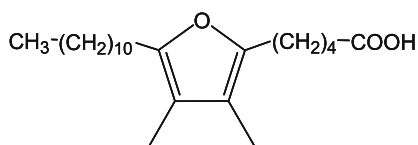
Chi-square tests: (1) Using the two overall totals for pheromone and control, test of pheromone effect (H_0 : Pheromone and control trap catches are equal): $\chi^2 = 199$, 1 df , $P < 0.001$. (2) Using the 12 treatment totals for the individual beetle categories (6 for pheromone and 6 for control), test of treatment by beetle-category interaction (H_0 : Pheromone trap catch, as % of total, is the same for all beetle categories): $\chi^2 = 7.88$, 5 df , $P = 0.16$.

significantly different ($\chi^2 = 76.3$, 1 *df*, $P < 0.001$). Given that substantial numbers of females were caught in both places, we suggest that females of both species were caught during the study, although the actual numbers of each were not determined. An important question for future research would be whether trap catches reflect actual species compositions around the trap sites. Establishing a correlation of this sort would enhance the value of the pheromone as a monitoring tool. A final pattern in the trap data was that females represented a significantly smaller percentage of the trap catch at Powderhorn Lake (34%) than at Hyde Lake (56%) ($\chi^2 = 14.7$, 1 *df*, $P < 0.001$). The reason for this difference is unknown, but may reflect the slightly later phenology at Powderhorn Lake.

Biological Activity and Future Research. A surprising finding was that both *G. californiensis* and *G. pusilla* produce and respond to the same compound. The two species often occur at the same location and at the same time of year in their native Europe (Manguin et al., 1993). It was suggested by Manguin et al. (1993) that the species might have different pheromones, but the evidence so far is the opposite. It is unclear how interspecific competition/confusion is avoided, whether there are subtle differences in diel activity periods of each species, additional pheromone components that have yet to be discovered, or still other mechanisms for long-range species recognition and reproductive isolation.

Related Compounds from the Literature. Several other furan-containing fatty acids derivatives that are structurally related to pheromone **1** have been reported. The example shown in Figure 5 is found in minor amounts in grasses,

A "furan fatty acid"



Furan fatty acid metabolite

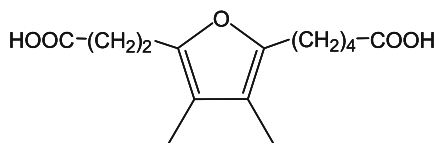


FIG. 5. Natural compounds chemically related to **1**: A "furan fatty acid" and the product obtained from it after metabolism by a rat (see text).

potato leaves, and birch tree leaves (Hannemann et al., 1989) and in olive oil (Boselli et al., 2000). The same compound is also a major lipid in the testes of a fish, the northern pike (*Esox lucius* L.) (Glass et al., 1975). Related lipids in these and other organisms have different numbers of methylenes in the side chains, unsaturation in a side chain, and/or have one of the ring methyls replaced by hydrogen. When fed to rats, furan fatty acids were partially metabolized and excreted in the urine; one such example is shown in Figure 5 (Sand et al., 1983). Interestingly, this metabolite differs from **1** only in the oxidation state at the end of the 5-carbon side group and in the absence of a closed lactone ring.

Other insects with macrocyclic lactone pheromones are known. Examples include the beetle *Oryzaephilus surinamensis* (L.) (Pierce et al., 1985), the beetle *Cryptolestes ferrugineus* (Stephens) (Wong et al., 1983), and the true bug *Piezodorus hybneri* (Gmelin) (Leal et al., 1998). Insect macrolides have a range of ring sizes, may have double bonds or branches, and may be chiral or achiral. However, none was previously found that includes the furan structure. Compound **1** is unique as a natural product and further extends the known chemical diversity of pheromones within the Chrysomelidae.

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Effects of Genetic Modification on Herbivore-Induced Volatiles from Maize

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Abstract Large-scale implementation of transgenic crop varieties raises concerns about possible nontarget effects on other organisms. This study examines the effects of genetic modification on plant volatile production and its potential impact on arthropod population dynamics. We compared herbivore-induced volatile emissions from *Bacillus thuringiensis* Berliner (Bt) maize plants to those from a non-transformed isolate following exposure to various types of leaf damage. When equal numbers of *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) larvae fed on Bt and non-Bt maize, volatile emissions were significantly lower in the transgenic plants, which also exhibited less leaf damage. When damage levels were controlled by adding more larvae to Bt plants, the plants' volatile emissions increased but displayed significant differences from those of nontransgenic plants. Significantly higher amounts of linalool, β -myrcene, and geranyl acetate were released from transgenic maize than from non-Bt plants. Manipulating the duration of feeding by individual larvae to produce similar damage patterns resulted in similar volatile profiles for Bt and non-Bt plants. Controlling damage levels more precisely by mechanically wounding leaves and applying larval regurgitant likewise resulted in similar emission patterns for Bt and non-Bt maize. Overall, changes in the herbivore-induced volatile profiles of Bt maize appeared to be a consequence of altered larval feeding behavior rather than of changes in biochemical plant defense pathways. The implications of these findings for understanding the impacts of plant-mediated cues on pest and natural enemy behavior in transgenic crop systems are discussed.

Keywords Transgenic crops · Bt · Herbivore-induced volatiles · *Zea mays* · Maize · *Helicoverpa zea* · Corn earworm

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Introduction

Genetically modified (GM) crops designed to resist insect herbivores are rapidly being adopted in many parts of the world. Transgenic maize (*Zea mays* L.) engineered to express delta-endotoxins from the soil bacterium *Bacillus thuringiensis* Berliner (Bt) has been commercially available since 1996 and now accounts for 35% of all maize grown in the US (National Agricultural Statistics Service, 2005). Large-scale implementation of transgenic crop technology has sparked substantial debate over the social, economic, and ecological implications of GM agriculture. Concerns about the ecology of insect-resistant transgenic crops have often focused on resistance development by pests and potential negative effects on natural enemies (Gould, 1998; Shelton et al., 2002). These issues are intricately linked to one another because natural enemies can influence the rate that pest populations adapt to resistant plants (Gould et al., 1991). The mechanisms underlying possible unintended ecological effects of transgenic crops warrant increased research attention.

One way in which GM crops might affect pest and natural enemy population dynamics is by altering plant defense mechanisms, including herbivore-induced plant volatiles. Volatile compounds released by plants can influence the behavior of herbivores searching for oviposition sites (Anderson and Alborn, 1999; De Moraes et al., 2001) and foraging natural enemies (Dicke et al., 1990; Dicke and van Loon, 2000; Turlings et al., 1990; De Moraes and Mescher, 2004). The ecological interactions mediated by these induced plant volatiles are often complex and can be quite sophisticated; for example, volatile blends can be keyed to particular herbivore species and attract species-specific parasitoids (De Moraes et al., 1998). Thus, these interactions may be sensitive to any changes in plant biochemistry that alter plant responses.

Volatile responses might be altered in GM crops through unanticipated phenotypic changes in plant defense systems because of pleiotropic effects or insertional mutations caused by incorporating a foreign gene (Schuler et al., 1999a). Significant phenotypic changes induced by genetic modification are not without precedent: the production of lignin, a major structural component of plants, increased in vascular tissues by 33–97% in Bt maize over non-Bt isolines (Saxena and Stotzky, 2001). Similar changes to complex biochemical pathways involved in plant defense might disrupt or alter the ability of plants to recognize and respond to herbivores, leading to significant differences between crop lines.

Induced volatile responses in transgenic crops might also be influenced by changes in interactions between herbivores and plants. The presence of Bt toxin changes the behavior of some insects. Lepidopteran larvae, for example, can detect and avoid plant parts or artificial diet containing varying forms of the toxin (Ashfaq et al., 2001; Gore et al., 2002, 2005). Such behavioral changes may alter herbivore feeding patterns, changing patterns and intensity of plant tissue damage and the exposure of the plant to oral secretions of herbivores. Plant volatile emissions are likely to be affected by such changes (Schmelz et al., 2003; Pare et al., 2005).

Whereas qualitative and quantitative changes in volatile emissions have been shown for undamaged Bt cotton and mechanically damaged GM tomatoes (Smith et al., 1996; Yan et al., 2004), the effects of genetic engineering on herbivore-induced plant volatiles remain largely unexplored. No change in parasitoid attraction was

found between Bt and non-Bt oilseed rape plants equally damaged by Bt-resistant herbivores, suggesting a similarity in the volatiles produced by each plant, but no mechanism was elucidated (Schuler et al., 1999b). Further understanding of these effects will be accomplished most readily in plant species, such as maize, where the molecular and biochemical bases of induced volatile production are well understood. In response to herbivore feeding on maize, specific blends of compounds from the lipoxygenase, terpenoid biosynthesis, and shikimic acid pathways are released both locally and systemically (Turlings and Tumlinson, 1992). Whereas many of these compounds are not released in response to mechanical damage, the application of herbivore regurgitant induces a volatile response that closely resembles actual feeding (Turlings et al., 1990).

In this study, we investigated how induced volatiles varied between GM maize with the Bt *cryIA(b)* gene inserted and a nontransformed isoline. The endotoxin produced by the modified gene is targeted toward the European corn borer (*Ostrinia nubilalis* Hübner), but is also effective at increasing developmental times and mortality rates in other lepidopteran larvae, including the corn earworm (*Helicoverpa zea* Boddie) (Wiseman et al., 1999). *H. zea* is an economically important pest of maize kernels, but can also cause damage to whorl-stage maize plants by foliar feeding (Capinera, 2000). We first compared volatiles from undamaged plants to determine if Bt and non-Bt maize plants differed in their constitutive release of chemicals. Then, we performed several experiments comparing volatile induction by *H. zea* feeding on Bt and non-Bt maize plants, manipulating larvae numbers, damage amounts, and feeding patterns. To further control damage levels and patterns, we mimicked herbivore feeding by mechanically wounding leaves and applying regurgitant collected from larvae fed either with Bt or non-Bt maize.

Methods and Materials

Plants and Insects

Both Bt and non-Bt maize seeds were obtained from Dekalb (Monsanto Co., St. Louis, MO, USA). The Bt maize DKC61-25 contains the Monsanto Event 810, with the *cryIA(b)* gene. The nearest isoline of the Bt hybrid, DKC61-24, was used for the non-Bt plants. Plants were grown in a growth chamber (14-hr photophase; 25:22°C day/night, 65% RH) in pots (16 × 17-cm diam) filled with a peat-based general-purpose potting soil with micronutrients added.

H. zea larvae were reared from eggs (USDA/ARS, Tifton, GA, USA) on an artificial casein-based diet in plastic diet cups (30 ml, Solo Cup Co., Urbana, IL, USA) in incubators (14-hr photophase, 25°C, 60% RH). Larvae were starved for 24 hr before experiments. Third instar larvae were placed on plants for all experiments.

Volatile Collection and Analysis

We collected volatiles in a greenhouse from potted, intact 2- to 3-wk-old maize plants using a closed push/pull system (Analytical Research Systems, Gainesville, FL, USA). About 3 in. above the soil line, a Teflon® base consisting of two sliding

plates (that when pushed together left a hole for the stalk to pass) was secured around the plant. A cotton ball was wrapped around the stalk to fill space between the base and plant and to allow air to exit. A cylindrical glass chamber (46 cm tall, 8-cm diam) was placed over the plant, resting on the Teflon[®] base. Air was pumped into the chamber (3.0 l/min) through Teflon[®] tubing and pulled out of the chamber (1.0 l/min) through side ports and across traps containing 40 mg SuperQ[®] (Alltech, Deerfield, IL, USA). Two separate volatile collections were made daily: one during the light phase for 12 hr, and the other during the dark phase for 8 hr.

Traps were eluted with 150 μ l of dichloromethane, and *n*-octane (80 ng) and nonyl acetate (400 ng) were added to each sample as internal standards. Volatiles were analyzed by gas chromatography with a Hewlett-Packard model 5890 GC. Samples were injected in 1- μ l aliquots with a splitless injector held at 240°C. The column (15 m \times 0.25 mm i.d., HP-1) was maintained at 60°C for 2 min, and then increased 4°C/min to 180°C, where it was held for 10 min. Quantifications of compounds were made relative to the internal standard using Enhanced Chem-Station software (Agilent Technologies, Palo Alto, CA, USA). Identifications were confirmed with mass spectrometry and by comparing retention times with those of known standards.

Undamaged Plants

Volatiles were collected from undamaged Bt and non-Bt maize to determine baseline emissions. Collections were made from four plants of each type. Means for Bt and non-Bt maize for this and all other experiments were compared with Student's *t* test using Minitab v. 14.1 (State College, PA, USA) (Sokal and Rohlf, 1995).

Equal Number of Larvae Per Plant

We tested the induced responses of maize when exposed to an equal number of larvae on each plant type to approximate likely field conditions. Two third instars were added to each chamber containing individual Bt or non-Bt plants and allowed to feed freely on plants during 4 d of volatile collecting. Each day, dead or molting larvae were replaced with ones that had been starved for 24 hr. These comparisons were made on six Bt and six non-Bt plants.

To avoid disturbing the plants while larvae were feeding, we assessed herbivore damage while plants remained inside volatile collection chambers. Each morning during the 4-d feeding experiment, we estimated the size of damage holes on unfurled leaf blades and through the enumerated layers of the whorled cylinder by comparison with a guide composed of five different-sized circles of known area. From the number and estimated size of holes, we calculated the total leaf area removed.

Equal Amount of Herbivore Damage

To determine if the amount of larval feeding affects induced responses of Bt and non-Bt maize, we added *H. zea* larvae to Bt plants daily to achieve equivalent damage on both plant types. On the first day of the experiment, 8–10 third instars were added to each Bt maize plant, and one or two larvae were added to each non-Bt plant. Each day, more third instars (~5) were added to Bt maize through the tops

of chambers to replace ones that had died. By the end of the 4-d sampling period, most larvae had reached their fourth instar. As a control, the tops of the non-Bt chambers were vented for an equal time. Each day, we assessed and estimated the amount of plant damage as above. Three plants of each type were used for comparisons.

Equal Pattern and Amount of Herbivore Damage

Individual larvae on non-Bt plants feed longer and cause greater localized damage. Because the feeding pattern (i.e., the number and size of damage holes) may influence induced responses, we manipulated the duration of larval feeding, resulting in the consumption of an equal amount of leaf tissue in a similar pattern on each plant type. Third instars placed on Bt and non-Bt maize plants were monitored continuously as they fed, and individual larvae were removed once they consumed an area of leaf tissue equivalent in size to the damage a Bt plant would typically incur from one larva ($\sim 5 \text{ mm}^2$). The number of larvae on each plant was manipulated to achieve similar amounts of damage across plants ($\sim 60 \text{ mm}^2$). After all larvae were removed, usually about 3 hr after the first larvae were placed on the plants, we collected volatiles in a growth chamber for the next 3 d by using a collection system similar to the one used in the other experiments (Analytical Research Systems). Air was pumped into the chambers at 2 l/min and pulled out through traps at 0.8 l/min. A total of five Bt plants and four non-Bt plants were used as replications in this experiment. When volatile collections were finished, we cut plants at the base and digitally scanned leaves to quantify leaf area removed (SigmaScan Pro v. 5.0, SPSS Inc., Chicago, IL, USA).

Mechanical Damage and Regurgitant Application

To control the pattern and amount of damage more precisely than was possible using natural herbivory, we artificially simulated herbivory by mechanically damaging leaves and applying larval regurgitant. We collected regurgitant from fourth instar *H. zea* by probing the preoral cavity with a pipette tip and drawing in the fluid released from the mouth. Larvae in diet cups had either been fed with foliage from non-Bt maize for 24 hr or foliage from Bt maize for 6 hr after having been starved for 18 hr prior to collecting. The shorter feeding time was necessary for those fed with Bt maize because the larvae would become inactive soon after eating the transgenic leaves, at which point regurgitant could not be obtained. Regurgitant was kept on ice while being collected and boiled for 3 min to stop enzyme activity (Mori et al., 2001). Samples were stored at -80°C until needed. Preliminary experiments showed no clear differences between the volatile profiles of Bt or non-Bt plants induced by regurgitant collected from larvae fed with either Bt leaves or non-Bt leaves (data not shown). Because of this similarity in responses and the difficulties involved in collecting regurgitant from larvae exposed to the Bt toxin, regurgitant collected from non-Bt fed larvae was used to compare volatiles from Bt and non-Bt plants with artificial herbivory.

Volatiles were collected from mechanically damaged plants with and without regurgitant. Plants were damaged by scraping leaf tissue from the upper side of

leaves with a razor blade in a 1 × 2 cm rectangle. Each day, the glass volatile collection chambers were temporarily removed, and three leaves on each plant were damaged, with regurgitant (10 µl) applied to each damaged spot. The chambers were replaced, and volatiles were collected. Three Bt and three non-Bt plants were used for each experiment (with and without regurgitant).

Results

Undamaged Plants

We found no differences in the total amount of volatiles released or in quantities of individual compounds in the emissions from undamaged Bt and non-Bt maize (Table 1).

Equal Numbers of Larvae Per Plant

When equal numbers of *H. zea* larvae were fed on both maize isolines, non-Bt plants suffered 4 to 22 times more damage by the end of the experiment (Student's *t* test, $t_6 = -3.53$, $P = 0.012$). By the third day, the amount of damage as well as the amount of volatiles released was significantly greater on non-Bt plants (Table 1). Damage to Bt maize typically occurred only on the first day larvae that were placed on the plants, because after initial feeding, larvae often would be inactive but still alive for one or two more days. On non-Bt plants, larvae would continue to feed for the 4 d of the experiment, causing extensive damage.

Equal Amount of Herbivore Damage

When we equalized the amount of herbivory each day, there was a significant difference in the total amount of volatiles released by day 3 (Table 1). Four

Table 1 Total volatiles (Mean ± SE) released during and damage incurred (mm²) preceding day 3 from Bt and non-Bt maize

Treatment	Type	Total volatiles (ng/hr)	<i>P</i> value	Total damage (mm ²)	<i>P</i> value
Undamaged control	Bt	180.7 ± 41.6	0.834	0	n/a
	Non-Bt	193.5 ± 41.7		0	
Equal number of larvae per plant	Bt	652.7 ± 236.7	0.003	39.4 ± 23.3	0.006
	Non-Bt	3118.6 ± 482.9		302.4 ± 59.8	
Equal amount of herbivore damage	Bt	3869.3 ± 413.5	0.049	159.7 ± 25.3	0.519
	Non-Bt	2275.7 ± 274.8		139.3 ± 6.8	
Equal pattern and amount of herbivore damage	Bt	1192.0 ± 65.3	0.419	62.1 ± 6.2	0.913
	Non-Bt	1300.0 ± 115.5		61.0 ± 7.3	
Mechanical damage	Bt	949.6 ± 293.1	0.959	600.0	n/a
	Non-Bt	925.0 ± 330.5		600.0	
Mechanical damage + regurgitant application	Bt	1521.2 ± 460.1	0.908	600.0	n/a
	Non-Bt	1614.7 ± 587.2		600.0	

Bolded numbers signify statistically significant differences between Bt and non-Bt values (Student's *t* test, $P < 0.05$).

Table 2 Major compounds released from Bt and non-Bt maize receiving an equal amount of herbivore damage on day 3^a

Compound	Bt	Non-Bt	<i>P</i> value
(<i>E</i>)-β-Farnescene	867.4 ± 148	632.6 ± 150	0.347
Bergamotene	177.6 ± 31	124.8 ± 25	0.275
(<i>E</i>)-β-Caryophyllene	23.3 ± 2	16.6 ± 5	0.308
Geranyl acetate	205.2 ± 31	75.9 ± 17	0.034
Indole	257.6 ± 20	293.4 ± 154	0.839
Linalool	1000.9 ± 89	443.4 ± 94	0.023
β-Myrcene	46.6 ± 5	22.58 ± 4	0.035
(<i>E</i>)-4-8-Dimethyl-1,3,7-nonatriene	606.7 ± 112	318.6 ± 89	0.138
(<i>E,E</i>)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	151.3 ± 27	82.2 ± 19	0.131
Unknown 1	23.1 ± 9	5.5 ± 1	0.182
Unknown 2	57.1 ± 2	40.5 ± 22	0.530
Unknown 3	45.0 ± 8	29.7 ± 7	0.244
Unknown 4	35.4 ± 6	23.2 ± 5	0.231
(<i>Z</i>)-3-Hexenyl acetate	83.7 ± 19	21.6 ± 10	0.066

^a Values represent means ± SE in ng/hr.

Bolded numbers signify statistically significant differences between Bt and non-Bt values (Student’s *t* test, *P* < 0.05).

compounds constituted a major proportion of this difference: (*Z*)-3-hexenyl acetate, linalool, β-myrcene, and geranyl acetate (Table 2). The pattern of damage inflicted by each larvae differed between plant lines, however, with larvae on Bt plants feeding in short bouts and leaving many small, scattered holes, whereas larvae on non-Bt plants fed more or less continuously, creating large areas devoid of leaf tissue (Fig. 1).



Fig. 1 Sample of feeding patterns created by *Helicoverpa zea* larvae on Bt and non-Bt maize leaves

Table 3 Characteristics of damage holes created by *Helicoverpa zea* larvae while controlling duration of feeding to achieve an equal amount and pattern of damage

	Bt	Non-Bt	<i>P</i> value
Number of holes	15.5 ± 3.6	17.0 ± 3.7	0.789
Area (mm ²)	62.1 ± 6.2	61.0 ± 7.3	0.913
Perimeter (mm)	122.6 ± 11.0	126.9 ± 9.4	0.793

Equal Pattern and Amount of Herbivore Damage

Manipulating the amount of time larvae were allowed to feed resulted in plants with similar numbers of damage holes, leaf area removed, and perimeters of damaged areas (Table 3). In this case, the two maize isolines emitted similar blends in terms of total volatiles released and proportions of individual compounds (Table 1).

Mechanical Damage and Regurgitant Application

The volatiles released by Bt and non-Bt plants after applying larval regurgitant did not differ substantially from one another quantitatively or qualitatively (Table 1).

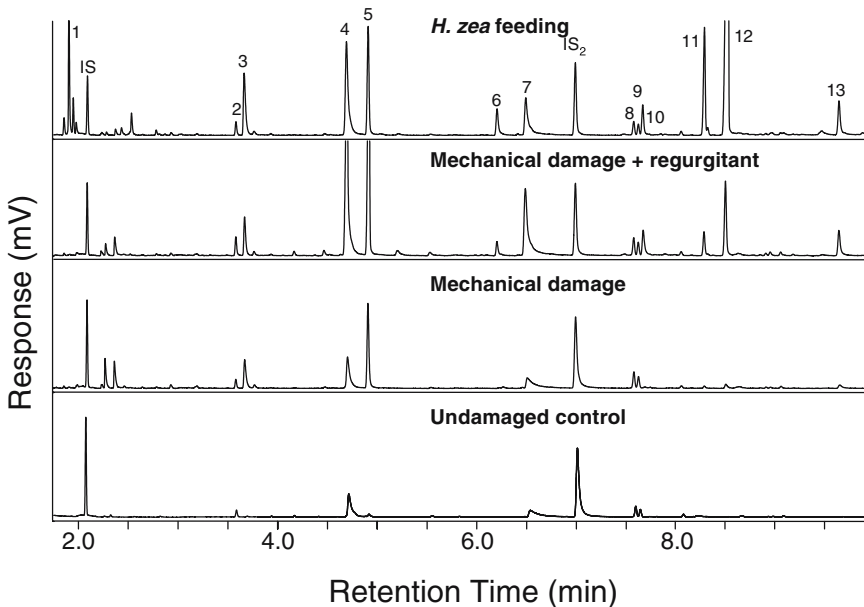


Fig. 2 Chromatograms of volatile compounds emitted on day 3 from non-Bt maize plants subjected to feeding by *H. zea* larvae, mechanical damage, mechanical damage with regurgitant applied, and no damage. Major compounds (with a threshold emission of 2 ng/hr) are labeled as follows: (1) unknown 1; (2) β -myrcene; (3) (*Z*)-3-hexenyl acetate; (4) linalool; (5) (*E*)-4-8-dimethyl-1,3,7-nonatriene; (6) unknown 2; (7) indole; (8) unknown 3; (9) unknown 4; (10) geranyl acetate; (11) bergamotene; (12) (*E*)- β -farnescene; (13) (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene; IS and IS₂, internal standards (*n*-octane and nonyl acetate)

The volatile profile of maize plants with artificial herbivory closely approximated that of plants exposed to actual larvae damage (Fig. 2). However, regurgitant-treated plants did not release some “green leafy volatiles” (Turlings et al., 1998) and had a weaker induction of β -farnescene and bergamotene (Fig. 2). Plants that were wounded with a razor blade appeared to release fewer volatile compounds and smaller quantities of the ones that were emitted relative to those damaged by herbivores.

Discussion

In our experiments, genetic modification did not appear to alter the volatile profile of undamaged maize (Table 1). However, undamaged Bt cotton plants emitted unique compounds and different proportions of typical compounds when compared to non-Bt cotton (Yan et al., 2004), hinting at the diversity of effects genetic modification can have on different plant species.

Undamaged Bt maize plants do not appear to influence the ovipositional preferences of some Lepidopteran pests (Hellmich et al., 1999; Liu et al., 2002), suggesting that an equal number of larvae would initially be expected on each plant type. When we allowed equal numbers of larvae to feed on plants continuously for 4 d, non-Bt maize received up to 22 times more damage and emitted significantly more volatiles than Bt maize (Table 1). In the field, similar reductions in induced volatile emissions could cause areas planted with Bt maize to be less attractive to natural enemies that rely on herbivore-induced plant cues to find suitable hosts. Sked (2003) showed that fields sown with Bt maize had one third the numbers of *Macrocentrus cingulum*, a parasitoid specialist on the European corn borer, as non-Bt plots. Such reductions in population size could conceivably lead to local extirpation of natural enemies. At the same time, reduced volatile emissions could attract herbivores seeking a suitable oviposition site. For example, *O. nubilalis* oviposits in the field preferentially on undamaged maize plants compared to those infested with conspecific larvae (Harmon et al., 2003). More pest egg masses laid on Bt plants emitting fewer volatiles than severely damaged non-Bt maize could increase the possibility of resistance emerging within pest populations (Hellmich et al., 1999).

If an ovipositional preference for Bt plants did arise in the field, the greater numbers of larvae might produce an amount of damage closer to that incurred by non-Bt plants. Our results indicate that even with an equal amount of damage, volatile emissions still varied between Bt and non-Bt plants (Table 2). Linalool, β -myrcene, and geranyl acetate were released in significantly greater quantities from Bt maize. These compounds have been implicated in natural enemy attraction through behavioral studies and antennal electrophysiological responses (Turlings et al., 1991; Rose et al., 1998; Gouinguene et al., 2005).

Although damage amounts were equalized by increasing the number of larvae on Bt plants, these larvae exhibited a different feeding behavior, and thus created a different pattern of damage than larvae feeding on non-Bt plants. Damage holes were more numerous but smaller on Bt maize, increasing the perimeter of damaged areas and maximizing the contact zone of oral secretions and exposed plant tissue. When the feeding of individual larvae was manipulated to achieve similar damage

patterns, differences in volatile emissions between Bt and non-Bt maize were no longer evident, suggesting that these differences resulted from the altered feeding behavior of larvae on Bt plants, rather than physiological changes in plant response resulting from genetic modification.

When the damage pattern and amount were standardized, either by manipulating the feeding duration of larvae or by applying larval regurgitant to mechanically damaged leaves, the induced volatile emissions of Bt and non-Bt maize were similar. These results suggest that Bt and non-Bt fields subjected to similar amounts and patterns of feeding damage, as might occur if pest populations developed resistance to the Bt maize, would be equally attractive to natural enemies. Similarly, the parasitic wasp *Cotesia plutellae* was found to be equally attracted to Bt oilseed rape plants fed upon by Bt-resistant hosts (*Plutella xylostella*) and wild-type plants equally damaged by the same herbivore, suggesting no change in the composition of the induced volatiles (Schuler et al., 1999b).

In summary, observed differences in induced volatile profiles between Bt and non-Bt maize appear to result from different amounts and patterns of feeding damage inflicted. Individual larvae on transgenic plants fed in short bouts, causing less damage and leaving distinctive damage patterns relative to conventional plants. When we controlled for these differences in the amount and pattern of feeding damage inflicted, differences in the volatile profiles of Bt and non-Bt plants disappeared, suggesting that these differences are a consequence of altered larvae feeding behavior on Bt plants rather than of changes in biochemical plant defense pathways. As reduced herbivory is the goal of insect-resistant transgenic crops, the consequent reductions in volatile emissions may have important implications for sustainable pest management.

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Volatile Emissions from an Odorous Plant in Response to Herbivory and Methyl Jasmonate Exposure

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Abstract Induced volatile terpenes have been commonly reported among diverse agricultural plant species, but less commonly investigated in odorous plant species. Odorous plants synthesize and constitutively store relatively large amounts of volatiles, and these may play a role in defense against herbivores. We examined the effect of herbivory and methyl jasmonate (MeJA) exposure on the release of volatile organic compounds (VOCs) in the marsh elder, *Iva frutescens*, which contains numerous constitutive VOCs, mainly mono- and sesquiterpenes. Our specific goal was to test for the presence of inducible VOCs in a naturally occurring plant already armed with VOCs. The abundant, native specialist leaf beetle *Paria aterrima* was used in herbivore induction trials. VOCs were sampled from herbivore wounded and unwounded, and from MeJA treated and untreated *I. frutescens*. Total VOC emissions were significantly greater in response to herbivory and MeJA treatment compared to unwounded controls. Herbivore wounding caused a substantial shift in the emission profile (42 VOCs from wounded, compared to 8 VOCs from unwounded *I. frutescens*), and MeJA had a similar yet less substantial influence on the emission pattern (28 VOCs from MeJA treated compared to 8 VOCs from untreated *I. frutescens*). Constitutive VOC emissions predominated, but some VOCs were detected only in response to herbivory and MeJA treatment, suggesting *de novo* synthesis. Several VOCs exhibited a delayed emission profile in contrast to the rapid release of constitutive VOCs, and principal components analysis revealed they were not associated with constitutive emissions. While *I. frutescens* contains many constitutive VOCs that are released immediately in response to herbivory, it also produces novel VOCs in response to feeding by the specialist *P. aterrima* and MeJA treatment.

Keywords *Iva frutescens* · *Paria aterrima* · Herbivory · Methyl jasmonate · Volatile emission · Terpenes · Plant defense · Constitutive defense · Induced defense

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Introduction

Many plants from diverse lineages synthesize secondary metabolites that accumulate in specialized secretory cells, or whose production is induced by herbivory (Karban and Baldwin, 1997). Terpenoids are a diverse group of secondary compounds that serve a variety of functions in plants, ranging from heat stress resistance (Sharkey and Singaas, 1995) to serving as cell membrane components, plant hormones, membrane-bound sugar carriers, and photoprotective pigments (Gershenzon and Croteau, 1993). Terpenoids may also play a direct defensive role in plant species that produce these compounds, as well as an indirect defensive role, by attracting natural enemies of herbivores (Paré and Tumlinson, 1999; Theis and Lerdau, 2003). Stored volatile organic compounds (VOCs) may be volatilized into the atmosphere by a healthy unwounded plant depending on their concentration and physiochemical properties (Niinemets et al., 2004). These stored VOCs are volatilized in greater quantity into the atmosphere upon tissue breakage during herbivore attack (Karban and Baldwin, 1997). While many plants contain large amounts of stored VOCs, others do not synthesize and emit them until a stimulus (such as herbivory) is perceived. Here, we use the term “constitutive” to refer to VOCs that are stored in the leaf and can be immediately released by mechanical wounding, “induced” to refer to VOCs that are increased in response to herbivory (which may include up-regulated constitutive VOCs), and “novel” to refer to unique induced VOCs that are not normally stored by the plant, but whose synthesis is up-regulated in response to herbivory.

Induced VOCs may be emitted hours or days after an attack, both from the site of wounding as well as systemically from undamaged plant leaves (Paré and Tumlinson, 1997a; 1999; Mattiacci et al., 2001). The synthesis of novel compounds not normally produced and accumulated may also be induced by herbivore oral secretions (Alborn et al., 1997; Paré et al., 2005). $^{13}\text{CO}_2$ pulse-labeling experiments have demonstrated the *de novo* biosynthesis of novel VOCs emitted from herbivore-wounded plants (Paré and Tumlinson, 1997b).

Numerous studies have shown that third-trophic-level members use novel VOCs as indicators of prey location, thus facilitating a tritrophic interaction and an indirect defense to the wounded plant (Dicke et al., 1990; Drukker et al., 2000; De Boer and Dicke, 2004). For instance, the C_{11} homoterpene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (herein referred to as nonatriene), and the C_{16} homoterpene, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (herein referred to as TMTT), among other terpenes, are induced by a broad spectrum of herbivores feeding on their host plants, and these induced VOCs are attractive to foraging predators and parasitoids (Dicke et al., 1990; Turlings et al., 1991; Vanpoecke et al., 2001).

Novel VOCs induction has been a focal point for research in tritrophic interactions and indirect defense, while constitutive VOCs have garnered relatively less attention. Constitutive VOCs have been mainly studied for their roles in direct defense as toxins and herbivore deterrents (Wittstock and Gershenzon, 2002). However, herbivory may up-regulate the production of constitutive VOCs, and due to their volatility they may also play a role in indirect defense. In many coniferous trees, constitutive terpene synthase enzymes are up-regulated in response to herbivory (Litvak and Monson, 1998), and specialist bark beetle predators are attracted to both constitutive and induced VOCs from conifers (Raffa and Smalley, 1995). In angiosperms, constitutive VOCs from *Zea maize*, in addition to novel

VOCs, enhance the ability of predators and parasites to find herbivore hosts (Fukushima et al., 2002). These findings suggest that constitutive VOCs may play a similar ecological role to induced VOCs.

Emission of VOCs in response to herbivory, as well as attraction of third-trophic-level members, has been documented in many systems, most of which are crop species lacking large amounts of constitutive VOCs. While much progress has been made in understanding the function of VOCs and the mechanisms regulating VOC emissions, less emphasis has been placed on understanding herbivore stimulated VOCs in natural plant systems that can store relatively large amounts of constitutive VOCs compared to agricultural species. Baldwin and coworkers have pioneered efforts to elucidate the function of VOCs in nature by using native populations of tobacco (*Nicotiana attenuata*) and the odorous sagebrush (*Artemisia tridentata*) (Kessler and Baldwin, 2001). Southeastern salt marsh plant communities have a high occurrence of many largely constitutive VOC producing species such as the marsh elder, *Iva frutescens* (Asteraceae), and (southern) wax myrtle, *Myrica cerifera* (Myricaceae). The role of herbivore-induced VOCs in plant assemblages containing large amounts of constitutive VOCs is not known.

The objective of this research was to examine herbivore-induced VOC emissions in a native coastal plant, *I. frutescens*, which is armed with an array of constitutive VOCs (mainly mono- and sesquiterpenes). Our specific goal was to determine the potential for induced VOC emission in response to herbivory by a native herbivorous beetle, *Paria aterrima* (Chrysomelidae) (Wilcox, 1957). Plants containing a high amount of constitutive VOCs may immediately release stored VOCs, up-regulate constitutive VOCs, and may synthesize and emit novel VOCs in response to herbivory. We also tested for potential VOC induction by treating unwounded *I. frutescens* with the volatile methyl jasmonate (MeJA). VOC emissions (and other defense responses) are induced by the plant hormone jasmonic acid (JA), and its volatile methyl ester, MeJA (Dicke et al., 1999; Koch et al., 1999). Jasmonates are products of the octadecanoid pathway, which has been identified as one of the major signaling pathways in plant defense (Farmer and Ryan, 1990). Jasmonates may play a role in the up-regulation of VOCs, since treatment of plants with this compound can enhance both constitutive and novel VOC emissions (Martin et al., 2003). If *I. frutescens* contains herbivore-inducible VOC emissions, application of MeJA should stimulate the production and subsequent emission of novel VOCs, and potentially up-regulate constitutive VOC emissions.

Methods and Materials

Plants and Insects

Iva frutescens occurs naturally along the forest edge of salt marshes in southeastern North America. The salt marsh plant community shows a distinct zonation pattern with the grass, *Spartina alterniflora*, dominating the lower elevations, and rushes (*Juncus* sp.) occurring on the landward border. At higher elevations, the marsh transitions to a shrub zone that is dominated by *Borrchia frutescens*. *I. frutescens* occurs as scattered individuals throughout the shrub zone but dominates the landward edge. A *M. cerifera* zone occurs on the landward side of *I. frutescens*, and borders an inland oak–pine forest. A combination of physical stress and biotic

interactions are thought to limit the distribution of salt marsh plants, with physical stress playing a primary role at low elevations and competition playing a larger role on the landward edge (Pennings and Callaway, 1992; Bertness and Yeh, 1994; Pennings and Moore, 2001).

I. frutescens plants approximately 2 m in height were collected in June 2000 from the marsh edge of the forest transition zone on Goat Island, at the North Inlet Marsh, Belle W. Baruch Marine Laboratory in Georgetown, SC, USA. Plants were pruned to approximately 1 m in height, and transplanted into 3-gal pots in the field using a 3:1 soil/sand mixture (Baccto lite™ potting soil: play sand), transported back to the University of South Carolina (USC) (Columbia, SC, USA), where they were fertilized using Peter's® 20/20/20 (5 ml of fertilizer per 3.78 l water) and maintained in a greenhouse. Plants were pruned to a height of approximately 1 m, and fertilized on an annual basis. Plants were moved in early May 2002 to a growth chamber where environmental conditions could be controlled. All herbivore wounding and MeJA treatment experiments took place in June 2002.

Paria aterrima beetles were collected in June 2002 from *I. frutescens* plants at Goat Island and transported back to USC. Beetles were maintained in plastic storage containers fitted with a mesh screen on the lid in the same growth chamber as experimental *I. frutescens* plants. Beetles were offered cotton balls saturated with water and branches of *I. frutescens* leaves as a food source. Branches were collected from the coastal site and stored in a cold room at 4°C in sealed plastic bags with moist paper towels to maintain freshness. New branches were placed in the beetle containers daily.

Growth Chamber Conditions

Growth conditions were maintained on a 14-hr photoperiod from 7:00 A.M. to 9:00 P.M., with a daytime temperature of 22°C and a nighttime temperature of 16°C. These conditions were maintained throughout the course of the experiments. The light regime was chosen to allow all volatile measurements to be made under daylight conditions when volatile emissions are greatest (Loughrin et al., 1994). *I. frutescens* approximately 1 m in height were used for experiments. These plants were in the original pots in which they were initially transplanted in the field, and appeared to be root-bound to some extent. Plants were watered every 2 d to saturation.

Herbivore Wounding Trials

For the herbivore wounding trials, the volatile collection system was placed over a selected branch and loosely fastened around the stem with a cable tie. Twenty beetles were inserted into the collection system through a small hole in the top of each bag, which was subsequently sealed with a cable tie. Insects were randomly chosen for each replicate, and no insects were reused. Beetles designated for a wounding trial were placed in a separate container with water, but without food for 24 hr prior to each trial. Once beetles were placed inside the collection bag, VOC emissions were collected in 1-hr intervals for 4 hr, with the traps changed every hour. After the first four collections, beetles were removed and VOC emissions were collected in 4-hr sampling periods at 4–8, 20–24, 32–36, 44–48, 56–60, and 68–72 hr. This sampling scheme was chosen to characterize any rapid changes in VOC emission during herbivory, as well as changes following herbivory. New traps were used for each

sampling period. An unwounded plant was simultaneously sampled with a wounded plant. All wounding experiments were replicated with seven different plants, and initiated at the same time of day (11:00 A.M.).

Plant Volatile Collection

A push–pull volatile collection system was designed using Reynolds[®] oven bags. One top corner of a bag was fitted with a straight connector to serve as the inlet, while another straight connector was fitted to the opposite side of the bag at the base near the opening to serve as the exhaust. Connectors were fastened to the bags with cable ties. Compressed medical grade air was used as the inlet air supply. A gas regulator was connected to the gas cylinder to control the flow rate into the collection bag at 1.1 l min^{-1} . An SKC, Inc. Aircheck sampler (model # 224-44XR) was used to pull headspace air through the bag and onto a 250-mg Tenax TA (Alltech, Inc.) trap fitted to the exhaust line on the bag. Headspace VOCs were sampled at 1 l min^{-1} . Therefore, 90.9% of the air was sampled during each sampling period. A frame was constructed out of chicken wire and placed inside the bag to maintain a rigid structure around the branch being sampled to minimize VOC emissions due to artificial damage. For each wounding trial, the frame and bag were baked prior to use at 160°C for 30 min to eliminate any potential volatile contaminants. Control collections (bag and frame only) showed two artificial peaks from the bag and frame system (data not shown). These peaks were not included in *I. frutescens* VOC data analysis. For the herbivore wounding experiments, one branch from a plant was selected for wounding as described below. The frame and bag were placed over the branch, and volatiles were collected at intervals described above. VOC collections from collection bags containing only beetles revealed no detectable beetle-associated VOCs (data not shown).

Chemical Analysis

Each volatile trap was eluted with 2 ml of gas chromatography/mass spectroscopy (GC/MS) analytic grade pentane and stored in 2-ml auto sampler vials. Pentane was added in 1-ml aliquots and allowed to elute by gravity. A light stream of air was used to push the remaining pentane through the trap. The eluate was concentrated to 300 μl under a stream of compressed nitrogen. The concentrated sample was fractionated into two 150- μl samples. All samples were analyzed by using a Hewlett Packard 5890 series II gas chromatograph equipped with an HP-5 methyl-silicone capillary column (30 m \times 0.25 mm) with helium as a carrier gas at a rate of 1.22 ml min^{-1} . The injector temperature was set at 275°C . All injection volumes were 2 μl . The temperature program consisted of an initial hold at 50°C for 3 min, followed by an increase to a final temperature of 220°C at a rate of 5°C/min , followed by a hold at maximum temperature for 20 min. Samples were subsequently analyzed by mass spectroscopy with a Hewlett Packard 5971 series Mass Selective Detector. Volatile compounds collected from headspace emissions were identified by comparison of retention time and mass spectra to those contained in the National Bureau of Standards essential oil database, as well as the database of essential oil components identified by GC/MS (Adams, 1995). All VOCs were quantified by an external standard method based on two major constitutive VOCs from *I. frutescens*, the monoterpene limonene, and the sesquiterpene β -caryophyllene. Six-carbon volatiles,

Table 1 Volatile organic compounds (VOCs) detected by GC/MS from leaf extracts^a, herbivore^b wounded and unwounded, and methyl jasmonate^c treated and untreated *Iva frutescens*

Compound name	Leaf extract (ng · g ⁻¹ fr wt)	Herbivory (ng · cm ⁻² hr ⁻¹)		Methyl jasmonate (ng · cm ⁻² hr ⁻¹)	
		Wounded	Unwounded	Treated	Untreated
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
1 Tricyclene*	22 ± 2	0.32 ± 0.17			
2 α-Pinene ^{d,*}	3969 ± 369	48 ± 22	2 ± 0.8	43 ± 16	3.7 ± 1.3
3 Sabinene ^{d,*}	2182 ± 206	16 ± 7	0.6 ± 0.2	11 ± 2	2.2 ± 1.6
4 β-Pinene ^{d,*}	2432 ± 223	22 ± 10	0.6 ± 0.1	11 ± 5	3.7 ± 3.4
5 Limonene ^{d,*}	16861 ± 1341	204 ± 87	10 ± 1.6	133 ± 30	15 ± 5
6 β-Phellandrene	74 ± 13	0.5 ± 0.2		3.3 ± 1.6	
7 δ-Carene ^f	217 ± 23	3.5 ± 1.5			
8 (e)-Ocimene ^{f,*}				0.9 ± 0.6	
9 Isolimonene ^{f,*}				1.6 ± 1.4	
10 <i>p</i> -Cymene ^{d,e,*}		0.08 ± .03			
11 Terpinolene	40 ± 4	0.05 ± 0.03			
12 Nonatriene ^{d,*}	22 ± 4	0.27 ± 0.11		4.9 ± 1.6	
13 Neo- <i>allo</i> -ocimene	31 ± 7	0.14 ± 0.08			
14 Oxygenated monoterpene 1	60 ± 8	0.21 ± 0.06			
15 Bornyl acetate	48 ± 3	0.14 ± 0.06			
16 Unknown 1	37 ± 3	0.21 ± 0.07		0.5 ± 0.4	
17 Unknown 2	56 ± 4			2.4 ± 2.4	
18 2-Methyl-butylate ^{f,*}				4.6 ± 2.8	
19 Methyl salicylate ^{f,*}				0.5 ± 0.3	
20 Bergemal ^{f,*}				0.8 ± 0.5	
21 Hexenyl butylate ^{f,*}				1.7 ± 0.4	
22 Unknown sesquiterpene 1	35 ± 3	0.14 ± 0.06		0.7 ± 0.5	
23 Unknown sesquiterpene 2	160 ± 17	0.04 ± 0.02			
24 Copaene	41 ± 6	0.09 ± 0.07			
25 Daucene*	56 ± 6	1.36 ± 0.6			
26 Geranyl acetate ^{d,*}	481 ± 51	2.4 ± 0.8			
27 <i>cis</i> -Jasmone ^{f,*}				0.1 ± 0.1	
28 β-Cubebene ^{d,*}	217 ± 26	1.3 ± 0.3		3.9 ± 3	
29 β-Elemene ^f				4.5 ± 3.1	
30 Unknown sesquiterpene 3*	12 ± 4	0.24 ± 0.15		2.0 ± 0.7	
31 α-Gurjunene ^{d,*}	1261 ± 114	4.1 ± 1.7		2.0 ± 0.7	
32 β-Gurjunene*	61 ± 6			1.3 ± 0.8	
33 β-Caryophyllene ^{d,*}	3412 ± 341	8.2 ± 2.9	0.1 ± 0.03	4.4 ± 2.0	1.1 ± 0.6
34 1,7-Di- <i>epi</i> -α-Cedrene ^{d,*}	1596 ± 158	2.9 ± 0.9			
35 Bergamotene ^{d,*}	387 ± 39	3.8 ± 1.2		4.3 ± 2.7	
36 γ-Muurolene ^{d,*}	691 ± 80	1.1 ± 0.4			
37 α-Humulene ^{d,*}	1352 ± 139	2.7 ± 0.9	0.1 ± 0.04	1.1 ± 0.4	0.2 ± 0.1
38 Germacrene D isomer*		0.79 ± 0.23			
39 γ-Curcumene*	63 ± 8	1.1 ± 0.4			
40 β-Farnesene	138 ± 14				
41 Unknown sesquiterpene 4	482 ± 48				
42 α-Muurolene	103 ± 14				
43 Unknown sesquiterpene 5	9574 ± 1205				

Table 1 (continued)

Compound name	Leaf extract (ng · g ⁻¹ fr wt)	Herbivory (ng · cm ⁻² hr ⁻¹)		Methyl jasmonate (ng · cm ⁻² hr ⁻¹)	
		Wounded	Unwounded	Treated	Untreated
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
44 Germacrene D ^{d,*}	10402 ± 1670	31.3 ± 12.2	0.92 ± 0.03	42 ± 21	1.5 ± 0.9
45 <i>cis</i> -β-Guaiene ^{d,*}	1075 ± 97	7.3 ± 3.2	0.8 ± 0.1	1.0 ± 0.8	
46 γ-Cadinene ^f	267 ± 24	0.5 ± 0.01		1.5 ± 0.5	
47 <i>trans</i> -β-Guaiene ^{d,*}	1427 ± 143	2.9 ± 0.04			
48 β-Bizabolene	146 ± 17			0.7 ± 0.4	
49 Unknown sesquiterpene 6	78 ± 11	0.25 ± 0.01			
50 Unknown sesquiterpene 7	193 ± 11				
51 Unknown sesquiterpene 8	435 ± 43				
52 δ-Cadinene ^{d,*}	493 ± 51	0.75 ± 0.26			
53 β-Curcumene	135 ± 9				
54 Unknown sesquiterpene 9	84 ± 15	0.25 ± 0.07			
55 Unknown 4	32 ± 1				
56 Unknown 5	121 ± 26				
57 Germacrene D 4-ol	2232 ± 207				
58 Oxygenated sesquiterpene 1 ^{d,*}	54 ± 8	0.95 ± 0.24			
59 Oxygenated sesquiterpene 2	121 ± 26				
60 Unknown 6	65 ± 12	0.18 ± 0.04			
61 Oxygenated sesquiterpene 3 ^{d,e,*}		1.7 ± 0.4			
62 Oxygenated sesquiterpene 4	935 ± 68				
63 Oxygenated sesquiterpene 5	1252 ± 96				
64 Oxygenated sesquiterpene 6	84 ± 12				
65 β-Eudesmol	97 ± 17				
66 Oxygenated sesquiterpene 7	60 ± 5				
67 Oxygenated sesquiterpene 8	81 ± 11				
68 α-Bizabolol ^{d,*}	50 ± 5	3.4 ± 1			
69 Oxygenated sesquiterpene 9 ^{d,*}	698 ± 68	0.98 ± 0.2			
70 Oxygenated sesquiterpene 10	49 ± 0				
71 Unknown 7	66 ± 12	0.46 ± 0.1			
72 Oxygenated sesquiterpene 11	72 ± 10				
73 Oxygenated sesquiterpene 12	269 ± 21				
74 Oxygenated sesquiterpene 13	190 ± 14				

^a Five g fresh weight leaf material extracted in pentane; *N* = 5.

^b Values are grand mean ± SE of all sampling intervals from entire study; *N* = 70.

^c Methyl jasmonate exposure of 18 hr, followed by 1 hr VOC collection; *N* = 5.

^d Compounds used in principal components analysis.

^e VOCs detected only in response to herbivory.

^f VOCs detected only in response to methyl jasmonate treatment.

*Significant difference between treatments (*P* < 0.05); repeated-measures ANOVA (herbivory), *t* test (methyl jasmonate).

termed “green leaf volatiles” are produced from the catalytic activity of hydroperoxide lyase when cell membranes are damaged. They are among the most ubiquitous and earliest volatiles released in response to herbivory, and may have VOC-inducing capabilities (Frag and Paré, 2002). Green leaf volatiles, while presumably emitted in *I. frutescens* in response to herbivory, were not detected due to contamination from an unknown source in the portion of the chromatogram where these volatiles would have been detected.

Identification of Constitutive Leaf VOCs

Five g of fresh leaf material were collected from undamaged plants, and lipophilic components were extracted from the leaves in 20 ml GC/MS grade pentane by using a mortar and pestle to crush the leaves. Ten ml of the leaf extract were collected into a 50-ml polypropylene tubes and centrifuged for 10 min at 3600 rpm. One hundred and fifty μ l aliquots were collected and analyzed by the GC/MS protocol described above. The volatiles obtained from the leaf extraction were used as a reference for preformed VOCs contained within *I. frutescens*, and also to generate reliable mass spectra for the identification of VOCs emitted in smaller quantities. Tentative identification of the compounds was carried out as described above.

Methyl Jasmonate Treatment

Five *I. frutescens* plants were exposed to exogenous MeJA based on the protocol described by Rodriguez-Saona et al. (2001). Briefly, 50 μ l of a 9:1 (ethanol/MeJA) mixture were applied to two cotton-tipped applicators (100 μ l total) and placed at the base of a single *I. frutescens* branch without physically touching any leaf material. Branches to be exposed to MeJA were enclosed in collection bags. Control plants were maintained in a separate growth chamber, and exposed to 100 μ l of ethanol. Following an overnight 18-hr exposure period, cotton applicators were removed, new collection bags were placed over the treatment branch, and VOCs were immediately sampled for 1 hr.

Statistical Analyses

The experimental design consisted of a series of repeated measures on individual plants over time. Therefore, we used multivariate analysis of variance (MANOVA) and repeated-measures analysis of variance (ANOVA) to test for main effects on emissions of multiple or single VOCs, respectively. Individual VOCs with significant main effects ($P < 0.05$) were compared across time using Tukey tests to correct for multiple comparisons. VOC emissions in response to MeJA treatment were analyzed using a *t* test. All statistical tests were carried out using SAS V8.2 (SAS Institute, Cary, NC, USA). We also compared VOC emissions across time and treatment by using principal components analysis of VOC emissions. Twenty-two individual VOCs, comprising >0.5% at any sampling time, were used in the analysis. Principal components analysis reduces multivariate data to a smaller set of orthogonal, uncorrelated components that account for the maximum amount of variability. The first principal component accounts for the greatest amount of variation in the data, while additional components account for successively smaller amounts of variation.

Results

Constitutive VOCs

Iva frutescens leaves contain a complex mixture of secondary compounds dominated by monoterpenes and sesquiterpenes (Table 1). Sixty-one compounds comprising $63.2 (\pm 5.5) \mu\text{g g fr wt}^{-1}$ of material were recovered by pentane extraction. Fifty-three compounds were identified either to a specific compound or, at the least, a class of terpene based on molecular ions in mass spectra, while eight compounds could not be reliably identified to any class. Sesquiterpenes and oxygenated sesquiterpenes were the most diverse class recovered, followed by the monoterpenes. The major constituents (each >5%) of the total mixture in order of decreasing concentration were limonene, germacrene D, unknown sesquiterpene 3, β -caryophyllene, and α -pinene (Table 1).

Herbivore-Induced VOC Emissions

Unwounded *I. frutescens* plants constitutively emitted a mixture of eight detectable VOCs consisting of the monoterpenes α -pinene, sabinene, β -pinene, limonene, and the sesquiterpenes β -caryophyllene, α -humulene, germacrene D, and *cis*- β -guaiene, all of which occur in high concentration within leaf material (Table 1). The production of these VOCs in unwounded plants did not differ over the 72-hr sampling period. Table 1 also contains the mean for all compounds recovered in response to herbivory across the sampling periods, which on average were higher than unwounded plants.

Statistical analysis of the dataset by MANOVA and repeated-measures ANOVA yielded similar results (Table 2). Herbivore wounding has a significant effect on the emission of VOCs over time ($P < 0.001$) (Table 2, Fig. 1). Significant interactions between treatment and sampling period were detected in repeated-measures ANOVA, indicating that the treatment effect varied significantly with time (Table 2). No significant nested effects of plant within treatment were observed (Table 2). Beetles feeding on *I. frutescens* caused total VOC emissions to increase in

Table 2 Manova test (A) of treatment effect on all volatile organic compounds over the entire study and repeated-measures anova (B) on total volatile emissions from herbivore-wounded and unwounded *Iva Frutescens*^a

	Value	F value	df	df	P value
(A) MANOVA					
Wilks' lambda	0	8.25	387	411.7	<0.001
Pillai's trace	6.472	3.1	387	468	<0.001
(B) Repeated-measures ANOVA					
Treatment		91.55	1	10	<0.001
Time		11.31	9	86	<0.001
Treatment \times Time		10.56	9	86	<0.001
Plant (Treatment)		1.77	10	86	0.078

^aForty-two VOCs from seven wounded and unwounded plants used in analysis. Individual VOCs determined significant by repeated-measures ANOVA were analyzed for differences over time using Tukey multiple comparisons.

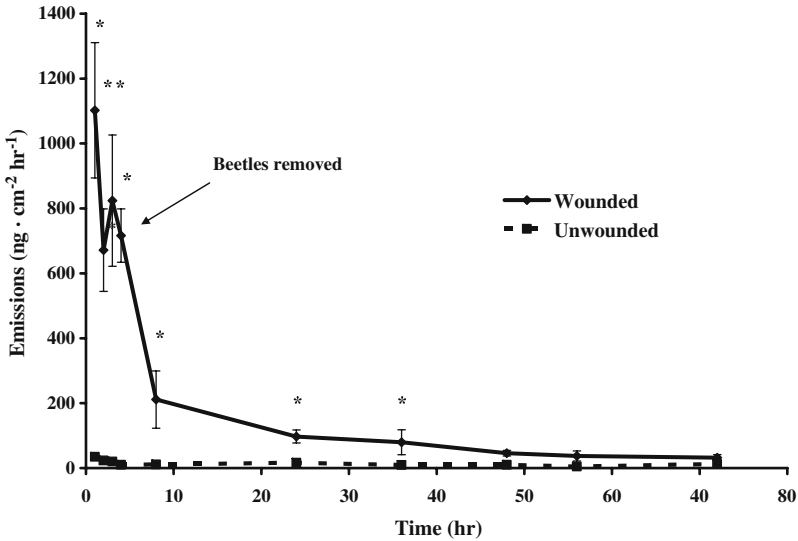


Fig. 1 Total emissions collected from herbivore wounded and unwounded *Iva frutescens* over 72 hr in response to 4 hr of herbivory by *P. aterrima*. Sample periods 1–4 collected during wounding. Sample periods 5–10 collected after wounding. Data points represent mean \pm SE of seven plants. Asterisk (*) indicates significantly increased emissions in the herbivore wounded treatment (Tukey, $P < 0.05$)

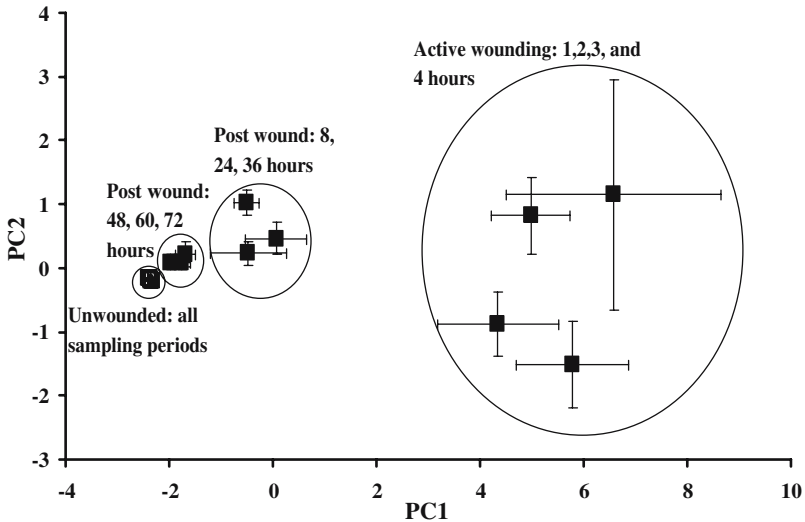


Fig. 2 Clustering pattern of sampling periods for wounded and unwounded *I. frutescens* in principal component space. Data points are mean \pm SE of principal component scores for seven wounded and unwounded plants at each time period

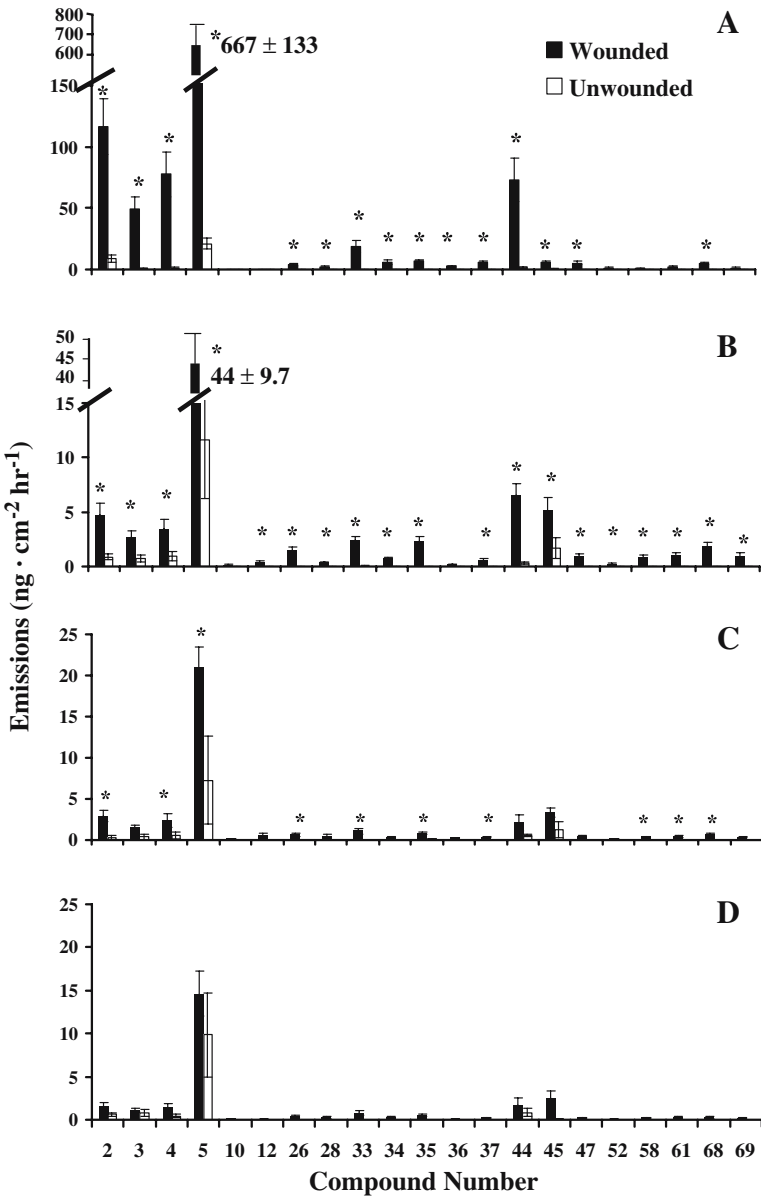


Fig. 3 Volatile organic compound (VOC) blend emitted from wounded (closed bars) and unwounded (open bars) *I. frutescens* during the first hour of herbivory (A), 24 hr (B), 48 hr (C), and 72 hr (D) after initial wounding by *P. aterrima* beetles. Twenty-two VOCs greater than 5% of the total blend are plotted. Values are mean ± SE of seven plants. Identities of compounds are listed numerically in Table 1. Asterisk (*) indicates significant difference from unwounded controls (Tukey, $P < 0.05$)

the first 4 hr during feeding compared to unwounded controls ($P < 0.05$) (Fig. 1). Herbivory caused a 22-, 14-, 16-, and 14-fold increase in total VOC emissions during hours 1, 2, 3, and 4, respectively. Following the 4hr feeding period, total VOC emissions declined dramatically, but remained elevated up to 48 hr after initial wounding ($P < 0.05$) (Fig. 1).

The influence of herbivory on VOC emissions over time is also displayed in principal component space (Fig. 2). Mean principal component scores at each sampling period are plotted for wounded and unwounded *I. frutescens*, and describes the influence of time and wounding on the variation in VOC emissions. Principal component 1 accounts for 61% of the total variation in the data, while component 2 accounts for 10%. Wounded plants show a steady relaxation toward unwounded plants, and four distinct clusters are seen over time (Fig. 2). The clusters indicate sampling periods with similar variation in VOC emissions. Active herbivore wounding sampling periods 1–4 hr, postwounding sampling periods 8, 24, and 36 hr, postwounding samplings periods 48, 60, 72 hr, and all unwounded principal component scores show distinct associations over time in principal component space (Fig. 2).

Herbivory caused a distinct compositional change in the total blend by stimulating the emission of 42 compounds including most constitutive VOCs. VOCs comprising greater than 0.5% of the total blend are displayed in Fig. 3 (22 total compounds). With the exception of the monoterpenes α -pinene, sabinene, β -pinene, and limonene and the sesquiterpenes β -caryophyllene and Germacrene D, all other VOCs were present in extremely small quantities (all less than 1% of the total blend). However, most of these VOCs remained significantly elevated 2 d after wounding, and persisted for the 72hr duration of the experiment. Two novel compounds were detected in the herbivore treatment, *p*-cymene, and an unidentified oxygenated sesquiterpene 3 (Figs. 3 and 4). These two compounds were unique in that they were not detected in *I. frutescens* leaf extracts, nor in MeJA-induced emissions (discussed below). Significant emissions of the unique oxygenated sesquiterpene 3 were immediately detected in response to herbivory (Tukey, $P < 0.05$), and these emissions remained significantly elevated 24 hr after herbivory (Fig. 4).

p-Cymene, nonatriene, and unidentified sesquiterpene 3 exhibited a delayed emission pattern that was distinct from VOCs released immediately upon wounding (Fig. 4). These compounds were not detected in wounded *I. frutescens* plants during the initial 2 hr of herbivory. Beginning at the third hour, they were detected in only one *I. frutescens* plant, which accounts for the large amount of variability in emission at that collection period. Significant emissions of unidentified sesquiterpene 3, *p*-cymene, and nonatriene were not detected until 4, 24, and 36 hr, respectively (Tukey, $P < 0.05$) (Fig. 4). Nonatriene and *p*-cymene emissions rapidly waned 48 hr after herbivory, but remained detectable throughout the experiment (Fig. 4). Unidentified sesquiterpene 3 emissions were not detected in *I. frutescens* after 24 hr (Fig. 4). The relationship between the constitutive and novel VOCs in *I. frutescens* is also shown in the plot of VOC loading factors in principal component space 1 and 2 (Fig. 5). Loading factors indicate the similarity between individual VOCs and the principal components on which they are plotted. All 22 VOCs used in the analysis have positive loadings on principal component 1, and most of the major constitutive mono- and sesquiterpenoids are closely associated in principal component space (Fig. 5). The herbivore novel compounds [*p*-cymene (10) and oxygenated

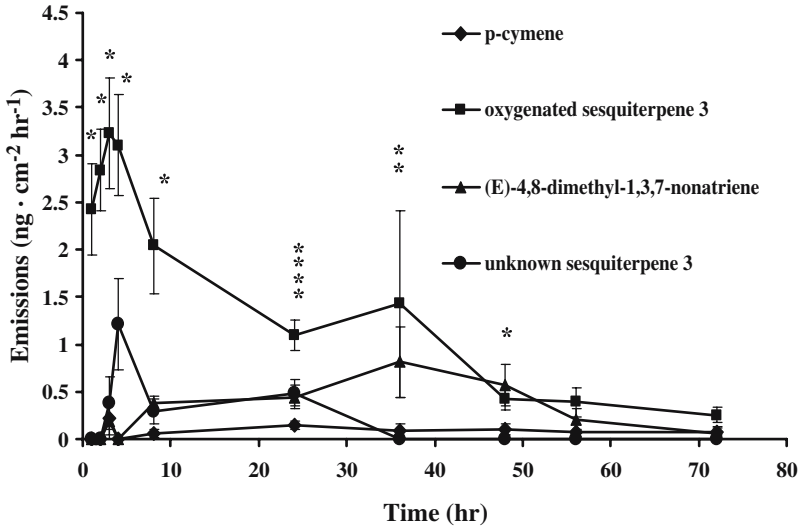


Fig. 4 Delayed volatile organic compound (VOC) emissions [*p*-cymene, oxygenated sesquiterpene 3, and (*E*)-4,8-dimethyl-1,3,7-nonatriene], and unique VOC emissions (*p*-cymene, and oxygenated sesquiterpene 3) from herbivore wounded *I. frutescens*. Asterisk (*) indicates significant difference from untreated controls (Tukey, $P < 0.05$). Values are mean \pm SE of seven plants

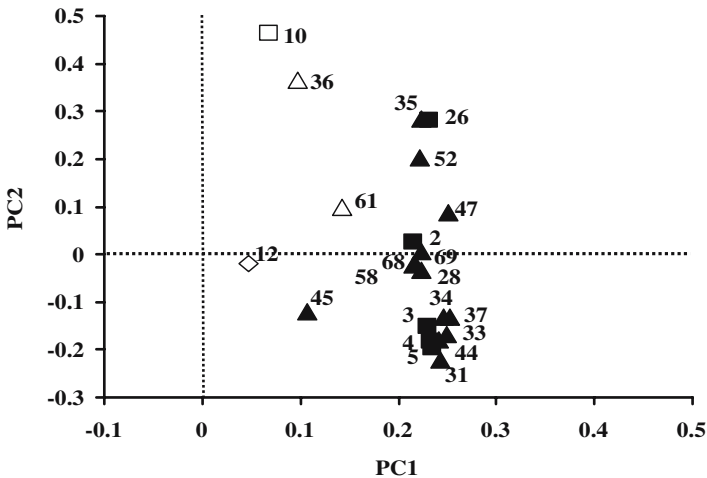


Fig. 5 Relationship of 22 major individual VOC loadings ($\geq 0.5\%$ of total blend) in principal component space. Numbers correspond to compounds listed in Table 1. The intersection of the dotted lines represents the origin of the VOC loading factors. VOC loadings were obtained from the factor pattern matrix of the principal components analysis, and represent the correlations between VOCs and the principal components on which the values are plotted. Thus, the distance between any two VOCs is an indication of the relationship between them. Similar shapes represent a class of terpenoid. Squares = monoterpenoids, triangles = sesquiterpenoids, diamonds = homoterpenes. Closed shapes = constitutive VOCs. Open shapes = delayed/herbivore specific VOCs

sesquiterpene 3 (**61**)] and the delayed VOC, nonatriene (**12**), do not cluster with any of the major constitutive terpenoids (Fig. 5).

MeJA-Induced VOC Emissions

Exposure of *I. frutescens* to MeJA caused a 10-fold increase in total VOC emissions compared to untreated controls ($P < 0.01$) (Fig. 6). MeJA-elicited VOC emissions were similar to, yet distinct from, VOC blends emitted from herbivore-treated *I. frutescens*. The blend of VOCs emitted in response to MeJA was less complex compared to the blend released in response to herbivory. MeJA-treated plants emitted a total of 28 compounds compared to 42 released in response to herbivory (Table 1). Some compounds were unique to the MeJA-treated plants including (*E*)- β -ocimene, hexenyl butyrate, methyl butyrate, *cis*-jasnone, and methyl salicylate (Table 1). While MeJA elicited the emission of novel VOCs, it also stimulated significant emissions of constitutive VOCs (Fig. 7). MeJA stimulated the release of nonatriene, and caused a 14-fold increase in α -pinene emissions, a 5-fold increase in sabinene, and an 8-fold increase in limonene emissions (Fig. 7). The herbivore wounding and MeJA treatment results can not be statistically compared because they were conducted as separate experiments; however, it is interesting to observe the similarities in some of the VOCs emitted from *I. frutescens* 24 hr after wounding, and after 18 hr of MeJA exposure (Figs. 6 and 7).

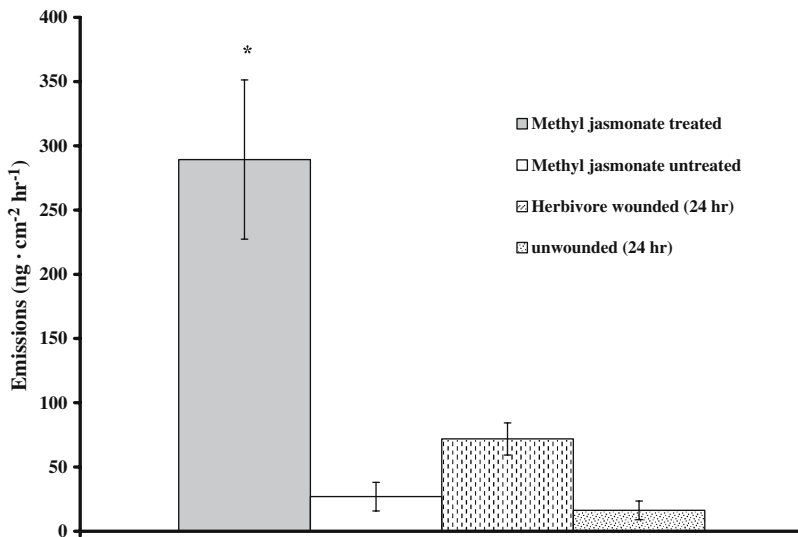


Fig. 6 Total volatile organic compound (VOC) emissions from methyl jasmonate treated and untreated; herbivore wounded and unwounded (24 hr after initial wounding) *I. frutescens*. Herbivore and methyl jasmonate experiments were not simultaneously conducted, preventing statistical comparison of the two treatments. Methyl jasmonate treated and untreated bars represent mean \pm SE of five plants. Asterisk (*) indicates significant difference from untreated controls (*t* test, $P < 0.05$). Herbivore wounded and unwounded bars represent mean \pm SE of seven plants

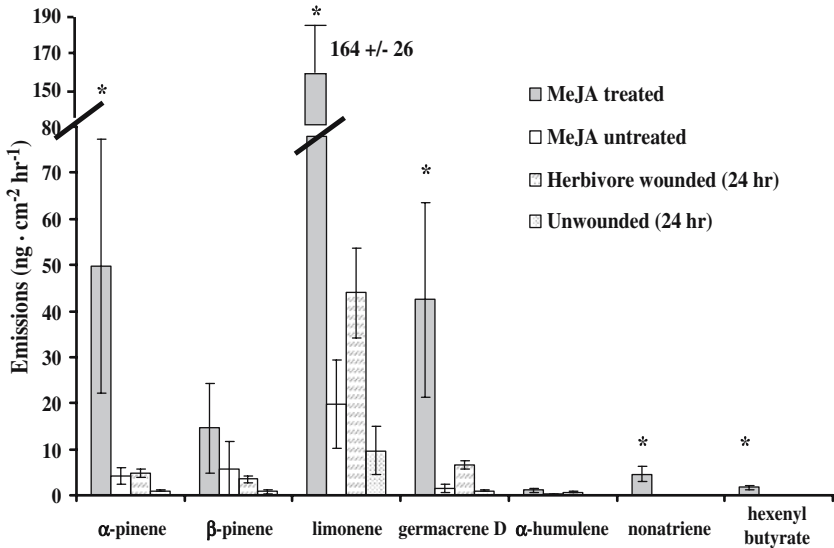


Fig. 7 Individual constitutive and nonconstitutive volatile organic compounds (VOCs) emitted from methyl jasmonate treated and untreated *I. frutescens*, and herbivore wounded and unwounded (24 hr after initial herbivory) *I. frutescens*. Methyl jasmonate treated and untreated bars represent the mean ± SE of five plants. Herbivore wounded and unwounded bars represent the mean ± SE of seven plants. Asterisk (*) indicates methyl jasmonate treatment is significantly different from methyl jasmonate untreated plants (*t* test, ****P* < 0.05)

Discussion

Many plants respond to insect attack by releasing VOCs that are known to attract natural enemies of herbivores (Paré and Tumlinson, 1999). A short 4-hr feeding period by *P. aterrima* on *I. frutescens* caused a dramatic quantitative and qualitative change in VOC emissions over time. Herbivory induced the emissions of both constitutive and novel VOCs, and dramatically changed the composition of the total blend released. Unwounded *I. frutescens* emitted a mixture of eight components that were detected by gas chromatography. Wounded *I. frutescens*, however, emitted a mixture of 42 compounds most of which are stored constitutively in the plant and only released in significant quantities in response to herbivory. Principal components analysis by treatment and time also shows the effect of wounding on VOC emissions. Sampling periods grouped into four main clusters, indicating the similarity in variation of VOC emissions at those time periods. The aggregation of postwounding sampling periods 8, 24, and 36 hr, separate from active wounding sampling periods, and the remaining postwounding sampling periods (Fig. 2), may be the result of the release of novel and delayed VOC emissions that persisted mainly during these sampling periods.

VOCs are produced by two main biochemical pathways. The cytosolic mevalonic acid pathway produces the sesquiterpenes, and the plastidal methyl erythritol phosphate pathway produces the monoterpenes (Lichtenthaler et al., 1997). Both pathways result in the formation of precursors that react with specific terpene synthases

to produce a wide variety of VOCs. Upon formation, VOCs are typically localized to specialized secretory cells where they may be stored. In this system, the quantitative and qualitative increase in VOC emissions in response to herbivory may be the result of several mechanisms. (1) The release of novel induced VOCs may be the result of *de novo* synthesis. (2) The increased emissions of constitutive VOCs may be the result of increased synthesis due to herbivore/MeJA up-regulation of constitutive terpene synthases. (3) Increased emission of constitutive VOCs may be the result of passive release from stored pools upon tissue breakage. The high emission during active feeding suggests mechanical release can be an important means for herbivore-stimulated VOC emissions. Casual observation of *I. frutescens* leaves indicates an abundance of nonglandular stellate trichomes. No obvious specialized storage structures were detected in cross sections of leaf material; however, it was observed that simple abrasion of the leaves results in no release of odorous compounds. Mechanical disruption of the lamina (breaking leaves), however, produces an immediate burst of odorous material, suggesting internal storage of compounds. Therefore, when cells are disrupted, an immediate burst of odor indicates that mechanism (3) may be in operation. While all three mechanisms may be operating to some degree during herbivory, the results of the MeJA-treated plants suggest that mechanisms (1) and (2) are responsible for the sustained increase in VOC emissions. Constitutive as well as novel VOC emissions were significantly elevated in undamaged leaf tissue exposed to MeJA, suggesting that the elevated emissions are not simply due to passive volatilization from ruptured storage structures.

Jasmonates are potent regulators of several defense pathways in plants, and both JA and its methyl conjugate, MeJA, have been directly implicated in the regulation of VOCs in plants (Reymond and Farmer, 1998; Walling, 2000; Farmer et al., 2003). Our results indicate that *I. frutescens* responds to MeJA treatment by emitting several VOCs, some of which were detected from the herbivore-wounded *I. frutescens*, and some that were unique to the MeJA treatment. MeJA-induced multiple biosynthetic pathways in *I. frutescens* including the shikimic acid pathway (producing methyl salicylate), the octadecanoid pathway (producing *cis*-jasmonone), as well as the mevalonate-dependent and -independent terpenoid pathways (producing mono- and sesquiterpenes). MeJA stimulates these same pathways in agricultural species such as tomato, lima bean, and corn (Thaler et al., 1996; Dicke et al., 1999; Schmelz et al., 2003). Martin et al. (2003) recently demonstrated that MeJA application induced the activities of both constitutive and novel terpene synthases in Norway spruce. The up-regulation of constitutive and novel VOCs suggests that *I. frutescens* invests in the production of both constitutive and novel VOCs following herbivore attack.

The function of VOCs has been debated ever since their discovery. Terpenes may function as direct defenses by deterring herbivores (Karban and Baldwin, 1997). They may also protect vital photosynthetic material from oxidative stress, or provide thermal tolerance and UV-B protection to leaf material (Niinemets et al., 2004). VOCs can influence the behavior of herbivores as well as predators and parasitoids (Paré and Tumlinson, 1999; Heil, 2004). A large quantitative and qualitative increase in VOC emissions was detected in response to a short 4-hr feeding period. In nature, *P. aterrima* occur in high abundances. Larvae emerge in early spring (April), develop into adults by late spring (May), and continue feeding through midsummer (July). Because of these extended feeding periods by *P. aterrima*, field emissions of constitutive and induced VOCs may be even more dramatic than those detected in this laboratory assay. We speculate that while the production of large

amounts of constitutive VOCs may provide some direct defensive and/or physiological functions, the large variation in VOC emissions in response to herbivory provides a basis for trophic interactions in this system. How effective VOCs may be at serving as infochemicals to higher trophic levels has yet to be determined in this setting. However, it has been shown that constitutive VOC emissions play an important role in increasing the effectiveness of foraging parasitoid wasps (Fukushima et al., 2002). Many studies aimed at understanding the ecological function of VOCs in nature have focused on agricultural species planted in monoculture field plots. In these settings, the chemical landscape is relatively homogenous compared to a native plant assemblage. *I. frutescens* coexists with several odorous plant species that contain their own unique VOC profiles (Wang, 2001), and the background levels of VOCs in the atmosphere may be considerably more heterogeneous relative to an agricultural field. Whether herbivores and predators/parasitoids can exploit VOC emissions in this chemically complex landscape remains to be determined. *P. aterrima* specializes on *I. frutescens* (Wang, 2001), and may exploit components of *I. frutescens* VOC mixture to locate host-plant material.

While most compounds were immediately detected upon herbivory, some constitutive compounds (nonatriene) as well as the novel VOCs (*p*-cymene) exhibited a delayed emission pattern. Our results showed that nonatriene and *p*-cymene were not detected in significant quantities until 24 hr after herbivory, and peak emissions occurred 36 hr after initial herbivory. While we did not assay enzyme activity, the results suggest that novel compounds, and those showing delayed emissions may be the result of a herbivore-specific response, whereas the immediate release and increased emission of constitutive VOCs may indicate a general wound response. In some systems, herbivore oral secretions are required to stimulate emissions of novel VOCs, and these emissions are typically induced hours or days after herbivory (Alborn et al., 1997; Boumeester et al., 1999; Degenhardt and Gershenson, 2000). Furthermore, nonatriene, *p*-cymene, and oxygenated sesquiterpene 3 were not associated with the major constitutive VOC emissions in principal component space (Fig. 3). The placement of these compounds away from the major constitutive mono- and sesquiterpenes may be the result of a difference in the dynamics or regulation of their emissions. While many constitutive VOCs were released immediately in response to herbivory and rapidly waned thereafter, some constitutive and novel VOCs had a different dynamic, with prolonged sustained emissions following herbivory.

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Experience Induces a Phytophagous Insect to Lay Eggs on a Nonhost Plant

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Abstract Experience of nonhost plants by phytophagous insects may alter their foraging and oviposition responses to these plants. Using the diamondback moth (DBM) *Plutella xylostella* (L.), its host-plant Chinese cabbage, and a nonhost-plant *Pisum sativum* (pea) as a model system, we examined whether experience of the nonhost plant by adults can induce oviposition on the nonhost plant. Naive DBM females did not accept pea for oviposition in either no-choice or choice conditions, whereas females with prior experience of pea laid up to 20% of their eggs on this plant. Naive females reduced their oviposition, but experienced females did not refrain from laying eggs in a nonhost-plant environment. Such habituation to nonhost plants could lead to host range expansion in phytophagous insects and increase mortality of pest insects in diversified crop systems.

Keywords Experience · Habituation · Oviposition · Nonhost plant ·
Host range expansion · *Plutella xylostella* · *Pisum sativum*

Introduction

The modification of host selection behavior by experience is a widespread phenomenon in phytophagous insects (Papaj and Prokopy, 1989; Bernays, 1995) and insect parasitoids (Turlings et al., 1993). The experience-altered behavior allows the insects to maintain a rigid, genetically determined rank of hosts, with retained flexibility to more successfully deal with uncertainty in the environment (Papaj and Prokopy, 1989; Turlings et al., 1993; Cunningham et al., 2001). In phytophagous insects, experience-induced changes in host selection behavior by larvae, such as habituation to feeding deterrents, have been well documented (Jermy, 1987; Bernays, 1995); but experience-induced changes of oviposition behavior have been reported only in a few species (e.g., Liu et al., 2005). Using the diamondback moth

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(DBM), *Plutella xylostella* L., its host-plant Chinese cabbage, and a nonhost-plant *Chrysanthemum* as a model system, Liu et al. (2005) showed that experience of a nonhost-plant extract by DBM adults resulted in a higher attraction of moths to host plants treated with the nonhost-plant extracts. These females laid more eggs on host plants treated with the extract. They speculated that this kind of habituation or induced preference for chemicals from nonhost plants may increase the tendency of insects to accept nonhost plants for oviposition and thus play a key initial role in host range expansion and the evolution of insect–plant relationships. Experiments presented here demonstrate that adult experience of a nonhost plant by a specialist herbivore can alter its oviposition behavior to accept the nonhost plant for oviposition.

Methods and Materials

Insects and Plants

The DBM culture was established from a field collection on a cabbage farm in a suburb of Hangzhou, China, and maintained on common cabbage (*Brassica oleracea* L. var. *capitata*, cultivar Jing-feng no. 1) in a temperature-controlled room at $26 \pm 1^\circ\text{C}$, 60–70% RH, and 14-hr light/10-hr dark photoperiod. The host-plant Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*, cultivar Zao-shu no. 5) and the nonhost-plant pea (*Pisum sativum* L. var. *arvense*, cultivar Qiwangyiji) were used in the oviposition tests. All experiments utilized potted Chinese cabbage grown to the fifth to sixth fully extended true leaf stage and potted pea grown to the 20th–25th fully extended true leaf stage in greenhouses. In oviposition choice tests, care was taken to use host plants and nonhost plants that had similar leaf areas.

Survival of DBM on Host and Nonhost Plants

Two hundred DBM eggs, deposited on plastic sheets, were randomly taken from the DBM culture. They were randomly divided into two cohorts of 100 each. Eggs of one cohort were placed onto four plants of Chinese cabbage, and those of the other onto four plants of pea. The two cohorts were reared in a temperature-controlled room at $26 \pm 1^\circ\text{C}$, 60–70% RH, and 14-hr light/10-hr dark photoperiod. Daily observations were made on the development, feeding, and survival of the two cohorts.

Experience Treatments

We used the following three types of experience treatments for test insects:

- (1) No experience. Insects were reared on common cabbage from egg to pupation. Pupae were collected 2–3 d after pupation and placed in a clean, ventilated cage ($55 \times 55 \times 55$ cm) for adult emergence and mating. Adults were provided with 15% honey-water as food. We collected mated females at 2–3 d postemergence for oviposition tests.
- (2) Experience after emergence. Insects were reared the same way, and adults were collected at the same age as that in (1), except that 8 hr prior to the

oviposition tests, five potted pea plants were placed into the emergence cage for the adults to experience. We collected mated females directly from the cage for oviposition tests.

- (3) Experience during and after emergence. Insects were reared on common cabbage from egg to late fourth instar, and then the mature larvae were moved manually onto pea plants to force them to pupate on the nonhost plants. Insects were left to emerge naturally on the nonhost plants. Adults were provided with 15% honey-water and enclosed with the nonhost plants in a cage. We collected mated females at 2–3 d postemergence from the cage for oviposition tests. As only chemicals experienced during and after emergence can affect adult foraging and oviposition in DBM (Liu and Liu, 2006), the treatment here was termed “Experience during and after emergence,” despite the fact that insects were placed on pea from late fourth instar onward.

Oviposition Tests

We tested oviposition by DBM females from each of the three experience treatments in two no-choice tests and one choice test:

- (1) No-choice test using host plant. One plant of Chinese cabbage was placed in the center of a ventilated cage. The cage was constructed with a frame of metal wires covered on all sides with black plastic sheets. The cage had an internal volume of $40 \times 40 \times 40$ cm with two 15×15 cm screened windows on the front and back sides for ventilation. At 19:00 hr, four females were released into each cage (no food was provided). After 12 hr in darkness at 26°C, the moths were removed, and all eggs deposited on each plant, pot, and the inner surface of the cage were counted.
- (2) No-choice test using nonhost plant. The same procedure as in (1) was used, except that pea was used as test plant.
- (3) Choice test using host and nonhost plants. One Chinese cabbage and one pea were placed in diagonally opposite corners of a ventilated cage ($55 \times 55 \times 55$ cm), so that the foliage of the two plants was approximately 20 cm apart at the closest point. At 19:00 hr, four females were released into the center of each cage. After 12 hr in darkness at 26°C, the moths were removed, and the eggs deposited on each of the two plants, the pots, and inner surface of the cage were counted.

In all, there were nine experience \times plant treatments as shown in Table 1. Ten replicates (four moths per replicate) were conducted for each treatment.

Data Analysis

Proportional data were transformed by arcsine square root for analysis of variance (ANOVA) and were back-transformed to percentages for presentation. The mean number of eggs in the nine plant (three levels) \times experience (three levels) treatments was analyzed by a two-factor ANOVA. The mean proportions of eggs deposited on host or nonhost plants were compared between the three experience treatments by using one-way ANOVA. Means were compared pairwise using the least significant difference procedure.

Table 1 Mean number of eggs laid by diamondback moths (*Plutella xylostella*) in each of the nine plant × experience treatments

Plant	Level of experience of pea			Mean ± SE of three experience treatments
	No experience	After emergence	During and after emergence	
Cabbage	120.3 ± 17.8 a ^a	103.2 ± 13.3 a,b	68.6 ± 7.4 d,e	97.4 ± 8.7 a ^b
Pea	45.9 ± 7.6 e	105.3 ± 11.4 a	61.5 ± 8.8 d,e	70.9 ± 8.7 b
Cabbage and pea	83.3 ± 8.8 b,c,d	98.7 ± 8.2 a,b,c	71.7 ± 8.6 c,d,e	84.3 ± 8.7 a,b
Mean ± SE of three plant treatments	83.1 ± 8.7 b ^c	102.4 ± 8.7 a	67.2 ± 8.7 b	

^a Means ± SE of the nine plant × experience treatments followed by the same letter do not differ significantly ($P < 0.05$).

^b Means ± SE of this column followed by the same letter do not differ significantly ($P < 0.05$).

^c Means ± SE of this line followed by the same letter do not differ significantly ($P < 0.05$).

Results

Survival of DBM on Host and Nonhost Plants

On Chinese cabbage, 95 of the 100 eggs hatched, the larvae were fed and pupated normally, and in 17 d, 68 moths in total emerged. On pea, 96 of the 100 eggs hatched but all died as first instar larvae within 2 d of hatching, although there was some feeding on the leaves by the larvae.

Number of Eggs Laid

Both test plant and experience had significant effects on the number of eggs laid (plants: $F = 4.57$, $df = 2,81$, $P = 0.013$; experiences: $F = 8.09$, $df = 2,81$, $P < 0.001$), as did the interactions of plants × experiences ($F = 3.90$, $df = 4,81$, $P = 0.006$). Females provided with Chinese cabbage laid the highest number of eggs, followed by females provided with both Chinese cabbage and pea, and then by those provided with only pea (Table 1). As for the effects of experience, females in the “After emergence” treatment laid significantly more eggs than females of the “No experience” or those of the “During and after emergence” treatments (Table 1).

The number of eggs laid on Chinese cabbage by females of the “No experience” treatment can be regarded as the normal level of oviposition by the test insects. When provided with Chinese cabbage, females of the “After emergence” treatment laid a similar number of eggs to the normal level, but the females of the “During and after emergence” treatment laid significantly fewer eggs (Table 1). When provided with pea, females in the “No experience” treatment laid only one third of the eggs, and the females of the “During and after emergence” treatment also laid significantly fewer eggs compared to the normal level; in contrast, females in the “After emergence” treatment laid a similar number of eggs to the normal level (Table 1). When provided with both Chinese cabbage and pea, females in both the “No experience” and “During and after emergence” treatments laid significantly fewer

eggs, but the females in the “After emergence” treatment again laid a similar number of eggs compared to the normal level (Table 1).

Egg Locations

In nonchoice tests using Chinese cabbage, females in the “No experience,” “Experience after emergence,” and “Experience during and after emergence” treatments laid on average 88, 77, and 82% of their eggs on the host plants, with the remainder on pots and cage surface (Fig. 1A). The proportions of eggs laid on cabbage did not differ among the three treatments ($F = 1.200, df = 2, P = 0.317$).

In nonchoice tests using pea, females in the “No experience,” “Experience after emergence,” and “Experience during and after emergence” treatments laid on average 4, 26, and 18%, respectively, of their eggs on the nonhost plants, with the remainder on pots and cage surface (Fig. 1B). The proportions of eggs deposited on pea differed among the three treatments ($F = 4.046, df = 2, P = 0.029$). The proportion of eggs laid on pea by females in the “Experience during and after emergence” treatment was higher than that of the “No experience” treatment ($P = 0.009$; Fig. 1B). The proportion of eggs laid on pea by females in the “Experience

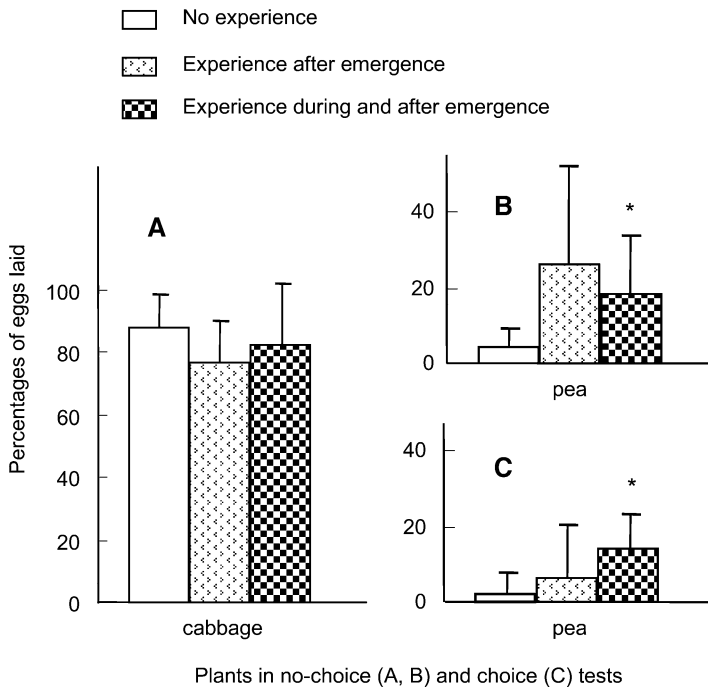


Fig. 1 Oviposition by naive and experienced *Plutella xylostella* females on host and nonhost plants in no-choice and choice tests. (A) Percentages of eggs laid on Chinese cabbage (of the total number of eggs laid on the plants and cages) in no-choice test. (B) Percentages of eggs laid on pea (of the total number of eggs laid on the plants and cages) in no-choice test. (C) Percentages of eggs laid on pea (of the total number of eggs laid on the Chinese cabbage and pea plants) in choice test. Error bars indicate standard deviation, and an asterisk above the column in a diagram indicates significant difference between that treatment and no experience ($*P < 0.05$)

after emergence” treatment was apparently higher than that of the “No experience” treatment, but the difference between the two treatments was not significant because of large variation among replicates ($P = 0.081$; Fig. 1B).

In choice tests using Chinese cabbage and pea, females in the “No experience,” “Experience after emergence,” and “Experience during and after emergence” treatments laid on average 83, 79, and 80%, respectively, of their eggs on the two plants, with the remainder on pots and cage surface. Of the eggs laid on the two plants, the average proportions of eggs laid on pea were 2, 6, and 14% for females in the three experience treatments, respectively, (Fig. 1C; $F = 3.457$, $df = 2$, $P = 0.046$). Compared to the proportion of eggs laid on pea by females of “No experience” treatment, the proportion of the “Experience during and after emergence” treatment was significantly higher ($P = 0.015$), but that of the “Experience after emergence” treatment was not ($P = 0.364$).

Discussion

Survival of DBM on the two plants indicated that the pea cultivar used in this study is a nonhost plant of the local DBM population. Naive DBM females generally did not accept pea for oviposition in either no-choice or choice conditions, whereas females with prior experience of pea laid a significant proportion of eggs on this nonhost plant (Fig. 1). Thus, experience of a nonhost plant by adults of a phytophagous insect can produce significant habituation and alter its foraging/oviposition responses to lay eggs on the plant.

The females in the “During and after emergence” treatment laid a lower number of eggs than those of the other two experience treatments. Pupae and adults were apparently smaller, but we missed the opportunity to take measurements of body size and lifetime fecundity of the test insects. The reduction in size and subsequently fecundity were apparently caused by insufficiency of food during the late fourth instar. Because of the experimental procedure, we had to move the fourth instars from cabbage onto pea before they completely stopped feeding. Thus, mature larvae were imposed to food limitation. It is possible that the negative impact of the partial food limitation on fitness of the test insects might have also contributed to the change of oviposition preference and the increased acceptance of the nonhost plant for oviposition. The effect of larval food availability and/or quality on fitness parameters, such as body size, fecundity, and survival, has been extensively studied for insects (Honěk, 1993; Boggs and Freeman, 2005). However, to our knowledge, the effect of larval food limitation on oviposition preference between plants has not been examined for DBM or any other insects. The latter effect warrants experimental investigation. In nature, pupation on nearby nonhost plants may occur. We have occasionally encountered pupae of DBM on pea and cucurbit plants next to a cabbage field, especially when all cabbage plants in a large plot surrounded by noncrucifer crops are suddenly harvested (unpublished data).

When provided with only the nonhost-plant pea, naive females drastically reduced their oviposition, whereas experienced females did not. They laid 26% of their eggs on the nonhost plant (Table 1, Fig. 1). These results indicate that females with prior experience of a nonhost plant are less likely to refrain from laying eggs in

a nonhost-plant environment than naive females, which constitutes part of the experience-induced physiological/behavioral changes conducive to oviposition on nonhost plants.

Many phytophagous insects are capable of including new plant species within their diets, although the causes and rates of host-plant range expansion are poorly understood (Strong, 1979; Schoonhoven et al., 1998; Liu et al., 2005). In nature, adult females may repeatedly encounter nonhost plants during host foraging, depending on the distribution and abundance of plants in the landscape and their movement behavior. The results from this study suggest that experience may induce some females to lay large number of eggs on nonhost plants. There is often substantial individual variability in larval survival (Zalucki et al., 2002), and in many species, larvae can feed and survive on plants that are not usually accepted as oviposition substrates by adult females (Schoonhoven et al., 1998; Janz et al., 2001). If some of the offspring show an increased acceptance for the novel host plants as a result of either an inherited or a conditioned behavior, further oviposition on novel hosts could be the first step of a host range expansion (Larsson and Ekblom, 1995). Numerous studies of insect–plant associations have provided a general pattern of strong predominance of specialists over generalists and a strong taxonomic conservatism in host-plant utilization (Schoonhoven et al., 1998). However, studies on the issue of directionality in host range evolution have produced mixed results (Termonia et al., 2001; Janz, 2002). Evidence has been accumulating to suggest that host range is dynamic, evolving through repeated phases of expansion and contraction (Janz et al., 2001; Janz, 2002). Learning in oviposition, including habituation to nonhost plants as demonstrated in this study, may play an important role in assisting insects to explore new host plants when the herbivores are in the evolutionary phase of host range expansion.

The ability of the Hangzhou DBM population to survive on pea was tested with a limited number of individuals of both insects and plants. The variation of survival among individuals of the local DBM population needs to be investigated with large number of field-collected individuals and a range of cultivars of pea. Gupta and Thorsteinson (1960) recorded a low level of survival on pea in a Canadian DBM population in the laboratory. Recently, Löhr and Rossbach (2004) found a field DBM population on pea crops in Kenya and demonstrated that this population could survive well on both pea and kale.

This study suggests that even the so-called specialist phytophagous insects may show strong experience-induced responses to nonhost plants, and it also questions the conserved nature of oviposition preference, which is fundamental to weed biological control. Experience-altered foraging and oviposition behavior must be carefully considered in host specificity tests for selecting weed biological control agents (Bellows and Headrick, 1999).

In agricultural ecosystems, increase of vegetation diversity has often resulted in reduction of abundance of monophagous insect pests (Andow, 1991). In polyculture crop systems, insect pests may lay large number of eggs on nonhost crops, and large number of eggs laid on nonhost crops can contribute to increased mortality of monophagous insect pests (Ampong-Nyarko et al., 1994). Depending on the spatial arrangement of plants, the scale of the intercrops, and the movement ambit of the insects, intercropping may lead to repeated encounter of nonhost plants by the insects. Experience-induced habituation or preference for nonhost plants may be an

important mechanism contributing to oviposition on nonhost plants in these circumstances. To reduce the likelihood of host range expansion by insect pests, different nonhost plants may be alternated between seasons to suppress any survival on the nonhost crops.

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Isothiocyanates Stimulating Oviposition by the Diamondback Moth, *Plutella xylostella*

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Abstract Recognition of cabbage as a host plant for the diamondback moth (DBM) has previously been shown to depend on compounds that are extracted by soaking intact foliage in chloroform. Analysis of such chloroform extracts by open column chromatography has now resulted in the isolation of highly active fractions that elicit oviposition on treated filter papers. Further separation of these fractions by high-performance liquid chromatography revealed the presence of two distinct groups of active compounds that may be classified as volatile and non-volatile. The two prominent volatile components were separated and identified by mass spectrometry as the isothiocyanates, iberin (3-methylsulfinylpropyl isothiocyanate) and sulforaphane (4-methylsulfinyl-3-butenyl isothiocyanate). Subsequent bioassays of a range of isothiocyanates showed that iberin and sulforaphane were the most active of those tested. Other isothiocyanates with sulfur in the side chain were also active, whereas alkyl and phenyl isothiocyanates had only limited activity. In electrophysiological experiments, electroantennograms (EAGs) indicated positive responses of moth antennae to the isothiocyanates that were most active in behavioral assays. Since sulforaphane has been identified as a major inducer of anticarcinogenic activity in mouse tissue, a synthetic analog (exo-2-acetyl-5-isothiocyanatonorbornane) that shows similar inducer activity was tested on DBM. This bicyclic analog was highly active in both behavioral and EAG assays, suggesting similarity in receptor sites for the two types of biological activity.

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Introduction

The diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera: Plutellidae), is a major pest of cruciferous crops on a worldwide basis and has developed resistance to most insecticides, including *Bacillus thuringiensis* (Talekar and Shelton, 1993). Efforts to find new natural approaches to control this insect have focused on understanding the plant chemistry that plays a major role in acceptance or rejection of potential host plants. The involvement of glucosinolates in host recognition has been suggested by several studies (Gupta and Thorsteinson, 1960; Reed et al., 1989; Renwick and Radke, 1990). Furthermore, a synergistic effect of leaf waxes with a glucosinolate or with cabbage homogenates was demonstrated by Spencer (1996) and Spencer et al. (1999). Also, the involvement of plant volatiles in attracting moths to their host plants was shown by Palaniswamy et al. (1986) and by Pivnick et al. (1990), but no attempts to identify active compounds were reported.

More recent studies have shown that potent oviposition stimulants for the DBM are extracted from cabbage foliage by soaking the intact leaves in chloroform (Hughes et al., 1997). A bioassay was developed whereby thousands of eggs were laid on filter papers treated with the extracts or with active fractions from an open column separation. However, sufficient separation was not obtained for identification of the active constituents at that time. Here, we report on the identification of two volatile compounds that account for much of the activity. Tests were performed to examine structure–activity relationships, and electrophysiological studies were conducted to determine the sensory mode of detection of these compounds by the moths.

Methods and Materials

Plants and Extracts

Cabbage plants, *Brassica oleracea* L. var. Golden Acre (Agway, Inc., Syracuse, NY, USA) were grown from seed in an air-conditioned greenhouse at 23/19°C day/night with a 16 hr photophase. Supplemental lighting was provided by 400-W multivapor, high-intensity discharge lamps (General Electric MV 400/VBU). Plants were generally 4–5 wk old when used for extraction. One kg of intact leaves was immersed in approximately 3 l of chloroform for a period of 1.5 hr. The resulting extract was separated from leaf water and evaporated to a small volume for chromatography.

Insects

P. xylostella moths used for behavioral assays were from a laboratory colony maintained in Ithaca, NY, as previously described (Hughes et al., 1997). For the

electrophysiological recordings in Switzerland, the insects came from the laboratory culture at Wädenswil that was established with approximately 200 individuals during spring of 2000 and reared as described by Marazzi and Städler (2004). Briefly, an equal number of male and female mature moths (approximately 100 per cage) was placed in mesh cages (50 × 45 × 45 cm) in a climate-controlled room (21 ± 1°C, 70% RH and 16 hr photophase), where they were allowed to mate and oviposit only on the leaves of potted *B. napus* cv CC-Cross F1 at the prebolt stage. The moths had access to a source of water and to 10% sugar water.

Behavioral Assays

Extracts and fractions were tested for activity using recently emerged moths (approximately 150 mixed sexes) in 31 × 31 × 31 cm screened cages. Moths in each cage were provided with a 10% sucrose solution. Test material representing 1 g leaf equivalent (gle) was applied in chloroform solution to 5.5-cm-diam. filter paper disks, and chloroform alone was applied to control disks. After air drying, the treated filter papers were weighed (±0.0001 g), and two treated and two control disks were placed on small plastic supports in opposite corners of each cage. After overnight exposure to the moths (approx. 15 hr), test filter papers were weighed to provide an estimate of the number of eggs laid, based on an average weight of 0.0000195 g per egg. Active fractions generally received more than 3000 eggs in this time period.

Known isothiocyanates were tested in the same way, using 250 µl of a 0.1 µg/µl chloroform solution to deliver 25 µg of test compound per disk. Relative activity of each isothiocyanate was determined in choice assays, using allyl isothiocyanate (ANCS) as control. Based on these comparative assays, activity was expressed as an oviposition preference index (OPI), where $OPI = 100 (T - ANCS) / (T + ANCS)$, where *T* is the number of eggs laid on the test compound and ANCS is the number of eggs laid on the control.

Chemicals

All of the common aliphatic and aromatic isothiocyanates for behavioral studies (Fig. 1) were obtained from Sigma-Aldrich Corp (St. Louis, MO, USA). Iberin and sulforaphane were from LKT Laboratories (St. Paul, MN, USA) and the sample of bicyclic ketal (Posner #23) was a gift from Dr. Gary H. Posner of Johns Hopkins University, Baltimore, MD, USA. For electroantennograms recorded in the Wädenswil Laboratory, analytical quality allyl (2-propenyl) as well as benzyl, butyl, and methyl isothiocyanates were obtained from Fluka Chemicals, Basel, Switzerland.

Isolation and Identification of Active Compounds

Chloroform extracts of cabbage were separated by open column and medium pressure chromatography on silica gel as previously described (Hughes et al., 1997). Using stepwise elution with increasing concentrations of chloroform in hexane, followed by methanol in chloroform, two active fractions, A and B, were obtained.

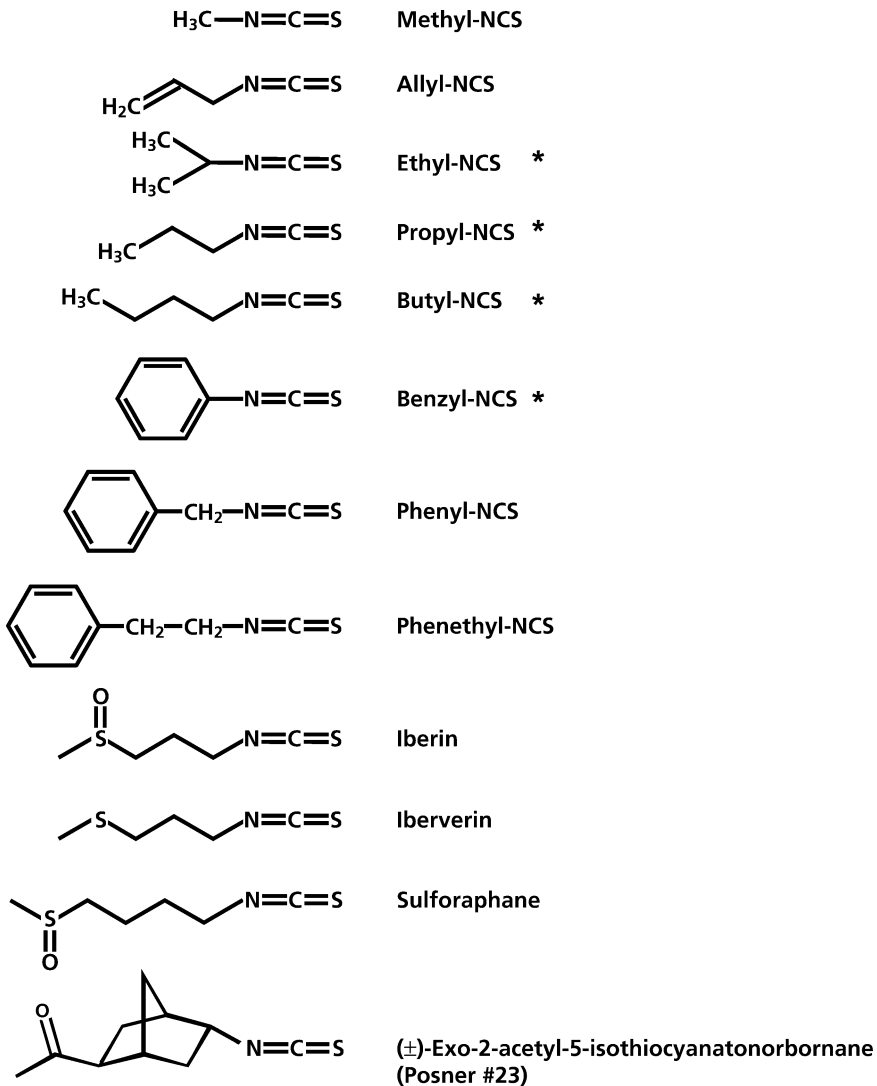


Fig. 1 Structures and common names of compounds tested for activity in stimulating oviposition and for electrophysiological responses of DBM. *: Not included in EAG experiments

Fraction A eluted in 30:70 chloroform/hexane, whereas B eluted in 4:96 methanol/chloroform. The active fractions were subjected to high-performance liquid chromatography (HPLC) using a Waters instrument fitted with a Phenomenex analytical column, Luna 5 μ silica, 1.0 \times 250 cm, with a flow rate of 1.0 ml/min. A diode array detector was used to monitor elution at 254 nm and to provide UV spectra of detected compounds. A solvent gradient of methanol in chloroform was used as follows: 0% methanol from 0 to 5 min, 3.0% MeOH at 17 min, 10.0% MeOH at 35 min, and 10.0% MeOH at 45 min.

The high-resolution electron impact mass spectra (HREIMS) and MS–MS were recorded on an AutoSpec (VG Analytical Instruments, Manchester, UK) instrument. The sample was introduced through a direct probe insert, and mass spectral data were collected at a temperature gradient of 70–350°C. The positive low-resolution electron spray ionization mass spectra (LRESIMS) and MS–MS were recorded on a VG Quattro (Micromass UK) instrument. The sample was introduced directly through a syringe pump at a flow rate of 200 $\mu\text{l/hr}$.

Electrophysiological Recordings

All isothiocyanates were dissolved in hexane (Fluka, GC grade, Buchs) to prepare six concentrations (1×10^{-5} , 1×10^{-4} , 1×10^{-3} , 1×10^{-2} , 0.1, 1 mg/ml).

Freshly emerged females were cooled in a refrigerator (5.5°C) for 15 min to reduce their activity. The wings and legs of the cooled insects were amputated, and the body was mounted ventral side up in the groove of a Plexiglas® holder and positioned so that the antennae were attached to a sticky wax layer, using strips of adhesive tape. The preparation was mounted under a stereomicroscope and continuously humidified with a water-saturated air stream (1 m s^{-1} , room temperature $\approx 22^\circ\text{C}$). These preparations showed good longevity, and it was possible to obtain strong responses for over 1.5 hr, allowing plenty of recording time. For the stimulation, we basically used the same method described by Guerin and Visser (1980). In brief, the airflow was split into continuous and stimulatory air streams in a 9:1 ratio, which converged prior to reaching the preparation. The stimulatory air stream passed through a Pasteur pipette (20 mm tip diam) containing the test compound in 100 μl paraffin oil spread on a folded filter paper ($15 \times 50 \text{ mm}$, from Schleicher and Schuell). The amount of an individual isothiocyanate in the air stream reaching the antenna is dependent on its partial vapor pressure, but this value can be kept constant by dissolution in paraffin oil (Kafka, 1970). It was injected into the continuous air stream upon activation of a valve. The indifferent electrode, filled with a saline solution (Kaissling, 1995), was inserted into the head, and the recording electrode, containing saline was brought into contact with the antennal tip. The EAG signal was recorded using a lab-built amplifier with high input impedance ($10^{13} \Omega$) and low bias current ($<10 \text{ pA}$). The signals were filtered (electronic high-pass with cornering frequency of 0.001 Hz), amplified (100 times), and digitized using Superscope II 3.0 Software (GW Instruments, Somerville, MA, USA) on a Macintosh computer. The EAG amplitudes were determined using PowerChrom v2.2.4 software (AD Instruments, Colorado Springs, CO, USA). Responses to the concentration series of each isothiocyanate were measured, from the lowest to the highest concentration; isothiocyanates were presented in a randomized order. We also tested the pure solvent to check that it did not elicit a strong olfactory response. Before and after running each concentration series, we recorded the response to 100 μg (*E*)-2-hexenal on filter paper (as described above). The data were analyzed by calculating the mean amplitude of the five responses to each tested combination of compound and concentration, then dividing this value by the average amplitude of the associated response to (*E*)-2-hexenal. This standardized the data to control for changes in preparation sensitivity over time.

Results

As previously reported, medium-pressure silica gel chromatography yielded two major active fractions that eluted with 30% chloroform in hexane (A) and 4% MeOH in chloroform (B). When these fractions were further separated by HPLC, fraction A revealed the presence of two major groups of UV-absorbing compounds that could not be completely separated, and, thus, no meaningful spectral data could be obtained. However, fraction B yielded clear peaks that were well separated (Fig. 2). Collection and assay of individual compounds and groups of compounds showed that the combined compounds eluting at 20.5–22.0 min as well as compounds **1** and **2** at 24.3 and 24.9 min were highly active in stimulating oviposition. Since the other groups of compounds could not be easily separated, further studies focused on compounds **1** and **2**.

High vacuum separation using a sublimation apparatus showed that compounds **1** and **2** are volatile, whereas other constituents of fraction B remained in the nonvolatile residue. The UV spectra of **1** and **2** were similar, with absorption maxima at 227 and 245 nm, which is typical of isothiocyanates. The positive HREMS of compound **1** gave a quasimolecular ion at m/z 164.020383 ($M + H$)⁺ and its sodium adduct at 186.002327 ($M + Na$)⁺, suggesting a molecular formula (MF) of C₅H₉S₂ON. The presence of sulfur was confirmed by a peak at m/z 166 ($M + 2 + H$)⁺ and its abundance in relation to m/z 164. In MS–MS, a molecular ion m/z 164 gave daughter ions at m/z 132, 130, 105, and 100. Similarly, the ion m/z 166 gave peaks at 134, 107, and 100. This suggested that fragments at 132 and 105 still retained at least one sulfur atom. On the basis of the fragmentation results, we identified this compound as iberin. This was further substantiated by comparing its NMR (in CDCl₃) spectrum with reported literature values (Kore et al., 1993) and by comparing its retention times on GC and HPLC with those of an authentic sample of iberin. The MS fragmentation pattern also matched that of the authentic sample.

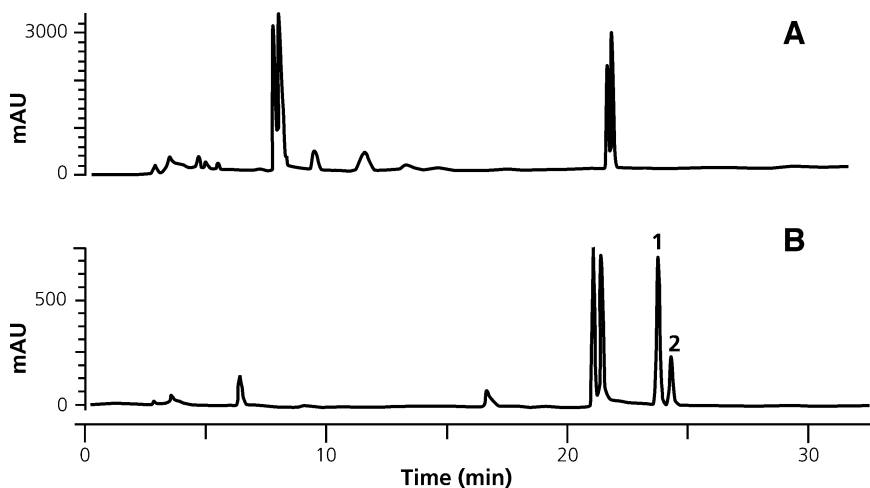


Fig. 2 HPLC separation of active fractions A and B from open column chromatography. Volatile compounds with strong activity are designated as **1** and **2**

The mass spectrum of compound **2** gave a quasimolecular ion at m/z 178.02 for $(M + H)^+$ in LRESIMS, and had a similar fragmentation pattern to that of **1**. The combined MS and UV spectral properties suggested that this compound was sulforaphane. The identity of compound **2** was confirmed by comparing its MS, as well as retention times, on HPLC and GC with those of authentic sulforaphane.

When authentic samples of iberin and sulforaphane were tested in bioassays, both isothiocyanates were highly active as stimulants. Comparative assays of a range of isothiocyanates were then conducted using allyl isothiocyanate (ANCS) as a standard. Tests of ANCS showed that this representative had limited activity when compared to iberin and sulforaphane. The comparative assays showed that methyl, ethyl, propyl, and phenyl isothiocyanates were considerably less active than ANCS (Fig. 3). Butyl and benzyl isothiocyanates had activity that was similar to that of ANCS, but sulforaphane, iberin, and iberberin were much more active than the standard (Fig. 3). Sulforaphane was previously identified as a major inducer of anticarcinogenic activity in mouse tissue (Zhang et al., 1992), and several isothiocyanate analogs with additional functional groups were subsequently found to have similar anticarcinogenic activity (Posner et al., 1994). One representative of these analogs, *exo*-2-acetyl-5-isothiocyanatonorbornane, was included in our oviposition assays, and this bicyclic ketal (referred to as Posner #23) also showed high activity when compared with ANCS (Fig. 3).

Electrophysiological recordings from DBM antennae provided EAGs that were in good agreement with behavioral responses. When tested at a medium dose of 10^{-3} to 10^{-2} mg, the most active isothiocyanates were the sulfur-containing iberin,

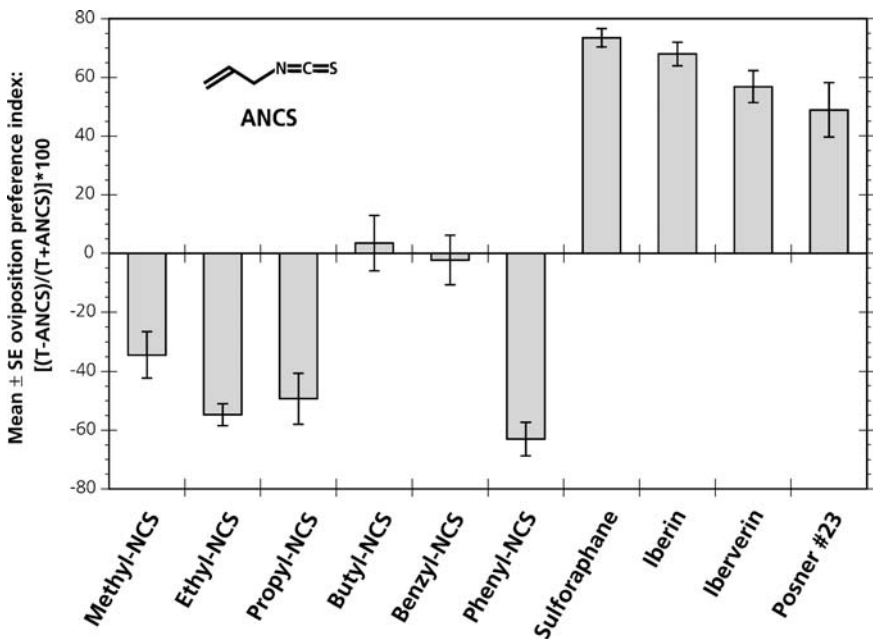


Fig. 3 Stimulation of DBM oviposition by isothiocyanates relative to that by allyl isothiocyanate (ANCS). Preference indices were calculated from egg distribution in separate choice tests between ANCS and each compound

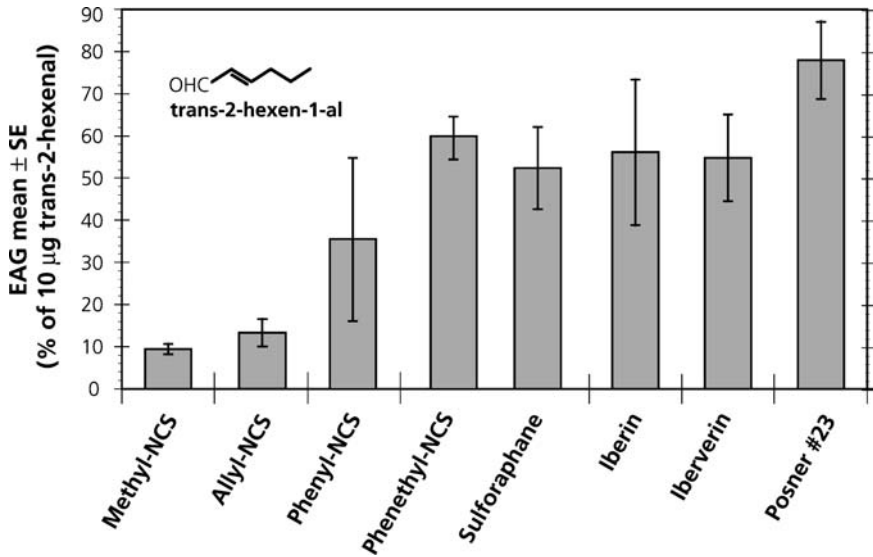


Fig. 4 EAG responses of DBM antennae to 10- μ g samples of isothiocyanates, in relation to a standard response to *trans*-2-hexenal

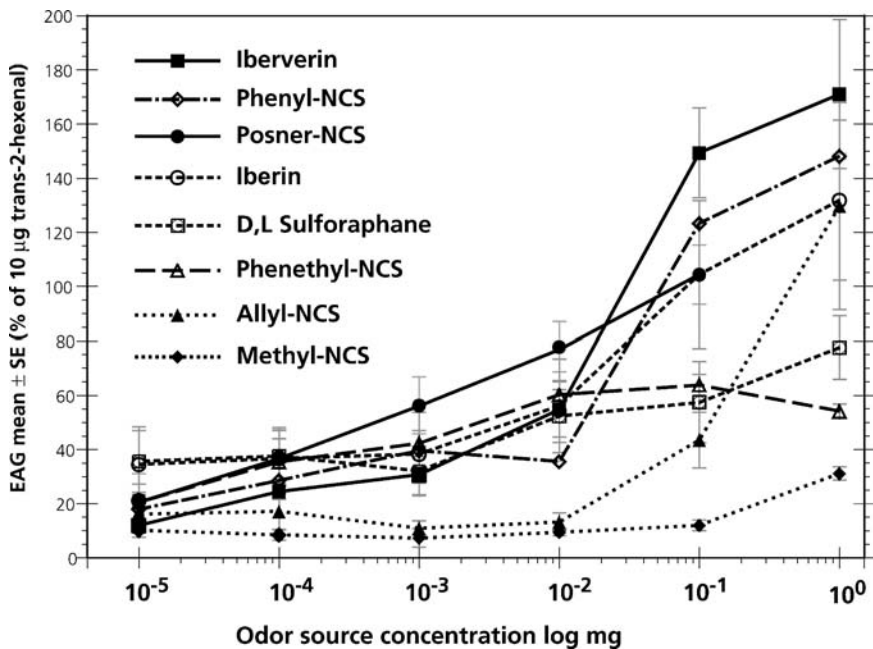


Fig. 5 Responses of DBM antennae to increasing doses of tested isothiocyanates

sulforaphane, iberverin, and the bicyclic keto analog (Posner # 23). The least-active representatives were methyl and allyl isothiocyanates, whereas phenyl isothiocyanate gave an intermediate response, and phenethyl isothiocyanate was highly stimulatory (Fig. 4). However, at higher doses of 10^{-1} to 10^{-0} mg, saturation of olfactory neurons by some compounds was apparent (Fig. 5). The almost perfectly linear relationship of EAG response to increasing concentrations of Posner # 23 was particularly striking, although the highest dose (1 mg) of this compound was not available and could not be tested.

Discussion

Recognition of cabbage as a host plant by ovipositing DBM females is dependent on perception of a combination of chemicals at or near the surface of foliage. However, the results of this study show that highly active individual constituents may be just as effective as total extracts. Although nonvolatile constituents are involved (Renwick and Radke, 1990; Spencer, 1996), volatile compounds apparently play a key role in stimulating oviposition, either before or after contact with the leaf surface. Our identification of the volatile isothiocyanates, iberin and sulforaphane, as the most prominent volatile compounds in active fractions provides additional evidence to support the idea that these moths may already recognize host plants while in flight (Bukovinszky et al., 2004).

The involvement of isothiocyanates in host recognition is not surprising. However, the presence of these compounds at the leaf surface or their release from intact foliage has not been reported previously. Isothiocyanates are released as hydrolysis products of glucosinolates, which are characteristic glycosides in crucifers. However, this hydrolysis is believed to occur only when foliage is disrupted by insect feeding or mechanical damage (Städler, 2002). The concentrations of isothiocyanates in the headspace above undamaged crucifer plants are known to be extremely low (Finch, 1978; Tollsten and Bergström, 1988). However, host recognition by another crucifer specialist, the cabbage root fly, has been shown to involve detection of these low levels of volatiles (De Jong and Städler, 1999). The presence of iberin and sulforaphane in chloroform extracts of intact foliage would now suggest that they are readily available at or near the leaf surface and may in fact be released in small quantities from foliage in the field. However, we cannot discount the possibility that compounds may leak from the cut petioles of the leaves, or that some kind of chloroform-induced tissue damage occurs.

The apparent synergy between a glucosinolate and wax in stimulating oviposition by DBM (Spencer, 1996; Spencer et al., 1999) may now be explained by the likely presence of small quantities of isothiocyanate from spontaneous degradation of the glucosinolate, sinigrin. The allyl isothiocyanate released might be adsorbed to the wax, which could then act as a slow release substrate and result in increased oviposition. On a test substrate, in the absence of wax, the stimulatory isothiocyanate would evaporate before significant oviposition could occur.

Preliminary behavioral assays of authentic isothiocyanates indicated intermediate activity for allyl isothiocyanate. When this representative was used as a standard for comparison, it was clear that iberin, sulforaphane, and iberverin were extremely active, whereas the alkyl representatives, methyl, ethyl, and propyl, were less active

than the standard. The most active compounds are characterized by the presence of sulfur, as thio, sulfinyl, or sulfonyl, in the side chain. When the bicyclic keto-isothiocyanate (Posner #23) was tested, this analog had high activity that was comparable to that of the sulfur-containing representatives. This compound is one of several bifunctional analogs of sulforaphane that were found to be potent inducers of anticarcinogenic detoxication enzymes in mouse tissues and murine hepatoma cells (Posner et al., 1994). These authors found that the most potent anticarcinogenic analogs were those isothiocyanates in which the isothiocyanate group was separated from a methyl sulfonyl or an acetyl group by three or four carbons. It appears, therefore, that the structure–activity relationship for DBM stimulation and for anticarcinogenic activity is remarkably similar.

EAG recordings in response to the various isothiocyanates verified the fact that olfaction is the mode of perception of these stimulants. Independent experiments have shown that volatiles from differently fertilized *Brassica* plants play an important role in host selection of ovipositing DBM females (Marazzi and Städler, 2004). Furthermore, the most active stimulants in our study evoked the highest levels of electrophysiological activity. The high activity of sulforaphane, iberin, iberberin, and Posner #23 mirrored the behavioral responses to these compounds. The response to phenyl isothiocyanate, which was relatively inactive in behavioral assays, and to phenethyl isothiocyanate (not available for behavioral assays) may be explained by the fact that EAG responses may be positive or negative. In addition, EAG responses are likely to be higher in response to more volatile members of the series. No attempt was made to compensate for differences in volatility of the different compounds. The most active compounds in behavioral tests are actually among the least volatile of the group. It is likely, therefore, that the EAG results would be more dramatic if the actual dose reaching the antenna were to be more precisely controlled. Lower activity of the most volatile compounds in behavioral assays might be expected as a result of rapid dissipation during the test period, but no evidence of reduced activity after a 15 hr exposure was observed.

The correlation between structural features required for oviposition stimulating activity and anticarcinogenic activity is remarkable. The extensive structure–activity experiments for anticarcinogenic activity that were conducted by the Johns Hopkins Group (Posner et al., 1994) showed the superiority of the sulfur-containing compounds that, we now find, are most active as oviposition stimulants. Although only one of the synthetic bicyclic analogs was available for our experiments, the behavioral and electrophysiological responses to this compound appear to confirm that the same molecular characteristics are required for activity. The close similarities in requirements for the two types of biological responses suggest that the receptors involved have features in common.

Our study was restricted to a continuation of previous work on cabbage, *B. oleracea*. However, subsequent investigations have shown that similar soaking of foliage of several other crucifers in chloroform provides highly stimulatory extracts. Extracts of those species that produce glucosinolates with sulfur in the side chain appear to be particularly active (Renwick, unpublished data). Since the glucosinolate composition of crucifers varies widely, it is likely that other isothiocyanates are responsible for most of the activity in other plants. Recent studies on the attractiveness of *Barbarea vulgaris* to DBM have focused on the possible use of this plant as a trap crop, since the hatching larvae do not survive (Badenez-Perez et

al., 2005). Although the involvement of volatiles has been suggested (Lu et al., 2004), the compounds responsible for high levels of oviposition and the types of isothiocyanates released from this plant have yet to be determined. Glucosinolate analyses of *Barbarea vulgaris* have shown that phenethyl is the only glucosinolate that would yield an isothiocyanate upon hydrolysis (Agerbirk et al., 2001; Windsor et al., 2005), and autolysis of foliage results in the release of traces of phenethyl as well methyl thioalkyl and allyl isothiocyanates (Cole, 1976). However, we have no evidence to indicate that the isothiocyanates released into chloroform are dependent on the glucosinolate composition of the plant.

The involvement of sulfur-rich compounds in DBM oviposition has been further indicated by fertilization experiments. *Brassica napus* plants that received higher rates of sulfur fertilization were preferred for oviposition by the moths. Methanolic surface extracts of the *B. napus* foliage produced EAG responses that were higher in the case of fertilized plants (Marazzi et al., 2004), although isothiocyanates were not detected within the threshold limits of the GC-MS system that was used.

Future studies on chloroform extracts of foliage from *Barbarea vulgaris* and other highly attractive crucifers are likely to reveal additional isothiocyanates that play a role in host recognition by the DBM. However, the involvement of sulforaphane and related compounds that have important physiological activity in mammals would suggest that comparative results from assays might reveal structure–activity relationships that could have wider biological applications. Additional work on the DBM will be required to identify nonvolatile constituents of host plants involved in the sequence of events leading to host selection by this important worldwide pest.

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Defensive Secretion Components of the Host *Parastizopus armaticeps* as Kairomones for the Cleptoparasite *Eremostibes opacus*

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Abstract The subsocial tenebrionid *Parastizopus armaticeps* Pér. is parasitized by the closely related *Eremostibes opacus* Koch (Coleoptera: Tenebrionidae). We found that the pygidial defensive secretions of both species are similar and contain a mixture of 1,4-benzoquinones, 1-alkenes, and monoterpene hydrocarbons. The 1-alkenes are dominated by 1-undecene, with admixtures of 1-tridecene in both species and 1-pentadecene in *P. armaticeps* only. Methyl- and ethyl-1,4-benzoquinone are the major quinones of the secretions of both species. The monoterpene fractions consist of (–)- α -pinene, (–)-camphene, sabinene, (–)- β -pinene, and (–)-limonene. Volatiles trapped with Porapak Q at the entrance to the breeding burrows of *P. armaticeps* were identified as components of the defensive secretion. However, in contrast to the secretion, the 1,4-benzoquinones were almost completely absent in the volatiles. Bioassays investigating attraction showed that the cleptoparasite *E. opacus* was drawn to the monoterpene hydrocarbons, produced by *P. armaticeps*, and deterred by the 1,4-benzoquinones. The 1-alkenes had no effect. Among the monoterpenes, only (–)-camphene was attractive to *E. opacus*. This is one of the rare cases of chemical exploitation of defensive allomones, and the first based on odor homology. We have drawn an evolutionary scenario

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including various functional changes in the defensive secretion compounds, leading to the kairomonal exploitation.

Keywords *Parastizopus armaticeps* · *Eremostibes opacus* · Tenebrionidae · Coleoptera · Defensive secretion · Monoterpene · (–)-Camphene · Host-finding kairomone

Introduction

“...predatory and parasitic arthropods are able to intercept the sex [and aggregation] pheromone signals of their prey or hosts” (Stowe et al., 1995), and there are a few cases where less specific allomones (e.g., defensive secretions) are known to be used as kairomones (Eisner et al., 1991; Mattiacci et al., 1993; Aldrich and Barros, 1995; Krell et al., 1997). Defensive secretions of arthropods, however, may have various additional functions in intra- and interspecific communication (Blum, 1996) and could, therefore, be eavesdropped by predators or parasites.

The nocturnal detritivorous desert tenebrionid *Eremostibes opacus* Koch lives in close association with the larger subsocial *Parastizopus armaticeps* Pér. of the same subtribe Stizopina (Rasa, 1994). During the dry season, both species are found together in burrows that the bigger *P. armaticeps* has excavated. The smaller *E. opacus* has either no effect or a positive effect on *P. armaticeps* by reducing the average transpiration rate per beetle in the burrow (Helsberg, 1994). During the breeding season of *P. armaticeps*, however, which starts after heavy rainfalls, *E. opacus* becomes a cleptoparasite (Rasa, 1996). This is based on the specific reproduction behavior of the host species.

P. armaticeps shows biparental brood care and division of labor (Rasa, 1990). A pair digs a breeding burrow together, and, after larval hatch, the female collects detritus at night and deposits it at the entrance to the burrow. The male stays in the burrow and pulls the food down into the burrow to form a food cache on which the larvae feed and continues to excavate the burrow and guard the entrance. The individuals of *E. opacus* gain admittance to breeding burrows by chemical mimicry based on cuticular hydrocarbons Geiselhardt et al., (in press). They feed on the food cache, and invading females lay their eggs there. The larvae inhabit the sand layer under the food cache and pull down items into the sand to feed.

E. opacus is specific to the host *P. armaticeps* and must find the burrows of *P. armaticeps* among the burrows of other fossorial arthropods, such as scorpions, millipedes, or other tenebrionids (Rasa, 1994). Choice experiments have shown that *E. opacus* is attracted to the odor of *P. armaticeps*, but *E. opacus* is unable to distinguish between the odor of *P. armaticeps* and conspecifics, whereas the odor of the sympatric tenebrionid *Gonopus agrestis* is avoided (Rasa, 1994). Rasa (1994) hypothesized that *E. opacus* host specificity is based on an odor homology within the tribe Opatrini.

To test this hypothesis as a basis for the cleptoparasitic relationship, we (1) analyzed and compared the defensive secretions of *P. armaticeps* and *E. opacus*, (2) adsorbed the volatiles from breeding burrows of *P. armaticeps* using Porapak Q, and (3) conducted a choice experiment to test the response of *E. opacus* to synthetic

blends and single volatiles of the *P. armaticeps* defensive secretion to identify the host-finding kairomone.

Methods and Materials

Beetles

P. armaticeps and *E. opacus* originated from Twee Rivieren, Kalahari Gemsbok National Park, South Africa, and were bred in the laboratory. Each species was kept in a separate terrarium containing Kalahari sand, hiding places, and a water source. They were fed with oat flakes or *Lebeckia linearifolia* (Fabaceae), which is their natural food plant.

Collection of Defensive Secretion

Defensive secretions were milked from beetles by holding a small piece of filter paper against the junction of the elytra and the anal sternite (Brown et al., 1992). The filter papers were extracted in 0.5 ml *n*-pentane.

Chemical Analysis

Samples for quantification were analyzed on an HP 6890 gas chromatograph (GC) equipped with a split/splitless injector (300°C) and an autosampler (injection of 1 μ l). A fused silica column (DB-1, 30 m \times 0.25 mm ID, 0.25 μ m, J&W Scientific, Folsom, CA, USA) was used with a helium flow of 1 ml/min. The oven temperature was programmed as follows: 2 min at 50°C, to 250°C at 6°C/min, to 300°C at 20°C/min. The FID was operated with 40 ml H₂/min and 450 ml air/min at 300°C.

Chemical identifications were performed on a coupled gas chromatography–mass spectrometer system (HP 6890 series GC-HP 5973 MSD) under the same GC conditions. Electron impact ionization was 70 eV.

To determine the configuration of the monoterpenes, authentic standards of (+)- or (–)-enantiomers [all from Fluka, except (+)-camphene from Merck] were added to the defensive secretions of *P. armaticeps* and *E. opacus*. Each standard was adjusted to the equivalent of the corresponding monoterpene in the defensive secretion. A β -DEX™120 fused silica column (30 m \times 0.25 mm ID \times 0.25 μ m, Supelco, D-Deisendorf) was used for enantiomeric separation. The oven temperature program was 2 min at 35°C, to 270°C at 5°C/min, held for 15 min.

Collection of Volatiles

Glass terraria (30 \times 20 \times 20 cm) were equipped with PVC inserts (27 \times 17 \times 10 cm) so that a 1-cm gap was left at each side. This gap was filled with moist Kalahari sand, and a thin layer of sand was spread on the top of the roof of the insert to provide an area over which the beetles could walk. A pair of *P. armaticeps* was placed in each terrarium and sprayed with water to stimulate them to dig a breeding burrow. The beetles were supplied with *Lebeckia ad libitum*.

The collection of volatiles was started once the beetles had dug a burrow at least 10 cm long or when larvae were present. A glass tube (2 mm ID, 9 cm long), packed with 1 cm Porapak Q (80/100 mesh, Waters Corporation, Milford, MA, USA 01757), was placed at the burrow entrance, as near as possible without disturbing the beetles. Each tube was connected to a vacuum pump (DC12/08NK, Fürgut, D-Aichstetten), and the airflow was adjusted to 100 ml/min. Volatiles were collected for 23 hr, and 10 samples were obtained for each burrow over a period of 30 d. For quantification, we used the mean total amounts of the 10 samples from each burrow. To determine the amount evaporated during the bioassays, we adsorbed the volatiles with the same method as for breeding burrows, but this time at the entrance of the bioassay boxes ($N = 10$). The rubber septa in the boxes were spiked with defensive secretion of *P. armaticeps* at the concentration equivalent to that of one beetle. Collection time was 6 hr (duration of bioassay). All experiments were conducted in an environmental chamber (GEA AG, D-Bochum) with a constant temperature of 30°C and a 14:10 hr light/dark regime.

Trapped volatiles were eluted by dripping 0.5 ml of *n*-pentane through the tubes. To each sample, 10 μ l of an internal standard [*n*-tetradecane (Fluka), 100 μ g/ml] were added, which was then concentrated to 50 μ l in a N_2 stream.

Synthetic Blends for Bioassays

“Terpene blend”: (–)- α -Pinene, (–)-camphene, (–)- β -pinene, and (–)-limonene (all from Fluka, purity >99%) were mixed in a ratio of 1:5:8:21, respectively, and 35 μ l of the mixture were added to 965 μ l of *n*-pentane. The concentrations of individual monoterpenes were 1 μ l/ml for (–)- α -pinene, 5 μ l/ml for (–)-camphene, 8 μ l/ml for (–)- β -pinene, and 21 μ l/ml for (–)-limonene.

“Alkene blend”: 1-Undecene (Sigma), 1-tridecene (Sigma), and 1-pentadecene (Fluka) were mixed in a ratio of 10:1:1, respectively, and 175 μ l of the mixture were added to 825 μ l of *n*-pentane.

“Quinone blend”: 1,4-Benzoquinone (Fluka), methyl-1,4-benzoquinone (Merck), and ethyl-1,4-benzoquinone (synthesized according to Peschke and Metzler, 1982) were mixed in a ratio of 1:3:5, respectively, and 120 mg of the mixture were dissolved in 1 ml of *n*-pentane.

In 5 μ l of each of the above blends, each component has the equivalent amount as in the defensive secretion of one *P. armaticeps*.

Bioassays

Choice experiments were conducted in glass terraria (50 \times 50 \times 20 cm) kept in an environmental chamber at 26–34°C and a 12:12 hr light/dark regime. Dusk and dawn (30 min) were simulated by an additional 40-W bulb. The terraria contained two inverted circular black plastic boxes (6 cm OD; 2 cm high), each with a small entrance, positioned in the corners 3 cm away from the walls. These hiding places contained water, food, and a rubber septum (violett 11 mm, Analyt, D-Müllheim) onto which the substances to be tested or the solvent for the control was applied. In the middle of the terraria, a dish filled with moist gravel provided high humidity. Trials were run with individual beetles. Five microliters of extract or solvent (*n*-pentane) were added to each rubber septum 4 hr before the beginning of the

photophase, and the beetles' choices were registered 6 hr after treatments were applied to the septa.

Results

Chemical Composition of the Defensive Secretions

The defensive secretion of *P. armaticeps* was composed mainly of 1-alkenes, 1,4-benzoquinones, and monoterpene hydrocarbons (Table 1; Fig. 1a). The 1-alkenes were dominated by 1-undecene, followed by 1-tridecene and 1-pentadecene. The most abundant quinones were ethyl-1,4-benzoquinone, methyl-1,4-benzoquinone, and 1,4-benzoquinone. Small quantities of vinyl-, isopropyl-, and propyl-1,4-benzoquinones were also identified. The major monoterpene hydrocarbons, in order of greatest abundance, were (–)-limonene, (–)- β -pinene, (–)-camphene, and (–)- α -pinene. Sabinene was only detectable in traces.

Table 1 Chemical composition (median, % total peak area) of extracts of the pygidial defensive secretion, and of volatiles trapped with porapak Q at the entrances to breeding burrows of *P. armaticeps* or bioassay boxes spiked with defensive secretion of *P. armaticeps*

Peak no.	Substance	Defensive secretion extracts		Breeding burrows (<i>N</i> = 6)	Bioassay boxes (<i>N</i> = 10)
		<i>P. armaticeps</i> (<i>N</i> = 50)	<i>E. opacus</i> (<i>N</i> = 50)		
1	1,4-benzoquinone	2.4	1.0	–	–
2	1-nonene	tr	0.4	–	–
3	(–)- α -pinene	0.3	0.3	4.2	3.0
4	(–)-camphene	1.4	1.4	8.0	9.2
5	sabinene	tr	0.1	–	–
6	(–)- β -pinene	2.1	10.6	9.7	14.5
7	methyl-1,4-benzoquinone	15.8	10.3	–	–
8	1-decene	tr	0.2	–	–
9	(–)-limonene	5.9	11.4	14.6	23.6
10	ethyl-1,4-benzoquinone	21.8	21.1	1.7	1.7
11	vinyl-1,4-benzoquinone	0.3	0.3	–	–
12	1-undecene	40.7	40.8	51.9	47.8
13	isopropyl-1,4-benzoquinone	tr	tr	–	–
14	propyl-1,4-benzoquinone	tr	tr	–	–
15	2-undecanone	tr	tr	tr	–
16	2,2'-bifuran	tr	tr	tr	–
17	1-tridecene	3.8	0.2	3.9	0.4
18	1-pentadecene	3.9	–	3.2	0.2

tr = <0.1%.

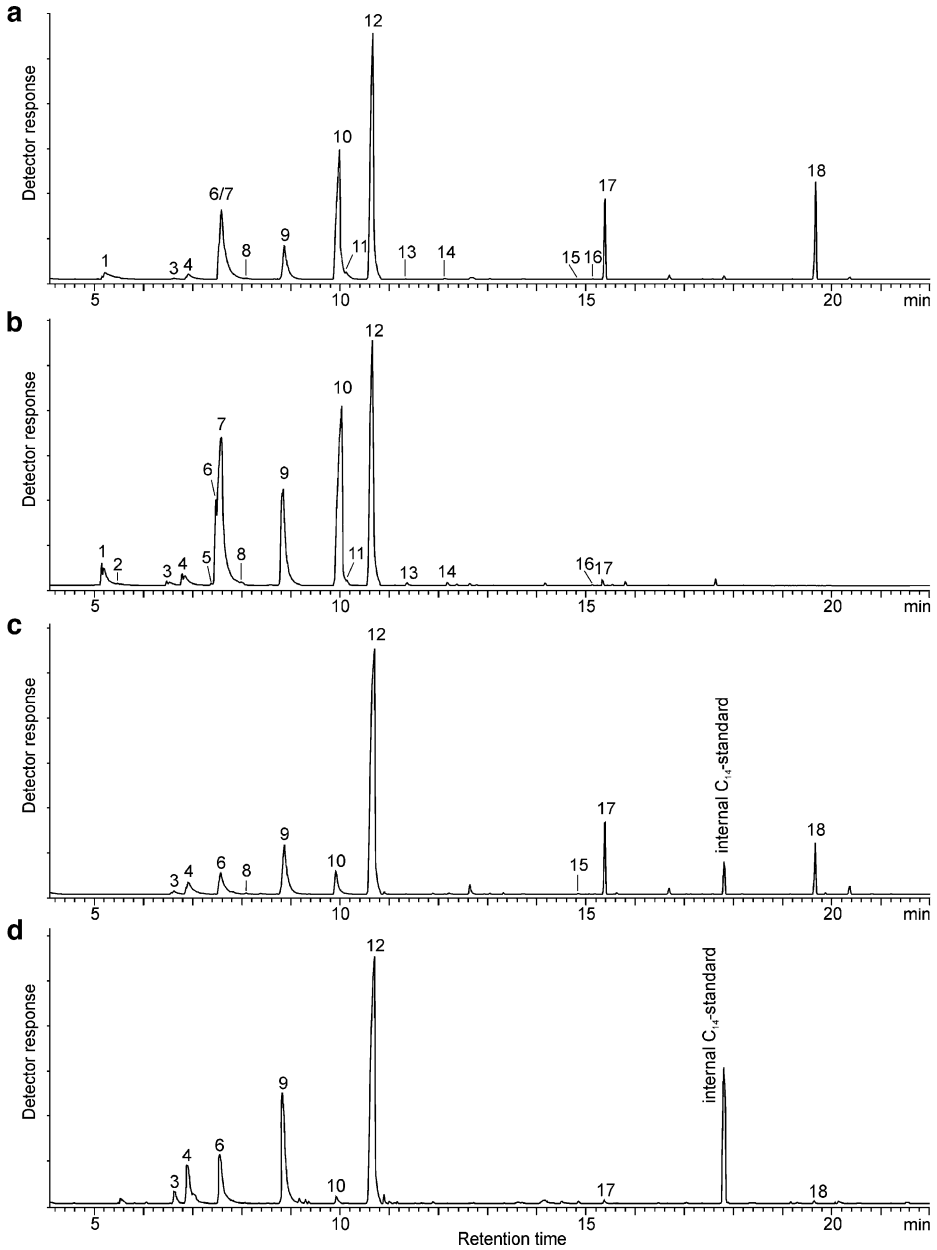


Fig. 1 Total ion chromatograms of pygidial defensive secretion extracts of (a) *P. armaticeps* and (b) *E. opacus*, and of volatiles trapped with Porapak Q at the entrances of (c) breeding burrows of *P. armaticeps* or (d) bioassay boxes with spiked defensive secretion of *P. armaticeps*. Key to peak numbers is given in Table 1

The composition of the defensive secretion in *E. opacus* was similar to that of *P. armaticeps*, except that the secretion of *E. opacus* contained no 1-pentadecene, and 1-tridecene was present only as a minor component (Table 1, Fig. 1b). The quinones and monoterpenes were the same in both species. The proportion of the monoterpenes in *E. opacus* (24.1%) was higher than in *P. armaticeps* (9.8%), based on the higher proportions of (–)-β-pinene and (–)-limonene.

Source and Chemical Composition of Burrow Volatiles

The comparison of the volatiles trapped with Porapak Q at the entrance to burrows with the defensive secretion of *P. armaticeps* showed that all volatiles derived from the defensive secretion (Table 1, Fig. 1c), the major 1-alkenes, and all monoterpene hydrocarbons were present. No volatiles were detected that might have come from the food or any other source. The main difference between the composition of volatiles from breeding burrows and defensive secretions was the low proportion of the 1,4-benzoquinones in the volatiles, with ethyl-1,4-benzoquinone being the only detectable quinone. The volatiles trapped at the entrance to breeding burrows and bioassay boxes (Table 1, Fig. 1d) showed a similar composition; only the quantities of 1-tri- and pentadecene were somewhat higher in breeding burrows.

Quantification of Burrow Volatiles

The total amount of volatiles adsorbed with Porapak Q at the entrance to breeding burrows of *P. armaticeps* was 167 ng/hr (median, range 42–1599 ng/hr, *N* = 6). The amounts measured from the same burrow varied considerably during the collection period, but there was no obvious dependency on the age of the larvae or pupae. For example, the total amounts of volatiles from one burrow varied between 5 and 5451 ng/hr, with a mean of 1599 ng/hr. When a rubber septum was spiked with one beetle equivalent of defensive secretion, the amounts trapped at the entrance of the bioassay boxes showed a smaller range of variation (median 428 ng/hr, range 174–

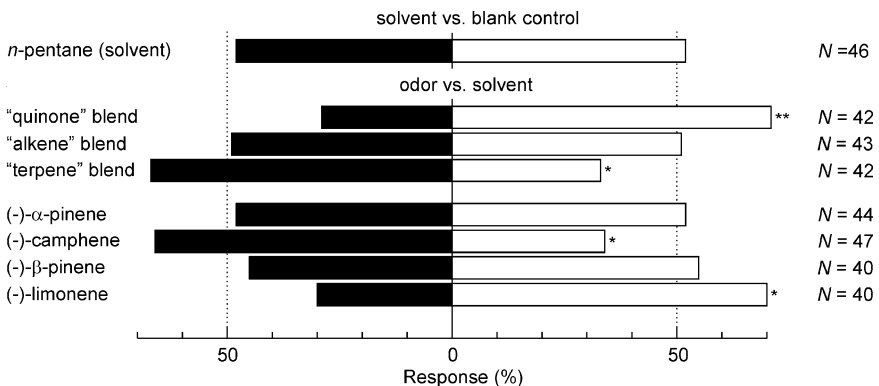


Fig. 2 Bioassay responses of *E. opacus* to solvent and different blends of volatile classes or single monoterpene hydrocarbons emitted from breeding burrows of *P. armaticeps*. The bars show the percentage of individuals choosing a bioassay box. The asterisks indicate significant attraction/avoidance to treatments (two-tailed binomial test, **P* < 0.05, ***P* < 0.01)

905 ng/hr, $N = 10$). The median was somewhat higher than that adsorbed from breeding burrows (Mann–Whitney U test, $U = 10.0$, $P = 0.03$), but the amounts were all within the range of variability of the latter.

Bioassay for Attraction

The response of *E. opacus* toward the different blends (Fig. 2) showed that the “terpene blend” attracted the beetles significantly ($N = 42$, two-tailed binomial test, $z = 2.01$, $P < 0.05$). The “quinone blend” deterred *E. opacus* ($N = 42$, $z = 2.62$, $P < 0.05$), whereas the “alkene blend” produced no effect ($N = 43$, $z = 0.00$, n.s.). Of the monoterpene hydrocarbons, only (–)-camphene was attractive to *E. opacus* ($N = 47$, $z = 2.04$, $P < 0.05$); (–)- α -pinene ($N = 44$, $z = -0.15$, n.s.) and (–)- β -pinene ($N = 40$, $z = -0.47$, n.s.) showed no effects, and (–)-limonene was avoided by the beetles ($N = 40$, $z = -2.37$, $P < 0.05$).

Discussion

“...it is impossible for a species to completely avoid emitting chemical signals” (Stowe et al., 1995). In the case of *P. armaticeps*, chemical signals originate from the secretion of the pygidial gland and mediate the interspecific communication with its cleptoparasite. Besides the primary function as defensive secretion, the compounds adopt secondary functions in the different stages of its lifetime. We developed an evolutionary scenario of these functional changes, leading to the kairomonal exploitation of the secretion by the cleptoparasite *E. opacus*.

Composition of the Defensive Secretion

Our chemical investigations on the defensive secretions of *P. armaticeps* and *E. opacus* agreed with the results on many tenebrionid beetles (Happ, 1968; Tschinkel, 1975; Markarian et al., 1978; Gnanasunderam et al., 1981; Attygalle et al., 1991; Brown et al., 1992). The secretions of all these species contained methyl- and ethyl-1,4-benzoquinone, in most cases together with 1-alkenes. Besides the two major 1,4-benzoquinones, the secretions of *P. armaticeps* and *E. opacus* comprised 1,4-benzoquinone and other substituted 1,4-benzoquinones, of which vinyl-1,4-benzoquinone was identified here for the first time in tenebrionids. Tschinkel (1975) has analyzed the defensive secretion of *Parastizopus balneorum* Pér. (= *P. armaticeps* Pér.) and detected only methyl- and ethyl-1,4-benzoquinone, together with 1-alkenes. As those beetles originated from a different population, this could be the reason why we found no 1-heptadecene and a lower proportion of 1-pentadecene in the secretion of *P. armaticeps*. In contrast to previously studied tenebrionid species (Tschinkel, 1975), the defensive secretions of *P. armaticeps* and *E. opacus* contained substantial proportions of monoterpene hydrocarbons. Monoterpenes have been detected in defensive secretions of numerous insects, e.g., termites (Moore, 1968; Gush et al., 1985), ants (Brand et al., 1974), and bugs (Aldrich et al., 1990). They have also been reported in defensive secretions of some Australian and New Zealand tenebrionids from different subfamilies (Gnanasunderam et al., 1981; Brown et al., 1992). As yet, only α -pinene, limonene, and α -phellandrene have been

reported in the defensive secretions of tenebrionids. Camphene, sabinene, and β -pinene are new to the family. Although Tschinkel (1975) found no monoterpene hydrocarbons in the defensive secretion of *P. balneorum*, the presence of (–)- α -pinene, (–)-camphene, sabinene, (–)- β -pinene, and (–)-limonene seems to be typical for defensive secretions of Stizopina, as all investigated species, except *Psammogaster malani* Koch, contained these monoterpenes (unpublished data).

Burrow Volatiles and Possible Functions

The volatiles adsorbed on Porapak Q from burrows of *P. armaticeps* are components of the defensive secretion. This contradicts a study of odor profiles of undisturbed *P. armaticeps* and *E. opacus*, which rejected the pygidial defensive secretion as a source of volatiles in resting odor profiles (Hein et al., 1996). The peaks were only described by their retention times, and no information was given about relative amounts or mass spectra, limiting further comparison with our results. However, we found no evidence for a source of volatiles other than the defensive secretion.

The main difference between the composition of emitted defensive secretion and volatiles trapped with Porapak Q was the low proportion of 1,4-benzoquinones in the volatiles, regardless of whether they were collected at the entrances of breeding burrows or bioassay boxes. We concluded that the low amounts of 1,4-benzoquinones were not a result of a reduced production of quinones during breeding; rather, they were the result of the physical conditions during the experiment. 1,4-Benzoquinones are polar substances and soluble in water, in contrast to 1-alkenes and monoterpene hydrocarbons. Therefore, their adsorption on the moist sand could be the reason for their underrepresentation.

The adsorbed 1,4-benzoquinones might have a secondary function as antimicrobial agents during breeding. We did not observe any microbial infection in the food caches of breeding burrows if parent beetles were present, but an infection occurred within 1 or 2 d after a pair was removed (unpublished data). Prendeville and Stevens (2002) have shown that 1,4-benzoquinones from the defensive secretion of the tenebrionid *Tribolium* inhibit the growth of yeast and bacteria. In addition to the 1,4-benzoquinones, monoterpene hydrocarbons are known to have antimicrobial activities. For example, in the termite genus *Nasutitermes*, the frontal gland secretion, containing α -pinene, β -pinene, and limonene, reduces fungal infection of soldiers and spore germination in the nests (Rosengaus et al., 2000). The 1-alkenes not only serve as solvents for the 1,4-benzoquinones, but they may also have a repellent effect on insects, e.g., the ant *Monomorium pharaonis* (Peschke and Eisner, 1987). Therefore, 1-undecene could prevent ants or other insects from entering breeding burrows of *P. armaticeps*.

Exploitation of Defensive Secretion as a Kairomone

The use of kairomones for host or prey location is a widespread phenomenon in arthropods (Weseloh, 1981; Vinson, 1984; Stowe et al., 1995; Aldrich, 1999; Powell, 1999). Besides the numerous examples of parasitoids and predators exploiting the pheromone system of their victims (Bowers and Borden, 1992; Teerling et al., 1993; Kielty et al., 1996; Zuk and Kolluru, 1998; Zhu et al., 1999), there are a few cases of

kairomonal exploitation of allomones. For example, Eisner et al. (1991) found that cleptoparasitic milichiid flies are attracted to defensive secretion of bugs caught by orb-weaving spiders. Moreover, the use of heteropteran defensive secretions for host or prey location has been shown by Mattiacci et al. (1993) for the egg parasitoid *Trissolcus basalus* (Hymenoptera) and by Aldrich and Barros (1995) for crab spiders. Fresh carcasses of juliform millipedes attract necrophagous *Onthophagus* dung beetles. Krell et al. (1997) have demonstrated that 1,4-benzoquinones from the defensive secretion were responsible for the attraction. In addition, Schmitt et al. (2004) have shown that only the mixture of the two major 1,4-benzoquinones, methyl- and 2-methoxy-3-methyl-1,4-benzoquinone, was attractive to *Onthophagus* dung beetles, but not the individual components. In all these cases, whether exploitation of pheromones or allomones, the species had to “decipher the language of their hosts or prey” before they could exploit them.

On the other hand, in *E. opacus*, the exploitation of (–)-camphene as a host-finding kairomone could have evolved as a consequence of “misinterpreting” their own aggregation pheromone. Rasa (1994) has shown that *E. opacus* are unable to distinguish between the odor of conspecifics and *P. armaticeps*, and that they are attracted to high odor concentrations. The larger *P. armaticeps* probably emit greater amounts of volatiles than *E. opacus*, so that *E. opacus* are more likely to be attracted to aggregations of *P. armaticeps* than to those of their own species. All Stizopina that have been investigated, except *Ps. malani*, which is lacking monoterpenes, form intraspecific aggregations (unpublished data). Therefore, (–)-camphene might be a basal aggregation pheromone within the Stizopina. This hypothesis remains to be proved. If it is true, it could be the reason why interspecific aggregations have evolved several times independently in this subtribe (Rasa and Endrödy-Younga 1997).

An additional function of defensive secretion components as aggregation pheromones is known from the tenebrionid genus *Blaps* (Tannert and Hien 1973). Low concentrations of the defensive secretion led to the formation of aggregations in this species. However, the components have not yet been identified. The deterrence of *E. opacus* by 1,4-benzoquinones could be the result of high concentrations in the bioassay boxes. However, 1,4-benzoquinones are universal within defensive secretions of Tenebrionidae (Tschinkel, 1975) and, therefore, they cannot be responsible for the discrimination of *E. opacus* between its host and other burrowing tenebrionids in the Kalahari, such as *Gonopus* (Rasa, 1994).

In summary, the subsocial *P. armaticeps* and its cleptoparasite *E. opacus* exhibit defensive secretions that are typical for tenebrionid beetles, except for high proportions of monoterpene hydrocarbons. As these monoterpenes characterize the defensive secretions of all members of the subtribe Stizopina, to which both species belong, they can be considered as a homologous feature. During the breeding period of *P. armaticeps*, defensive chemicals might adopt different secondary functions. Both monoterpenes and 1,4-benzoquinones are known for their antimicrobial properties, which prevent the food cache from bacterial or fungal infections, and the volatiles, monoterpenes as well as 1-alkenes, are repellents for potential arthropod intruders. However, *E. opacus* exploits just these monoterpenes as the host-finding kairomone. The evolution of cleptoparasitism by *E. opacus* might originate from mistaking volatiles from *P. armaticeps* aggregations for their own aggregation pheromone, (–)-camphene, leading to interspecific aggregations. During drought, such interspecific aggregations would be beneficial for both

partners by reducing their transpiratory water loss. At this evolutionary stage, (–)-camphene probably functioned as a synomone. After *E. opacus* was able to mimic its host's cuticular hydrocarbon pattern, and thus gained access to breeding burrows, (–)-camphene may have become a kairomone. This would represent the first case of volatile semiochemical exploitation based on odor homology.

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Ethanol and (–)- α -Pinene: Attractant Kairomones for Some Large Wood-Boring Beetles in Southeastern USA

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Abstract Ethanol and α -pinene were tested as attractants for large wood-boring pine beetles in Alabama, Florida, Georgia, North Carolina, and South Carolina in 2002–2004. Multiple-funnel traps baited with (–)- α -pinene (released at about 2 g/d at 25–28°C) were attractive to the following Cerambycidae: *Acanthocinus nodosus*, *A. obsoletus*, *Arhopalus rusticus nubilus*, *Asemum striatum*, *Monochamus titillator*, *Prionus pocularis*, *Xylotrechus integer*, and *X. sagittatus sagittatus*. *Buprestis lineata* (Buprestidae), *Alaus myops* (Elateridae), and *Hylobius pales* and *Pachylobius picivorus* (Curculionidae) were also attracted to traps baited with (–)- α -pinene. In many locations, ethanol synergized attraction of the cerambycids *Acanthocinus nodosus*, *A. obsoletus*, *Arhopalus r. nubilus*, *Monochamus titillator*, and *Xylotrechus s. sagittatus* (but not *Asemum striatum*, *Prionus pocularis*, or *Xylotrechus integer*) to traps baited with (–)- α -pinene. Similarly, attraction of *Alaus myops*, *Hylobius pales*, and *Pachylobius picivorus* (but not *Buprestis lineata*) to traps baited with (–)- α -pinene was synergized by ethanol. These results provide support for the use of traps baited with ethanol and (–)- α -pinene to detect and monitor common large wood-boring beetles from the southeastern region of the USA at ports-of-entry in other countries, as well as forested areas in the USA.

Keywords Cerambycidae · *Xylotrechus* · *Monochamus* · *Acanthocinus* · Curculionidae · *Hylobius* · *Pachylobius* · Elateridae · *Alaus* · Ethanol · α -Pinene · Kairomone · Exotics

Introduction

Introductions of bark and wood-boring beetles via the movement of solid wood packing materials used in crating and securing cargo for shipping are common (Hoebcke, 1994; Haack and Cavey, 2000; Haack, 2001). From 1996 to 1998, most

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interceptions of beetles at ports-of-entry in the USA were associated with solid wood packing material (USDA APHIS and Forest Service, 2000). Coleoptera accounted for 94% of all pest interceptions with the Cerambycidae accounting for 37% of all beetle interceptions. The risk of invasive species of Cerambycidae moving between continents and countries is particularly high because of the cryptic nature of adults and larvae (Allison et al., 2004).

The impacts of Cerambycidae can be varied, ranging from beneficial as primary agents of nutrient recycling within forested stands (Hanks, 1999; Allison et al., 2004) to damaging in terms of social, economic, and environmental values (Liebholt et al., 1995). The mining activities of larvae result in large-diameter holes and tunnels in wood that can translate into significant levels of degrading damage to forestry products (Safranyik and Raske, 1970; Cerezke, 1977). Several species of long-horned beetles (*Monochamus* spp.) are known vectors of a wilting disease caused by the pine wood nematode *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickel (Tylenchida: Aphenlenchoididae) (Wingfield et al., 1982; Linit, 1988). Introduction of the pine wood nematode into Japan in the early 1900s resulted in numerous epidemics of pine wilt on 28% of the pine forests by 2000 (Mamiya, 2003). Since 1996, the Asian long-horned beetle *Anoplophora glabripennis* (Motschulsky) has killed thousands of high-value urban trees in Illinois, New York, New Jersey, and Ontario with the cost of eradication and tree restoration expected to exceed US\$300 million by 2009 (USDA Forest Service, 2005). Similarly, the brown spruce borer *Tetropium fuscum* (Fabricius) has become established in Nova Scotia, killing significant numbers of red spruce *Picea rubens* Sargent in park lands near Halifax (Natural Resources Canada, 2005). It is likely that even more introductions will occur because of increases in the movement of solid wood packing material associated with expected increases in international trade (USDA APHIS and Forest Service, 2000).

One common trapping protocol used for wood borers at ports-of-entry and forest monitoring sites in the USA employs multiple funnel or intercept traps baited with devices releasing ethanol and (–)- α -pinene at fairly high rates (1–2 g/d at 20–25°C; USDA Forest Service, 2001). In Ontario, traps baited with ethanol and (±)- α -pinene were attractive to various species of Cerambycidae, including *Monochamus scutellatus* (Say) and *Xylotrechus undulatus* (Say) (Chénier and Philogène, 1989). In British Columbia, *Xylotrechus longitarsis* Casey was attracted to traps baited with ethanol and (–)- α -pinene (Morewood et al., 2002), whereas *Monochamus notatus* (Drury) and *M. scutellatus* were attracted to traps baited with ethanol and a blend of seven monoterpenes that included (–)- α -pinene (Allison et al., 2001).

My objective was to assess the efficacy of standard commercially available ethanol and (–)- α -pinene lures used with multiple-funnel traps in capturing the following common large (>8 mm in length) Cerambycidae in the southeastern USA: *Monochamus titillator* (Fabricius), *Xylotrechus sagittatus sagittatus* (Germar), *X. integer* (Haldeman), *Acanthocinus nodosus* (Fabricius), *A. obsoletus* (Olivier), *Arophalus rusticus nubilus* (LeConte), *Asemum striatum* (L.), and *Prionus pocularis* Dalman. The goal was to verify that traps baited with the combination of the two lures were as effective as, if not better than, traps baited solely with one lure over a broad range in the South: eight National Forests in five Southern states. Previous studies on the attractiveness of ethanol and monoterpenes to Cerambycidae in the USA have largely been restricted to Florida with some recent tests in South Dakota.

In Florida, Fatzinger (1985), Fatzinger et al. (1987), and Phillips et al. (1988) found that the combination of ethanol and turpentine was attractive to various species of

pine Cerambycidae; however, these studies did not incorporate blank controls. Moreover, the use of turpentine in these studies is a concern because the monoterpene composition of turpentine can vary widely depending on the species and location of pines used in producing turpentine (Mirov, 1961; Smith, 2000). The main monoterpene in one of the Florida studies (and likely the other two as well) was α -pinene (Phillips et al., 1988). In South Dakota, Costello (2005) found that traps baited with ethanol and α -pinene were attractive to the cerambycids *Acanthocinus obliquus* (LeConte), *A. spectabilis* (LeConte), *Acmaeops proteus* (Kirby), and *Monochamus clamator* (LeConte); however, traps baited solely with α -pinene were not tested.

I also monitored the responses of the following species of saproxylic beetles commonly found in southern pine stands: *Buprestis lineata* Fabricius (Buprestidae), *Alaus myops* (Fabricius) (Elateridae), and the root weevils (Curculionidae) *Hylobius pales* Herbst and *Pachylobius picivorus* LeConte. Larval *Alaus myops* prey upon the larvae of pine woodborers, whereas larvae of the weevils *Hylobius pales* and *Pachylobius picivorus* feed in the roots and stumps of stressed or dying pines, often feeding on seedlings after removal of mature trees in plantations (USDA Forest Service, 1985). Ethanol synergized attraction of *Pachylobius picivorus* to turpentine in Wisconsin (Hunt and Raffa, 1989), whereas in Florida, ethanol synergized attraction of *Hylobius pales* but not *Pachylobius picivorus* to turpentine (Phillips et al., 1988). Erbilgin et al. (2001) found that *Hylobius pales* and *Pachylobius picivorus* were attracted to flight traps baited with α -pinene and ethanol in Louisiana and Wisconsin, although neither compound was tested alone.

Methods and Materials

Chemicals and Release Devices

Phero Tech Inc. (Delta, British Columbia, Canada) supplied sealed ultrahigh-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 rates of ethanol and α -pinene from UHR pouches were approximately 1 and 2 g/d, respectively, at 25–28°C (determined by weight loss).

Experimental Design

Eight experiments were conducted in 2002–2004 to evaluate the attractiveness of ethanol and (-)- α -pinene to large bark- and wood-boring beetles in the southeastern region of the USA. All eight experiments used the same design with one experiment conducted in mature pine stands on each of seven National Forests (NF) and one Experimental Forest (EF) in the South (Table 1). Disturbances occurred in stands used in experiments 1–3 and 6–8 during the 6-mo period preceding trap deployment but not in stands used in experiments 4–5. Prescribed fire was the disturbance agent in experiments 1–3 and 8, whereas salvage logging was used to remove a spot infestation of *Dendroctonus frontalis* Zimmerman in stands used in experiment 6; stands in experiment 7 were thinned with removal of woody material.

I employed a behavioral choice type of design in all experiments with treatments within a block grouped within the same area and traps spaced 10–15 m apart. My expectation was that the plumes of volatiles from each treatment would blend

Table 1 National forest (NF) and experimental forest (EF) locations, forest types, and trapping dates for experiments (2002–2004)

Experiment	Location	Tree species	Trapping dates
1	Ocala NF near Salt Springs FL	<i>Pinus palustris</i> Miller	26 February–26 May 2002
2	Osceola NF near Lake City FL	<i>Pinus palustris</i> and <i>P. elliotii</i> Engelmann	25 February–25 May 2002
3	Oconee NF near Juliette GA	<i>P. taeda</i> L.	12 June–8 August 2002
4	Blue Valley EF near Highlands NC	<i>P. strobus</i> L.	20 June–20 August 2002
5	Bankhead NF near Grayson AL	<i>P. taeda</i> and <i>Tsuga canadensis</i> (L.)	28 April–10 July 2003
6	Nantahala NF near Murphy NC	<i>P. strobus</i> and <i>P. echinata</i> Miller	1 May–14 August 2003
7	Sumter NF near Union SC	<i>P. taeda</i>	15 April–16 July 2003
8	Apalachicola NF near Tallahassee FL	<i>Pinus palustris</i> and <i>P. elliotii</i>	30 March–16 June 2004

together and spread over a single catchment area. In all experiments, 16 8-unit multiple-funnel traps (Phero Tech Inc.) were set in four linear blocks of four traps per block at each of the two sites. Blocks within site, and traps within blocks, were spaced 10–15 m apart, whereas sites were spaced 50–500 m apart. Each trap was suspended between trees by rope such that the bottom of each was 0.2–0.5 m above ground level. No trap was within 2 m of any tree. 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). In each of the eight experiments, four treatments were randomly assigned to traps within each of eight replicate blocks as follows: (1) blank control; (2) ethanol alone; (3) α -pinene alone; and (4) ethanol + α -pinene. In all experiments, lures were replaced at intervals of 50–60 d.

Statistical Analyses

Data were analyzed only for locations where sufficient numbers of the following species were captured: *Acanthocinus nodosus*, *A. obsoletus*, *Arhopalus rusticus nubilus*, *Asemum striatum*, *Monochamus titillator*, *Prionus pocularis*, *Xylotrechus integer*, *X. s. sagittatus*, *Buprestis lineata*, *Alaus myops*, *Hylobius pales*, and *Pachylobius picivorus*. The 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). Trap catch data were transformed by $\ln(Y + 1)$ to remove heteroscedasticity (Pepper et al., 1997). Where possible, trap catch data in all experiments were subjected to two-way analysis of variance (ANOVA) using the following model components: (1) replicate, (2) ethanol, (3) α -pinene, and (4) ethanol \times α -pinene. In a number of locations, mean trap catches of some species to one or two treatments (control and traps baited with ethanol alone) were zero with zero variance and a clear violation of the assumption of homoscedasticity (Cobb, 1998). In such

instances, data were subjected to one-sided *t* tests (using a Bonferroni correction for multiple comparisons), testing that individual treatment means were greater than zero (Reeve and Strom, 2004). Trap catch data where variances were homoscedastic for all treatments at all locations were subjected to two-way ANOVA using the following model components: (1) replicate nested within location, (2) location, (3) treatment, and (4) locations × treatment.

In all experiments, the Holm–Sidak multiple comparison procedure was used to compare means within a location for each species when treatment effect was significant at *P* = 0.05. When two treatments had means of zero with zero variance, then the two remaining means were compared by two-sided *t* test at *P* = 0.05.

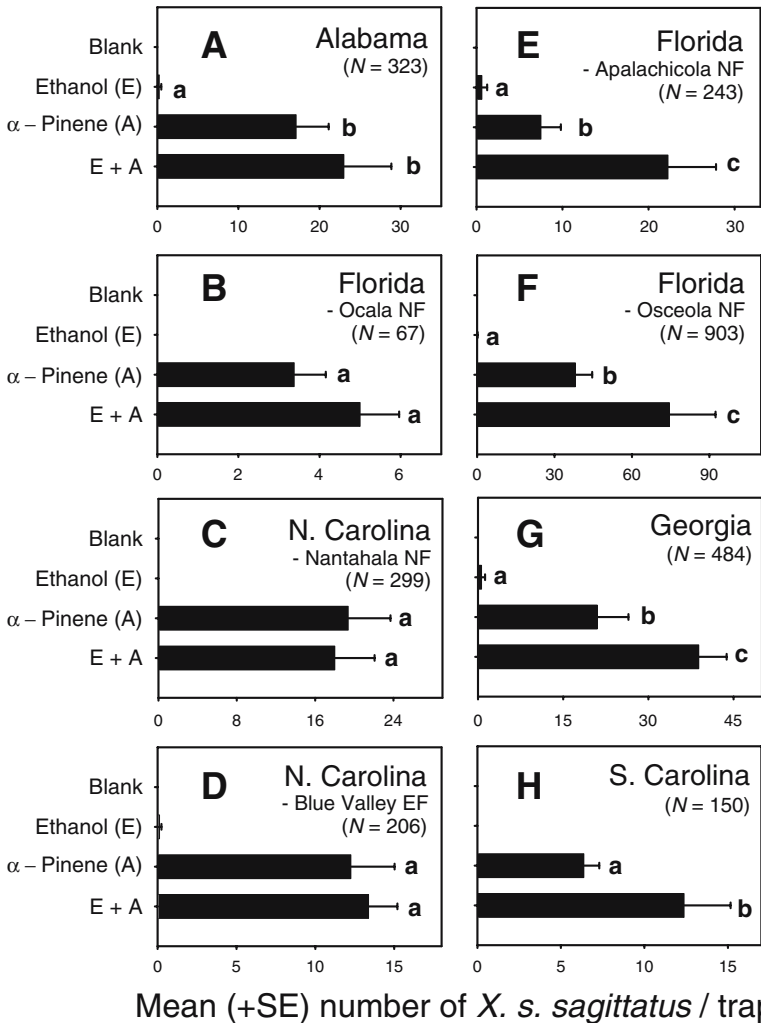


Fig. 1 Effects of ethanol and (–)- α -pinene on trap catches of *Xylocopa s. sagittatus* (Cerambycidae) in southeastern USA. Means followed by the same letter are not significantly different at *P* = 0.05 [Holm–Sidak multiple comparison test for three treatments (A, E, F, and G) or *t* test for two treatments (B, C, D, and H)]. Treatments without a letter had zero catches of beetles

Synergism between ethanol and (–)- α -pinene in attracting beetles was indicated by satisfying two statistical conditions. First, ethanol and/or (–)- α -pinene had to be benign alone in attracting beetles to traps. Second, the combination of ethanol and (–)- α -pinene had to result in higher trap catches than ethanol or (–)- α -pinene alone.

Results

A total of 2675 *Xylotrechus s. sagittatus* (about 66% of all Cerambycidae caught) were captured from all eight locations (Fig. 1). The data for *X. s. sagittatus* could not

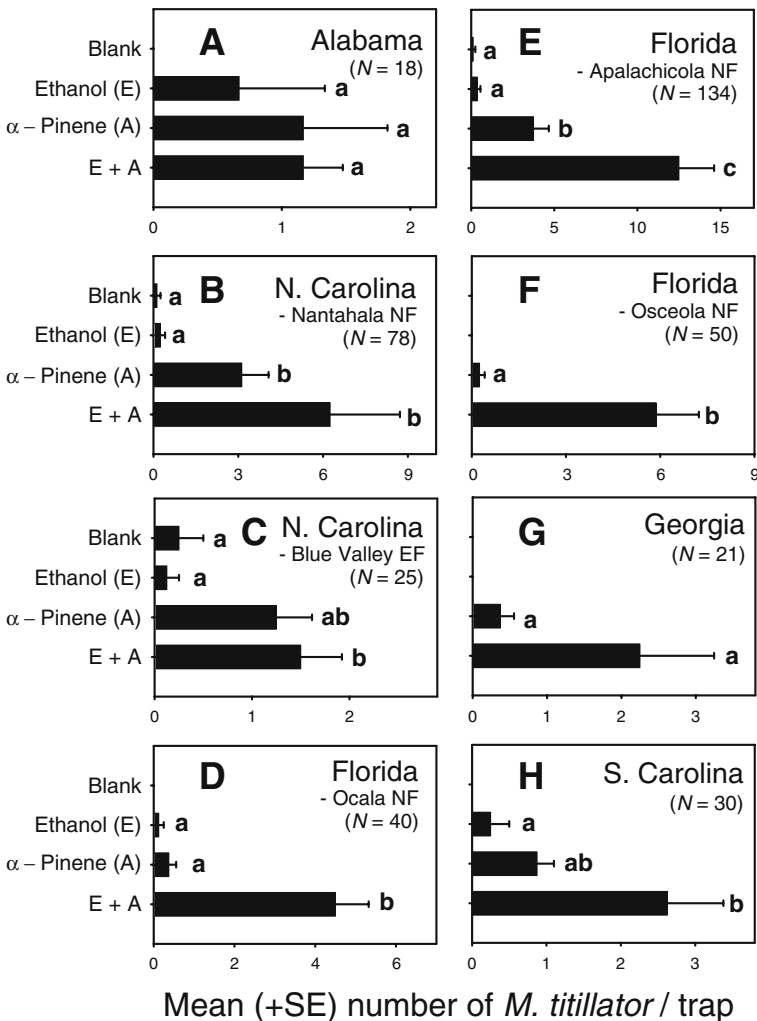


Fig. 2 Effects of ethanol and (–)- α -pinene on trap catches of *Monochamus titillator* (Cerambycidae) in southeastern USA. Means followed by the same letter are not significantly different at $P = 0.05$ [Holm–Sidak multiple comparison test for three to four treatments (A–E and H) or t test for two treatments (F and G)]. Treatments without a letter had zero catches of beetles

be analyzed by two-way ANOVA because no beetles were caught in control traps at any location. Catches of *X. s. sagittatus* in traps baited with (–)- α -pinene (with or without ethanol) were greater than zero at all locations (*t* tests, all *df* = 7, all *P* < 0.01). At some locations, ethanol seemed to affect trap catches of *X. s. sagittatus* as a synergist (Fig. 1). There were no beetles caught in traps baited solely with ethanol at four locations (Fig. 1), whereas catches in ethanol-baited traps at other four locations were not different from zero (*t* tests, all *df* = 7, all *P* > 0.50). However, when ethanol was added to traps baited with (–)- α -pinene, catches of *X. s. sagittatus* were significantly greater than those in traps baited with (–)- α -pinene in four locations: Georgia, South Carolina, and the Apalachicola and Osceola NF in Florida (Fig. 1E–H). At no location, was attraction of beetles to traps baited with (–)- α -pinene interrupted by ethanol.

Monochamus titillator was caught at all eight localities with a total capture of 396 beetles (about 10% of total Cerambycidae catch; Fig. 2). Trap catches of *M. titillator* were significantly affected by (–)- α -pinene but not ethanol in the Nantahala NF and Blue Valley EF in North Carolina, whereas both (–)- α -pinene and ethanol affected catches in the Apalachicola NF in Florida (Table 2). Two-way ANOVA could not be employed for *M. titillator* at the remaining five locations because no beetles were caught in control traps. Catches of *M. titillator* in traps baited solely with (–)- α -pinene were different from zero in South Carolina (*t* test, *df* = 7, *P* = 0.006) but not the remaining four locations (*t* tests, all *df* = 7, all *P* > 0.10). Ethanol had a synergistic effect on catches to traps baited with (–)- α -pinene at six of the eight locations (Fig. 2). Catches of *M. titillator* in traps baited with ethanol and (–)- α -pinene were significantly greater than those baited solely with (–)- α -pinene or ethanol alone at all three locations in Florida (Fig. 2D–F). In Alabama, Georgia, and South Carolina, catches of beetles in traps baited with ethanol and (–)- α -pinene were different from zero (*t* tests, all *df* = 7, all *P* < 0.04), whereas catches in traps

Table 2 Significant levels for ANOVA on effects of ethanol and (–)- α -pinene on beetle trap catches in southeastern USA

Species	Location	Rep	Ethanol (E)	α -Pinene (A)	E \times A
<i>Acanthocinus obsoletus</i>	FL Osceola NF	0.592	0.003	<0.001	0.001
<i>Alaus myops</i>	FL Apalachicola NF	0.267	0.074	<0.001	0.117
	FL Ocala NF	0.818	0.922	<0.001	0.425
	FL Osceola NF	0.085	0.186	<0.001	0.345
	SC Sumter NF	0.003	0.441	<0.001	0.978
	FL Osceola NF	0.001	0.074	<0.001	0.023
<i>Arophalus r. nubilis</i>	FL Osceola NF	0.001	0.074	<0.001	0.023
<i>Asemum striatum</i>	SC Sumter NF	0.045	0.666	<0.001	0.894
	FL Ocala NF	0.313	0.930	<0.001	0.621
<i>Buprestis lineata</i>	FL Ocala NF	0.141	0.268	0.001	0.565
	SC Sumter NF	0.141	0.268	0.001	0.565
<i>Hylobius pales</i>	FL Apalachicola NF	0.615	0.005	<0.001	<0.001
	FL Ocala NF	0.391	0.545	<0.001	0.545
	FL Osceola NF	0.610	<0.001	<0.001	0.001
<i>Monochamus titillator</i>	FL Apalachicola NF	0.778	0.001	<0.001	0.010
	NC Nantahala NF	0.291	0.470	<0.001	0.709
	NC Blue Valley EF	0.480	0.909	0.001	0.665
<i>Pachylobius picivorus</i>	FL Apalachicola NF	0.652	0.004	<0.001	0.005
	FL Ocala NF	0.688	0.009	<0.001	0.355
	FL Osceola NF	0.658	0.039	<0.001	0.187

baited with ethanol alone were not greater than zero (t tests, all $df = 7$, all $P > 0.50$). At no location, were catches in traps baited with $(-)\text{-}\alpha\text{-pinene}$ interrupted by ethanol (Fig. 2).

We caught a total of 187 *Acanthocinus obsoletus* at four locations (Fig. 3A–D). $(-)\text{-}\alpha\text{-Pinene}$ had a significant effect on *A. obsoletus* in the Osceola NF (Table 2) with catches in traps baited solely with $(-)\text{-}\alpha\text{-pinene}$ greater than those in blank

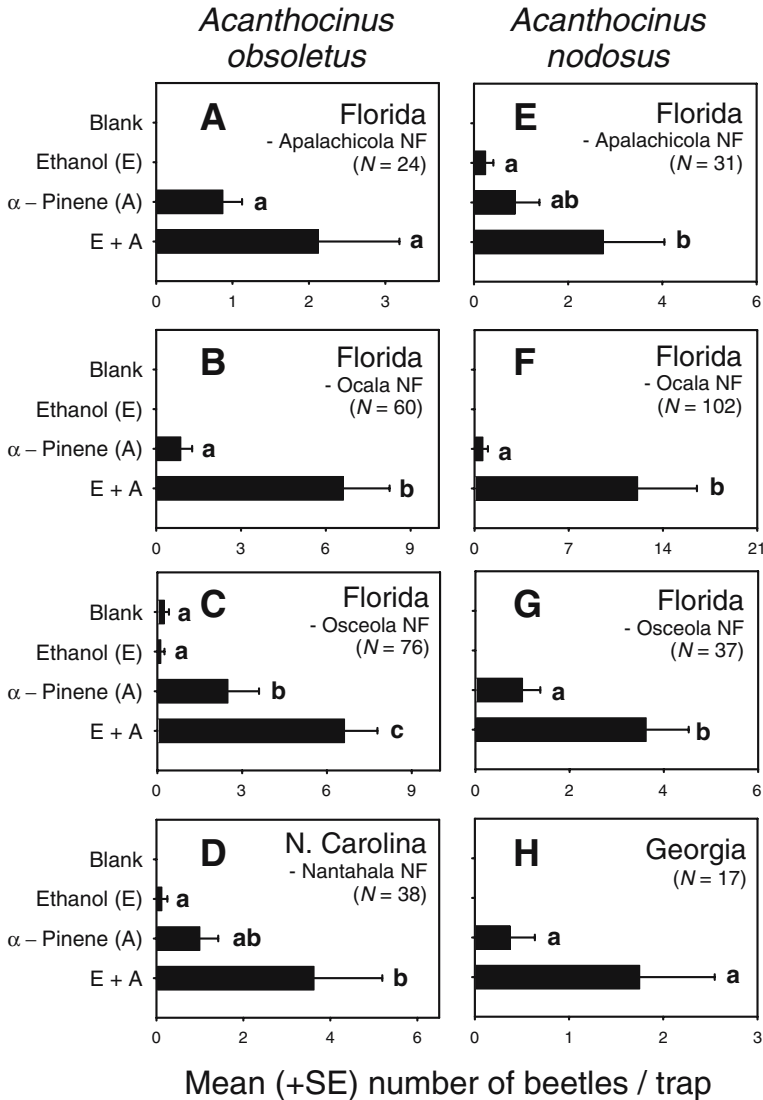


Fig. 3 Effects of ethanol and $(-)\text{-}\alpha\text{-pinene}$ on trap catches of *Acanthocinus obsoletus* (A–D) and *A. nodosus* (E–H) (Cerambycidae) in southeastern USA. Means followed by the same letter are not significantly different at $P = 0.05$ [Holm–Sidak multiple comparison test for three to four treatments (C–E) or t test for two treatments (A, B, and F–H)]. Treatments without a letter had zero catches of beetles

control traps (Fig. 3C). *Acanthocinus obsoletus* was not captured in control traps at the remaining three locations. Catches of *A. obsoletus* in traps baited with (–)- α -pinene were greater than zero in the Apalachicola NF (*t* test, *df* = 7, *P* = 0.03) but not in the Ocala and Nantahala NF (*t* tests, *df* = 7, *P* = 0.068 and 0.060, respectively). In contrast, catches of beetles in traps baited with ethanol and (–)- α -pinene were greater than zero in all three locations (*t* tests, all *df* = 7, all *P* < 0.04; Fig. 3A, B, D). Catches in traps baited solely with ethanol were not greater than zero in the Nantahala NF (*t* test, *df* = 7, *P* = 0.526); none were caught in ethanol-baited traps in the Apalachicola and Ocala NF (Fig. 3A, B). Catches of *A. obsoletus* in traps baited with (–)- α -pinene were synergized by adding ethanol lures at two of the three Florida locations (Fig. 3B, C).

We caught a total of 198 *Acanthocinus nodosus* at four locations (Fig. 3E–H). No beetles were captured in control traps at all four locations. Catches of *A. nodosus* in traps baited solely with (–)- α -pinene were greater than zero in the Osceola NF (*t* test, *df* = 7, *P* = 0.028) but not at the other three locations (*t* tests, all *df* = 7, all *P* > 0.15). As with *A. obsoletus*, ethanol had a synergistic effect on trap catches of *A. nodosus*. Catches of *A. nodosus* in traps baited with both ethanol and (–)- α -pinene were greater than zero at all four locations (*t* tests, all *df* = 7, all *P* < 0.02). Catches in traps baited solely with ethanol were not greater than zero in the Apalachicola NF (*t* test, *df* = 7, *P* = 0.2566); none were caught in ethanol-baited traps in the Ocala, Osceola, and Nantahala NF (Fig. 3F–H). Catches of *A. nodosus* in traps baited with (–)- α -pinene were increased by adding ethanol lures at two of the three Florida locations (Fig. 3F, G).

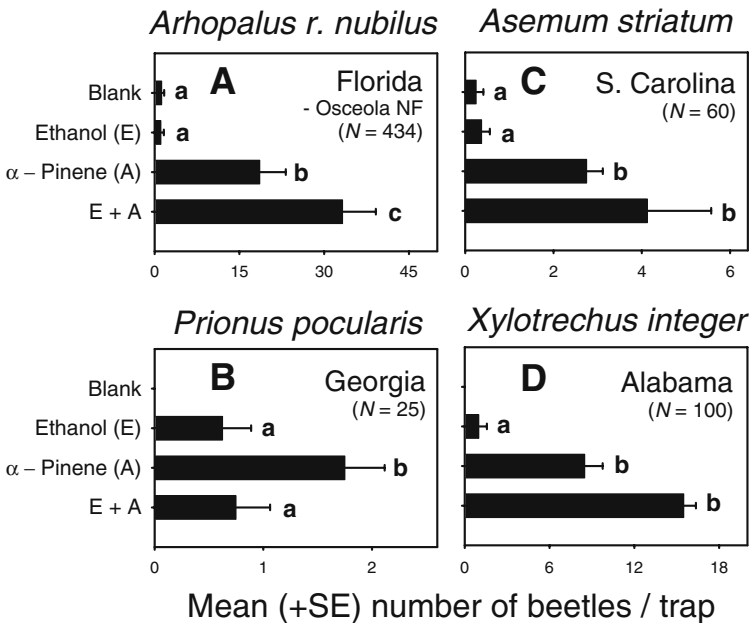


Fig. 4 Effects of ethanol and (–)- α -pinene on trap catches of *Arhopalus r. nubilus* (A), *Prionus pocularis* (B), *Asemum striatum* (C), and *Xylotrechus integer* (D) (Cerambycidae) in southeastern USA. Means followed by the same letter are not significantly different at *P* = 0.05 (Holm–Sidak multiple comparison test). Treatments without a letter had zero catches of beetles

Catches of *Arhopalus r. nubilus* and *Asemum striatum* were significantly affected by $(-)\text{-}\alpha\text{-pinene}$ in two separate locations (Table 2). For both species, catches of beetles in traps baited with $(-)\text{-}\alpha\text{-pinene}$ alone caught more beetles than control traps (Fig. 4A, C). Ethanol had a synergistic effect on *A. r. nubilus* with catches in traps baited with both ethanol and $(-)\text{-}\alpha\text{-pinene}$ greater than those in traps baited solely with $(-)\text{-}\alpha\text{-pinene}$; traps baited with ethanol alone were not attractive (Fig. 4A). Catches of *Xylotrechus integer* in traps baited with $(-)\text{-}\alpha\text{-pinene}$ (with or without ethanol) were greater than zero (t tests, all $df = 3$, all $P < 0.002$), whereas catches in traps baited solely with ethanol were not greater than zero (t test, $df = 3$, $P = 0.273$). Catches of *Prionus pocularis* in traps baited with $(-)\text{-}\alpha\text{-pinene}$ were greater than zero (t test, $df = 7$, $P < 0.001$), whereas catches in traps with the remaining treatments were not greater than zero (t tests, all $df = 7$, all $P > 0.059$), both of which were significantly less than catches in traps baited solely with $(-)\text{-}\alpha\text{-pinene}$ (Fig. 4B).

Buprestids were not common with only one species captured in numbers sufficient for analyses. *Buprestis lineata* were represented in trap catches from six locations with a total capture of 348 beetles (Fig. 5). In South Carolina and the Ocala NF in Florida, catches of *B. lineata* were significantly affected by $(-)\text{-}\alpha\text{-pinene}$, but not

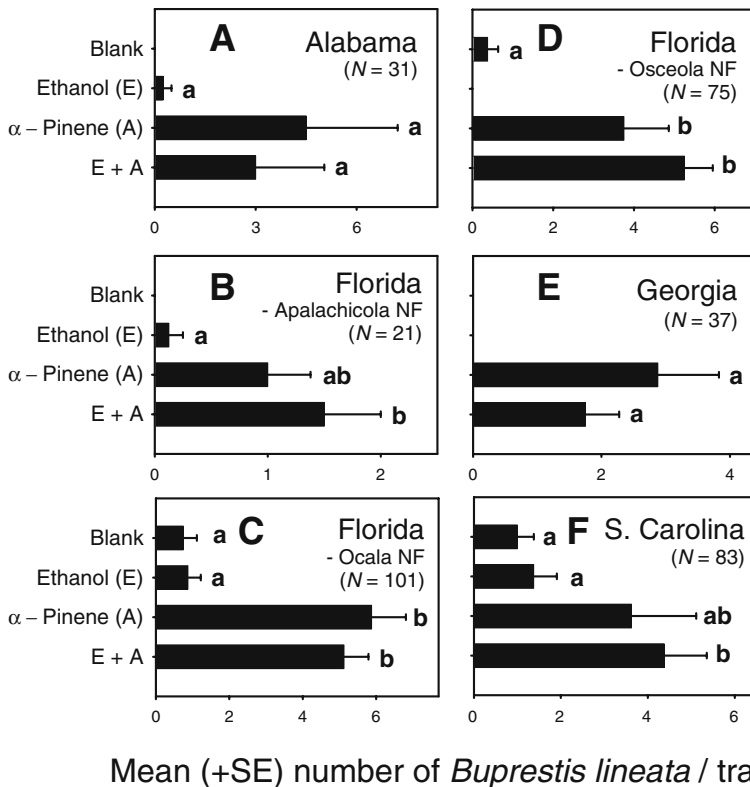


Fig. 5 Effects of ethanol and $(-)\text{-}\alpha\text{-pinene}$ on trap catches of *Buprestis lineata* (Buprestidae) in southeastern USA. Means followed by the same letter are not significantly different at $P = 0.05$ [Holm–Sidak multiple comparison test for three to four treatments (A–D, and F) or t test for two treatments (E)]. Treatments without a letter had zero catches of beetles

ethanol, with no significant interaction between ethanol and (-)- α -pinene (Table 2). In both locations, catches of beetles in traps baited with ethanol and (-)- α -pinene were greater than those in control traps (Fig. 5C, F). In the Apalachicola NF, catches of *B. lineata* in traps baited with ethanol and (-)- α -pinene were greater than zero (*t* test, *df* = 7, *P* = 0.038); the opposite was not true for catches in the remaining two treatments (*t* tests, all *df* = 7, all *P* > 0.056). In the Osceola NF (Florida), catches of *B. lineata* in traps baited with (-)- α -pinene (with or without ethanol) were greater than those in control traps (Fig. 5D). Catches of beetles in traps baited with (-)- α -pinene (with or without ethanol) were greater than zero in Georgia (*t* test, all *df* = 7, all *P* < 0.01) but not Alabama (*t* tests, all *df* = 3, all *P* > 0.17). At no location, was there a difference between catches of *B. lineata* in traps baited solely with (-)- α -pinene and those baited with both (-)- α -pinene and ethanol.

A total of 672 *Alaus myops* were captured in four locations (Fig. 6). Trap catches of *A. myops* in South Carolina and the three locations in Florida were affected by (-)- α -pinene (Table 2). At all four locations, traps baited with (-)- α -pinene (with or without ethanol) caught more beetles than control traps or traps baited solely with ethanol (Fig. 6). There was no significant interaction between location and treatment on catches of *A. myops* (*F* = 0.912, *df* = 9,84, *P* = 0.519). Traps baited with ethanol were not attractive to *A. myops*, although attraction of beetles to (-)- α -pinene was increased by ethanol at two of the Florida locations (Fig. 6A and C).

Two common pine root weevils *Hylobius pales* and *Pachylobius picivorus* were caught in sufficient numbers in Florida for analyses (total of 257 and 1616 beetles, respectively; Fig. 7). (-)- α -Pinene had a significant effect on catches of both species at all three locations in Florida (Table 2). Catches of *Pachylobius picivorus* in traps baited with (-)- α -pinene (with or without ethanol) were significantly greater than those in control traps at all three locations (Fig. 7A–C). The same was true for catches of *Hylobius pales* in the Osceola NF and the Ocala NF but not at the

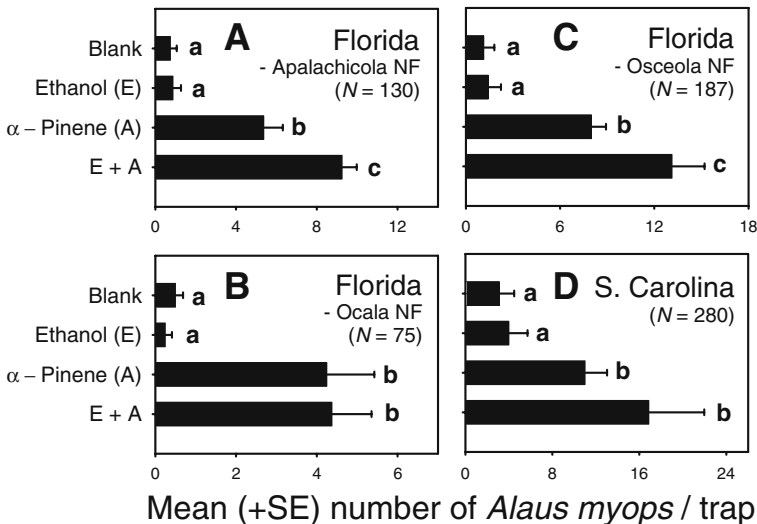


Fig. 6 Effects of ethanol and (-)- α -pinene on trap catches of *Alaus myops* (Elateridae) in South Carolina and northern Florida. Means followed by the same letter are not significantly different at *P* = 0.05 (Holm–Sidak multiple comparison test)

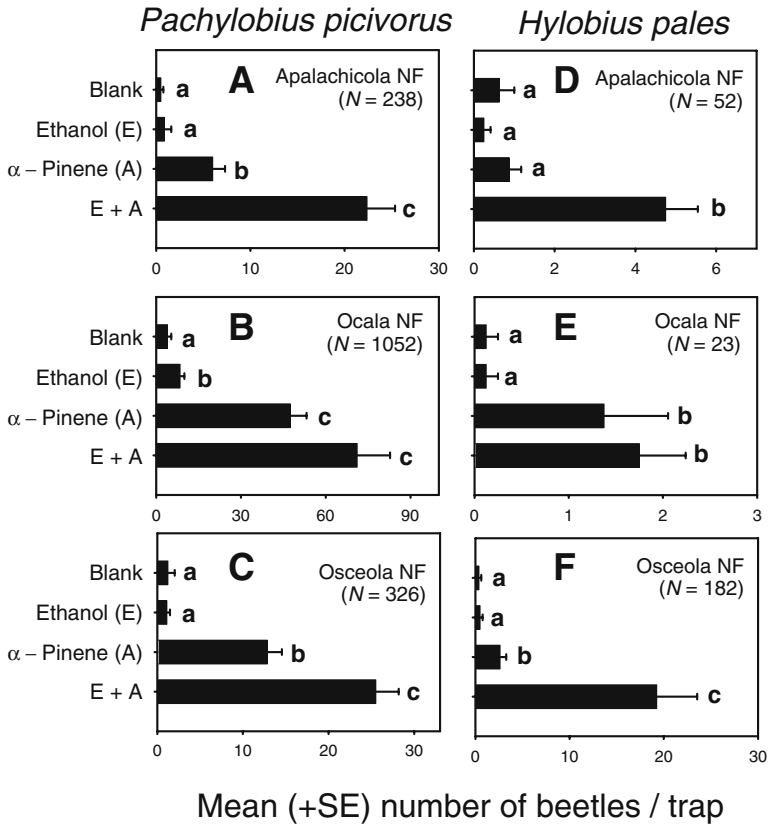


Fig. 7 Effects of ethanol and ($-$)- α -pinene on trap catches of *Pachylobius picivorus* (A–C) and *Hylobius pales* (D–F) (Curculionidae) in northern Florida. Means followed by the same letter are not significantly different at $P = 0.05$ (Holm–Sidak multiple comparison test)

Apalachicola NF (Fig. 7E and F). There was an interaction between treatments and location on catches of *Hylobius pales* in Florida ($F = 6.830$, $df = 6,21$, $P < 0.001$) but not on catches of *Pachylobius picivorus* ($F = 1.704$, $df = 6,21$, $P = 0.135$). At all locations, catches of both species in traps baited with ethanol and ($-$)- α -pinene were greater than those in control traps. However, the effect of ethanol on attraction of both weevils to ($-$)- α -pinene was synergistic in the Apalachicola and Osceola NF but not in the Ocala NF (Fig. 7A–F).

Discussion

Almost all of the wood-boring beetle species caught in this study breed in recently cut, wind-thrown, fire-killed, insect-killed, or dying pines as well as pine logs and stumps (USDA Forest Service, 1985; Yanega, 1996). One commonality to these situations is the release of resin by pines that thwarts further damage or invasions by insects or pathogens (Raffa, 1992; Trapp and Croteau, 2001). α -Pinene is a common and predominant monoterpene in the resin of most southern pines (Smith, 2000).

Xylotrechus integer breeds in stands of balsam fir *Abies balsamea* (L.) and eastern hemlock (Yanega, 1996); one of the sites used in Alabama contained a large component of mature eastern hemlock. However, α -pinene is a common monoterpene in eastern hemlock as well (Broeckling and Salom, 2003). Therefore, it is not surprising that traps baited with (–)- α -pinene were attractive to several species of pine-inhabiting wood borers throughout the southeastern USA. Selection should favor those individuals that capitalize on any cues that facilitate the quick invasion of suitable yet ephemeral and patchy resources (Atkins, 1966).

In many locations, attraction of four species of Cerambycids as well as the weevils *Hylobius pales* and *Pachylobius picivorus* and the wood-borer predator *Alaus myops* to traps baited with (–)- α -pinene was increased by the addition of ethanol (Figs. 1E–H, 2D–F, H, and 3B, C, F, G). The exceptions were *Prionus pocularis* (Fig. 4B), *Asemum striatum* (Fig. 4C), and *Xylotrechus integer* (Fig. 4D). Ethanol concentrations of woody tissues can rise dramatically in stressed or damaged trees because of anaerobic respiration (Moeck, 1970; Kelsey, 1996; Kelsey and Joseph, 2001). In cases such as those arising from fire damage, such cues are likely short lived and may be important for species that invade trees soon after injury (Kelsey and Joseph, 2003).

The synergistic effect of ethanol on the attraction of many of the species to traps baited with (–)- α -pinene was evident in many but not all locations. The apparent lack of synergy at some locations may be random events because of low numbers of beetles captured at these locations. Alternatively, it may represent geographical variation in the use of host attractants for these species, reflecting some variation in host preferences. Our studies were conducted in forests that varied widely in species composition (Table 1), and there is considerable variation in chemical and enantiomeric composition of resin among pine species in North America (Smith, 2000). The addition of other components, such as β -pinene or 3-carene, may be important in some locations. Enantiomeric composition of α -pinene may also be important (Allison et al., 2004). Erbilgin et al. (2001) found that *Pachylobius picivorus* were attracted equally to traps baited with ethanol and (+)- α -pinene as to traps baited with ethanol and (–)- α -pinene in Louisiana. However, they also found that catches of *H. pales* were lower in traps baited with ethanol and (+)- α -pinene than in traps baited with ethanol and (–)- α -pinene.

Managers of agencies responsible for interceptions of exotic species at ports-of-entry face a dilemma in choosing between general lures that attract a broad array of bark and wood-boring beetles and species-specific lures for a trapping program. Traps baited with commercial lures releasing ethanol and (–)- α -pinene at high rates are attractive to many common large wood-boring beetles from the southeastern USA (Figs. 1, 2, 3, 4, 5, 6, and 7) and other regions of North America (Phillips et al., 1988; Chénier and Philogène, 1989; Hunt and Raffa, 1989; Erbilgin et al., 2001; Allison et al., 2001; Morewood et al., 2002; Costello, 2005). Ethanol does not seem to interrupt attraction of these species to traps baited with (–)- α -pinene.

Species-specific attractants, such as pheromones, may be much more attractive than ethanol and (–)- α -pinene albeit to a smaller number of species (Billings and Cameron, 1984; Allison et al., 2003). The bark beetle pheromones ipsenol and ipsdienol are attractive to bark beetles as well as various species of sawyer beetles (*Monoctonus* spp.) in North America, but few other cerambycids (Billings and Cameron, 1984; Billings, 1985; Miller and Borden, 1990; Allison et al., 2001, 2003; De Groot and Nott, 2004; Miller and Asaro, 2005). However, as the absolute

efficiency of such lures in trapping populations of any species of bark- and wood-boring beetles is largely unknown, the relative increase in attraction to species-specific lures may not be that significant in forested areas. The relative increase in efficiency may be more important at ports-of-entry than in forested areas simply because of the lack of competing sources of attractants on average. Therefore, adding bark beetle pheromone lures to the combination of ethanol and (–)- α -pinene may provide an optimal solution by maintaining a low level of attraction to broad range of wood borers while maintaining a high level of attraction to some key species, such as bark beetles and sawyer beetles.

The issue of trap efficacy is an important one for risk assessments of exotic invasive species. We need to determine the proportion of a population caught by baited funnel traps to predict the real risk at that moment. We also need to know the variation in trapping efficacy caused by various environmental parameters, such as population levels, competing sources of attractants, and the lack of vertical silhouettes. Resolution of all these issues should lead to an increased ability in assessing risk and directing containment operations in an efficacious and timely manner.

The knowledge obtained by our work provides quarantine officials and forest managers in other countries with support for using traps baited with ethanol and (–)- α -pinene for capturing Cerambycidae native to the southeastern USA that may be inadvertently transported to other countries. In addition, such knowledge can be used at processing plants and ports-of-departure within the USA to ensure certification of products free of wood borers. Finally, such knowledge supports the use of traps baited with ethanol and (–)- α -pinene in monitoring the ecological impacts of wildfire and silvicultural treatments in southern forests (Hanula et al., 2002).

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Geographic and Seasonal Variation in Alkaloid-Based Chemical Defenses of *Dendrobates pumilio* from Bocas del Toro, Panama

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Abstract Poison frogs contain an alkaloid-based chemical defense that is derived from a diet of certain alkaloid-containing arthropods, which include mites, ants, beetles, and millipedes. Variation in population-level alkaloid profiles among species has been documented, and more than 800 different alkaloids have been identified. In the present study, we examine individual alkaloid variation in the dendrobatid poison frog *Dendrobates pumilio* among seven populations and between two seasons on Isla Bastimentos, located in the Bocas del Toro archipelago of Panama. Alkaloid profiles vary among populations and between seasons, illustrating that chemical defense in this species can vary on a small spatial and temporal scale. Alkaloid variation among populations is marginally correlated with geographic distance, and close populations have profiles more similar to each other than to distant populations. Individuals within populations also vary in alkaloid profiles. Differences are attributed to both spatial and temporal variations in the availability of alkaloid-containing arthropods. Many of the alkaloids present in the skin of *D. pumilio* appear likely to be of ant origin, supporting the importance of myrmecophagy in chemical defense among poison frogs. However, a variety of frog skin alkaloids was recently detected in mites, suggesting that mites may also play an important role in chemical defense.

Keywords Poison frogs · Dendrobatid · *Dendrobates pumilio* · Alkaloids · Arthropods · Ants · Mites · Chemical defense

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Introduction

Chemical defense in animals generally involves the manufacture or acquisition of defensive compounds from other organisms (Mebs, 2001). Although manufacturing defensive compounds is more common among animals, their acquisition from other organisms has convergently evolved in numerous lineages (Termonia et al., 2001). Animals commonly acquire defensive compounds from other organisms through the uptake, accumulation, in some cases modification, and storage of compounds (or precursors) that were originally present in other organisms, including microorganisms, plants, or other animals (Mebs, 2001; Termonia et al., 2001). Animals that employ this type of defensive strategy are dependent on symbiotic relationships with organisms capable of manufacturing defensive chemicals or a particular diet of chemically defended prey (Mebs, 2001).

Arthropods represent the largest group of animals known to acquire defensive compounds through diet, and this ability is known for lepidopterans, orthopterans, hemipterans, coleopterans, and hymenopterans (Boppre, 1990; Hartmann and Witte, 1995; Hartmann and Ober, 2000; Klitzke and Trigo, 2000). In addition to arthropods, flatworms (Kubaneck et al., 1995), nudibranch mollusks (Cimino and Ghiselin, 1998; Fahey and Garson, 2002), birds (Dumbacher et al., 2000, 2004), amphibians (Daly et al., 1994a,b, 2000, 2002, 2005; Jones et al., 1999; Saporito et al., 2003, 2004; Clark et al., 2005), and possibly snakes (Mori and Burghardt, 2000) have also been reported to acquire defensive compounds from dietary sources.

Poison frogs represent a group of organisms that are chemically defended from predation and possibly microorganisms by the dietary uptake of lipophilic alkaloids from arthropod prey (Daly and Myers, 1967; Daly et al., 1994a,b, 2000, 2002; Jones et al., 1999; Saporito et al., 2003, 2004; Clark et al., 2005; Macfoy et al., 2005). More than 800 alkaloids, representing at least 24 different structural classes, have been isolated from the skin of brightly colored poison frogs from four different anuran families (Dendrobatidae from Central and South America; Bufonidae from South America; Mantellidae from Madagascar; Myobatrachidae from Australia; Daly et al., 2005). The ability to uptake certain alkaloids through diet has been experimentally demonstrated in dendrobatid frogs of the genera *Dendrobates*, *Phyllobates*, and *Epipedobates* (Daly et al., 1994b), and mantellid frogs of the genus *Mantella* (Daly et al., 1997). It seems likely that bufonid toads of the genus *Melanophryniscus* will share a similar ability to uptake alkaloids (Garraffo et al., 1993a). Myobatrachid frogs of the genus *Pseudophryne* appear to biosynthesize pseudophrynamine alkaloids, yet are able to accumulate dietary provided pumiliotoxin alkaloids (Smith et al., 2002). Frogs of the genus *Dendrobates* have been shown to efficiently convert a dietary pumiliotoxin alkaloid (**251D**) to a more toxic allopumiliotoxin alkaloid (**267A**; Daly et al., 2003), and one species of the genus *Pseudophryne* appears to be able to metabolize a dietary pumiliotoxin (**307A**) by both reduction and hydroxylation (Smith et al., 2002), representing the only known incidents of alkaloid modification in poison frogs.

A variety of alkaloids are present in arthropods (for a review see, Jones and Blum, 1983; Numata and Ibuka, 1987; Braekman et al., 1998 and references within), and the ability of poison frogs to accumulate alkaloids through diet suggests that sequestration of these compounds from arthropod prey accounts for their presence in the skin of these frogs (with the exception of the pseudophrynamines of

myobatrachid poison frogs). Several alkaloid classes found in the skin of poison frogs have been identified in arthropods such as oribatid mites (Takada et al., 2005; Saporito et al., unpublished data), myrmicine, formicine, and ponerine ants (Daly et al., 1994a, 2000; Jones et al., 1999; Saporito et al., 2004; Clark et al., 2005), siphonotid millipedes (Saporito et al., 2003; Clark et al., 2005), and coccinellid and melyrid beetles (Ayer and Browne, 1977; Dumbacher et al., 2004). These arthropods represent the likely dietary sources for certain alkaloids found in poison frogs. Therefore, their presence in poison frogs is the result of uptake, accumulation, in certain cases modification, and storage of alkaloids from a diet of alkaloid-containing arthropods.

Alkaloid profiles (a measure of the number, type, and amount of alkaloids) are known to vary spatially and temporally among and within species of poison frogs (Myers and Daly, 1976, 1980; Daly et al., 1987, 1992, 1994a,b, 1996, 2002; Garraffo et al., 1993a,b; Myers et al., 1995; Clark et al., 2005; Mebs et al., 2005; and references within). In most cases, alkaloid variation has been described on a population level, in which samples of frogs collected from a certain population are pooled for chemical analysis (examples include Daly et al., 1987, 1996, 2002; Garraffo et al., 1993a,b; Mortari et al., 2004; and references within). Alkaloid variation among individuals within a population has been reported, but only in a few instances (see Daly et al., 1992, 1994a,b; Clark et al., 2005; Mebs et al., 2005; Myers et al., 1995). Although it is generally assumed that alkaloid variation within populations is less than variation among populations (see Daly et al., 1992), relatively few studies have directly addressed this issue. Changes over time in profiles have also been documented (Daly et al., 1987, 2002; Myers et al., 1995); however, no studies have specifically examined the question.

This study investigated the degree of geographic and temporal variations in alkaloid chemical defense within and among populations of the dendrobatid poison frog, *Dendrobates pumilio*, on Isla Bastimentos, located within the Bocas del Toro archipelago of Panama. The natural geographic range of *D. pumilio* extends from lowland Caribbean rainforests of southern Nicaragua through Costa Rica and into northwestern portions of Panama (Myers and Daly, 1983). Population-level alkaloid profiles have been shown to vary among populations of *D. pumilio* in the northwestern portions of Panama within and among islands in the Bocas del Toro archipelago, on the mainland bordering the archipelago, and in a few cases over time (Daly et al., 1987, 2002, unpublished data). In this study, we take a multivariate statistical approach to examine differences in individual alkaloid profiles within and among populations of *D. pumilio* over a small geographic range and between two different seasons.

Methods and Materials

Frog Collections

A total of 70 *D. pumilio* were collected from seven different populations located on Isla Bastimentos, Bocas del Toro Province, Panama (Fig. 1 and Table 1). Five *D. pumilio* were collected at each of the seven populations during the dry and wet seasons of 2003 (February 2–8 and August 20–23, respectively), for a total of 10

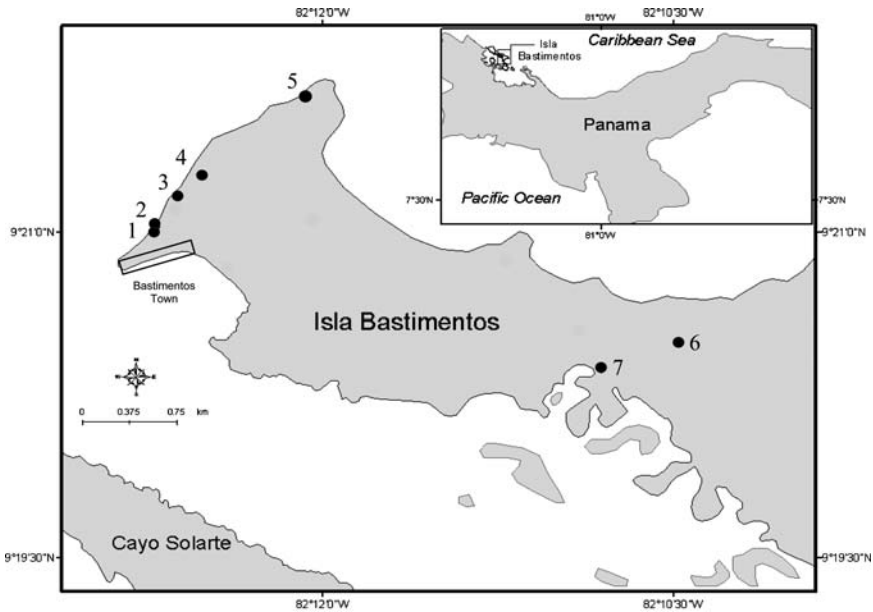


Fig. 1 Map of research sites on Isla Bastimentos, Bocas del Toro, Panama

frogs per population. At each of the seven sites, one 45×45 m plot was established. Frogs were captured by hand and the same plots were used in both seasons. Individuals were measured snout to vent (SVL) to the nearest 0.5 mm, and sex was determined. Only adult frogs were used (>20 mm SVL). All frogs were euthanized in the field laboratory, and individual *D. pumilio* skins were stored in uniquely marked 1.7-ml plastic vials containing 100% methanol at ambient temperature. All

Table 1 *Dendrobates pumilio* collection sites and descriptions on Isla Bastimentos, Bocas del Toro, Panama

Site	Name	GPS coordinates	Site description
1	Brust	9°20.996'N, 82°12.726'W	Numerous coconut palms; leaf-litter abundant
2	Cyclanthus slope	9°20.364'N, 82°10.807'W	Numerous <i>Cyclanthus</i> sp.; leaf-litter abundant
3	Tall Heliconia	9°21.169'N, 82°12.627'W	Numerous <i>Heliconia</i> sp.; leaf-litter sparse
4	Big Rock Forest	9°21.250'N, 82°12.519'W	Numerous <i>Cyclanthus</i> sp. and cacao; leaf-litter abundant
5	Faro	9°21.618'N, 82°12.074'W	Secondary forest; leaf-litter abundant
6	Red Frog Beach Hill	9°20.490'N, 82°10.486'W	Secondary forest; leaf-litter abundant
7	Shortcut	9°21.021'N, 82°12.704'W	Secondary forest; leaf-litter abundant

voucher specimens were deposited in the herpetological collection at Florida International University.

Alkaloid Extraction and Isolation

Individual alkaloid fractions were generated for all 70 *D. pumilio*; however, the protocol for preparing the individual fractions differed between seasons. Alkaloid fractions for the dry season samples was prepared as described by Daly et al. (1994b), whereas alkaloid fractions for the wet season samples were obtained from a modified version of this protocol (see below). The modified version was used to reduce analysis time.

Dry Season Samples

Each skin was cut into small pieces and macerated (with a mortar and pestle) \times 3, each time with 5 ml methanol. The combined methanol extract was diluted with 15 ml water and extracted \times 3, each time with 15 ml chloroform. Combined chloroform layers were concentrated to a small volume (\sim 5 ml) *in vacuo* at 35°C with a rotary evaporator. Following concentration, 20 ml *n*-hexane were added to the concentrated chloroform layer. This solution was extracted \times 3, each time with 15 ml of 0.1 N HCl. The combined 0.1 N HCl fractions were adjusted to pH 9.0 with 2 N aqueous ammonia, followed by extraction \times 3, each time with 15 ml chloroform. The combined chloroform extract was dried with anhydrous Na₂SO₄ and evaporated to dryness at 35°C *in vacuo* with a rotary evaporator. The resulting alkaloid residue was dissolved in sufficient methanol so that 100 μ l of this alkaloid fraction corresponded to 100 mg of the original wet weight of the skin. In the dry season samples, it appeared that the recovery of alkaloids was less than expected, although there was no apparent explanation for the poor yield.

Wet Season Samples

Each skin was cut into small pieces and macerated (with a mortar and pestle) \times 2, each time with 4 ml methanol. The combined methanol extract was immediately mixed with 8 ml of 0.1 N HCl and extracted \times 5, each time with 20 ml hexane. The pH was adjusted to 9.0 with 2 N aqueous ammonia, followed by extraction \times 3 with 15 ml chloroform. This solution was dried with anhydrous Na₂SO₄ and evaporated to dryness at 35°C *in vacuo* with a rotary evaporator. Following the original protocol, the resulting alkaloid residue was dissolved in sufficient methanol so that 100 μ l of this alkaloid fraction corresponded to 100 mg of the original wet weight of the skin.

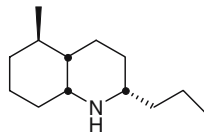
Alkaloid Identification (GC-MS)

All alkaloids within each fraction were identified by using gas chromatography in conjunction with mass spectrometry (GC-MS). Identification of individual alkaloids was based on comparison of retention times and mass spectral data with expected data for known anuran alkaloids. Anuran alkaloids have been assigned a code name, consisting of a bold-faced number corresponding to the nominal mass and a bold-faced letter for identification of individual alkaloids with the same nominal mass

(Daly et al., 1999). A revised, updated tabulation with code names for over 800 anuran alkaloids is now available (Daly et al., 2005).

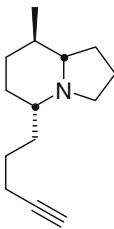
GC-MS analysis for all dry season samples was performed on a Finnigan GCQ instrument with a 25 m × 0.25 mm i.d. Rtx-5 amine fused-silica column (Restek). GC-MS analysis for all wet season samples was performed on the same Finnigan GCQ instrument, however with a 30 m × 0.25 mm i.d. DB-1 fused-silica column (J&W Scientific). GC separation of alkaloids was achieved by using a temperature program from 100 to 280°C at a rate of 10°C per min with helium as the carrier gas. Each extract was analyzed with both electron impact-mass spectrometry (EI-MS) and chemical ionization-mass spectrometry (CI-MS) with ammonia as the reagent

2,5-Disubstituted decahydroquinoline (DHQ):

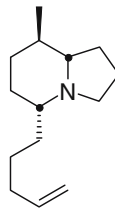


cis-195A

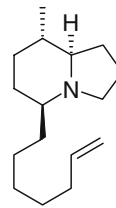
5,8-Disubstituted indolizidines (5,8-I):



205A

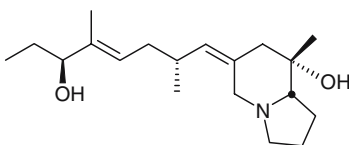


207A

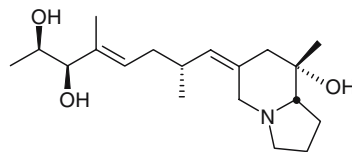


235B''

Pumiliotoxins (PTX):



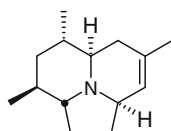
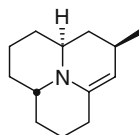
307A



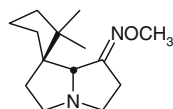
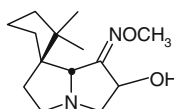
323A

Fig. 2 Structures of the most abundant alkaloids found in *Dendrobates pumilio* on Isla Bastimentos, Bocas del Toro, Panama

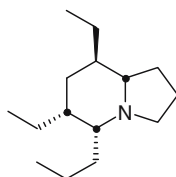
Tricyclics (TRI):

**205B****191B**

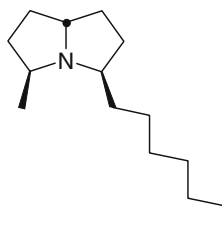
Spiropyrrolizidine oximes (Spiro):

**236****252A**

5,6,8-Trisubstituted indolizidine (5,6,8-I):

**223A**

3,5-Disubstituted pyrrolizidine (3,5-P):

**223H****Fig. 2** (continued)

gas. An injection volume of 2 μ l was used for each frog alkaloid fraction, corresponding to 2 mg wet weight frog skin.

Alkaloid Quantification (GC-FID)

All alkaloids within each fraction were assessed quantitatively by using gas chromatography in combination with a flame-ionization detector (GC-FID). GC was performed on a Hewlett Packard 5890 with a 6-ft 1.5% OV-1 packed column (2 mm i.d.). GC separation of alkaloids was achieved by using a temperature program from 150 to 280°C at 10°C per min with helium as the carrier gas. An injection volume of 2 μ l was used for each alkaloid fraction, corresponding to 2 mg wet weight frog skin.

In order to quantitatively assess the amount of alkaloids within individual frogs, a calibration curve for GC-FID was constructed by using a standard of the alkaloid, decahydroquinoline (DHQ) **195A**. Decahydroquinoline **195A** is the most common alkaloid found in all frog populations from the northwest coast of Isla Bastimentos. Each alkaloid within an individual frog was assigned to one of the following quantitative categories: (1) major alkaloid (present in an amount greater than or equal to 50 μg per 100 mg frog skin), (2) minor alkaloid (present in an amount between 5 and 50 μg per 100 mg frog skin), or (3) trace alkaloid (present in an amount less than 5 μg per 100 mg frog skin). Using this quantification scheme allows for the direct comparison of data from this study to those of previous and current studies conducted on other lipophilic alkaloid-containing frogs and toads.

Statistical Analyses

Variation in individual alkaloid profiles within and among populations of *D. pumilio* were graphically visualized for both seasons by using nonmetric multidimensional scaling (nMDS). Statistical differences in individual alkaloid profiles among populations for each season were detected with a one-way analysis of similarity (ANOSIM). Statistical differences in individual alkaloid profiles among the same populations between seasons were examined with a two-way ANOSIM. Both nMDS plots and ANOSIM results are based on Bray–Curtis dissimilarity matrices. A Mantel test was performed to examine the relationship between geographic distance and alkaloid profile variation among populations for the wet season data. All statistical analyses were performed with the software program PRIMER (version 5) and the PopTools add-in function in Microsoft Excel (written by G. Hood; available at <http://www.cse.csiro.au/poptools>).

Results

Alkaloids Present in *D. pumilio*

A total of 153 alkaloids, representing 16 different structural classes, were identified from *D. pumilio* skin extracts. Individual frogs contained an average of 17 different alkaloids (range 6–31 alkaloids). Populations also contained an average of 17

Table 2 The most abundant alkaloids present in each population of *D. pumilio* on Isla Bastimentos, Panama

Site	DHQ	3,5-P		5,8-I			5,6,8-I		Tricyclic		Spiro		PTX	
	195A	223H	205A	207A	235B	223A	205B	191B	236	252A	307A	323A		
1	+				+		+	+	+	+	+	+		
2	+		+	+	+		+	+	+	+	+	+		
3	+	+			+	+	+	+			+	+		
4	+	+			+		+				+	+		
5	+		+	+		+								
6	+		+	+										
7	+		+	+	+	+								

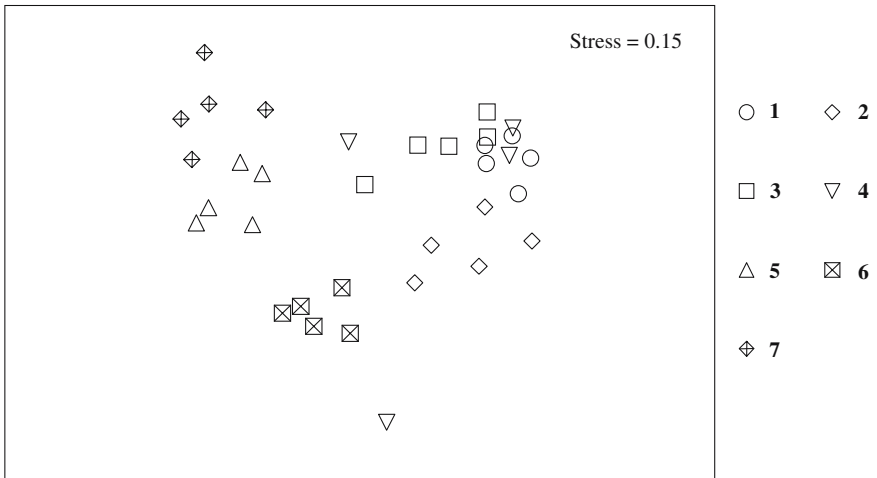


Fig. 3 Nonmetric multidimensional scaling (nMDS) plot of *D. pumilio* alkaloid profiles among seven populations on Isla Bastimentos during the dry season. nMDS plot is based on the presence/absence of alkaloids. Each symbol represents an individual frog from a specific population. Site numbers correspond to map in Fig. 1

different alkaloids (range 9–31 alkaloids). Decahydroquinoline **195A** was found in all frog skin extracts from each of the seven sites during both seasons, and represents the most common and widespread alkaloid among populations of *D. pumilio* on Isla Bastimentos. Decahydroquinoline **195A** is thought to be obtained from myrmicine ants that are found microsympatrically with dendrobatid frogs containing this alkaloid (Daly et al., 2000, 2002). Based on the presence of an

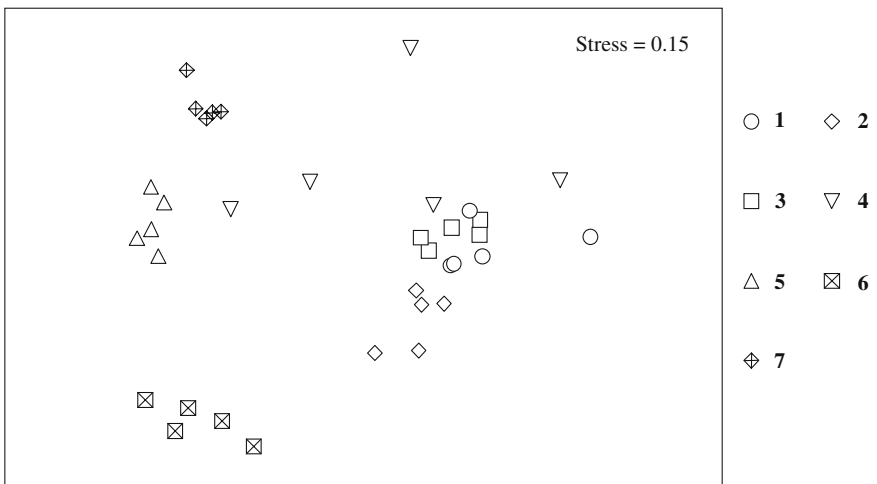
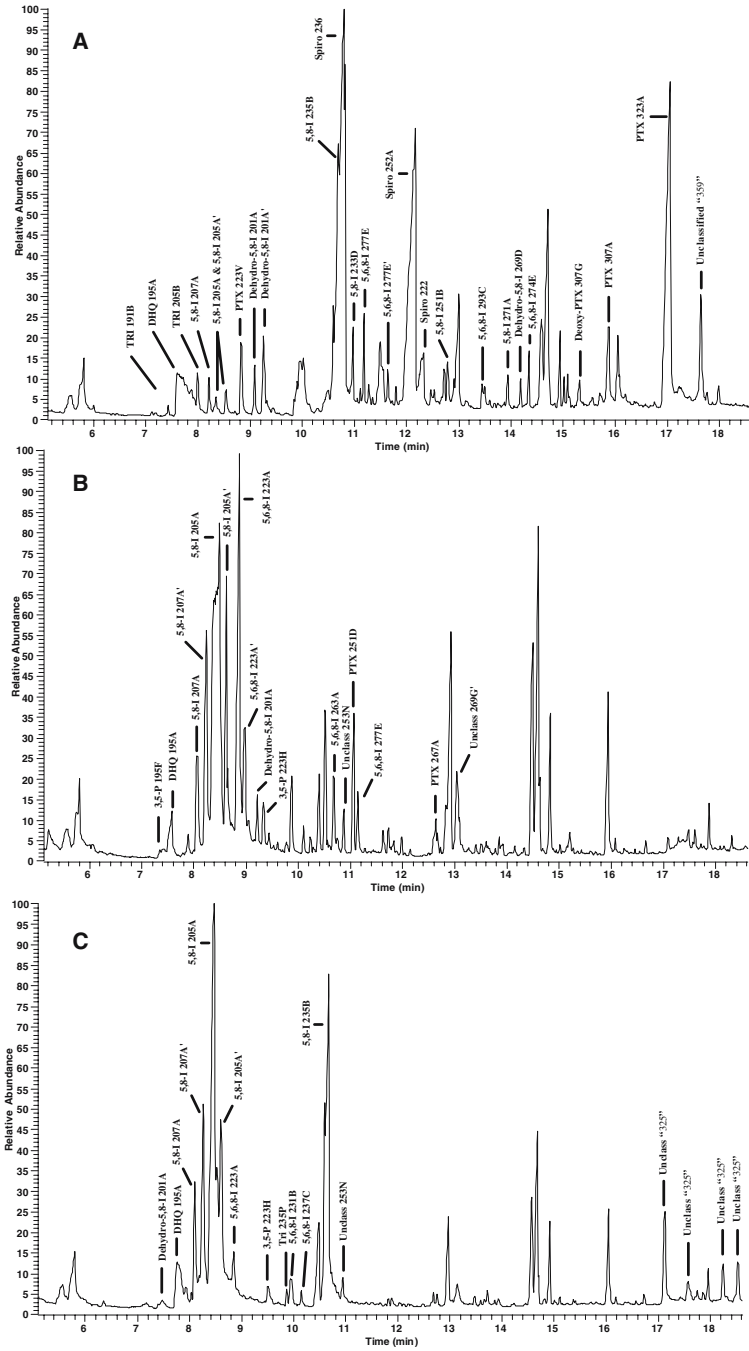


Fig. 4 Nonmetric multidimensional scaling (nMDS) plot of *D. pumilio* alkaloid profiles among seven populations on Isla Bastimentos during the wet season. nMDS plot is based on major, minor, and trace alkaloids. Each symbol represents an individual frog from a specific population. Site numbers correspond to map in Fig. 1



alkaloid in at least three out of the five frogs sampled from each site (excluding alkaloids present in trace amounts), the other most abundant alkaloids in populations of *D. pumilio* were 5,8-disubstituted indolizidines (5,8-Is) **205A**, **207A**, and **235B''**, pumiliotoxins (PTXs) **307A** and **323A**, tricyclics (TRIs) **205B** and **191B** (propyleine), spiropyrrrolizidines (Spiros) **236** and **252A**, a 5,6,8-trisubstituted indolizidine (5,6,8-I) **223A**, a 3,5-disubstituted pyrrolizidine (3,5-P) **223H**, and two unclassified alkaloids of apparent molecular weight “321” and “325.” Chemical structures for these are shown in Fig. 2 (structures have not yet been proposed for the two unclassified alkaloids).

The 5,8-Is **205A** and **207A** were detected as major or minor alkaloids in most frog skin extracts from sites 2, 5, 6, and 7, whereas the 5,8-I **235B''** was a major or minor alkaloid in most frog skin extracts from sites 1, 2, 3, 4, and 7. The PTXs **307A** and **323A** were detected as major or minor alkaloids in frog skin extracts from sites 1, 2, 3, and 4. The tricyclic **205B** was a major or minor alkaloid in most frogs from sites 1, 2, 3, and 4, whereas the tricyclic **191B** was a major or minor alkaloid in most frogs from sites 1, 2, and 3. The Spiros **236** and **252A** were the major or minor alkaloids in most frogs from sites 1 and 2. The 5,6,8-I **223A** was a major or minor alkaloid in most frogs from sites 3, 5, and 7. The 3,5-P **223H** was a major or minor alkaloid in most frogs from sites 3 and 4. Two unidentified alkaloids, “321” and “325,” were detected as major alkaloids in all frogs from site 7, but only during the wet season. Site locations and descriptions are in Table 1 and Fig. 1. The above information is summarized in Table 2.

Alkaloid Variation—Dry Season

Alkaloid profiles for individual frogs during the dry season were significantly different among the seven sites (Global $R = 0.77$, $P < 0.001$). Pairwise comparisons of the seven sites showed no statistical difference in alkaloid profiles between frogs from sites 3 and 4 (Global $R = 0.19$, $P < 0.063$). Differences within and among sites are graphically displayed with nMDS (Fig. 3). Due to low recoveries, profiles for individual frogs during the dry season were statistically analyzed based on untransformed presence/absence of alkaloids.

Alkaloid Variation—Wet Season

Alkaloid profiles for individual frogs during the wet season were significantly different among all seven sites (Global $R = 0.83$, $P < 0.001$). Differences within and among sites are graphically displayed with nMDS (Fig. 4). Profiles for individual frogs during the wet season were statistically analyzed based on untransformed categorical (i.e., major, minor, or trace) alkaloid data. Representative alkaloid profiles for an individual frog from sites 2, 5, and 6 are presented in Fig. 5. Alkaloid

Fig. 5 Gas chromatographs of alkaloid profiles of individual *D. pumilio* from three different populations on Isla Bastimentos, illustrating variation in alkaloids among populations. Only major and minor alkaloids are identified in the chromatographs. Peaks that are not identified represent trace alkaloids or nonalkaloid compounds (i.e., plasticizers, fatty acid methyl esters, etc. that were not removed during the fractionation process). (A) Individual *D. pumilio* from site 2, (B) individual *D. pumilio* from site 5, (C) individual *D. pumilio* from site 6. Site numbers correspond to map in Fig. 1

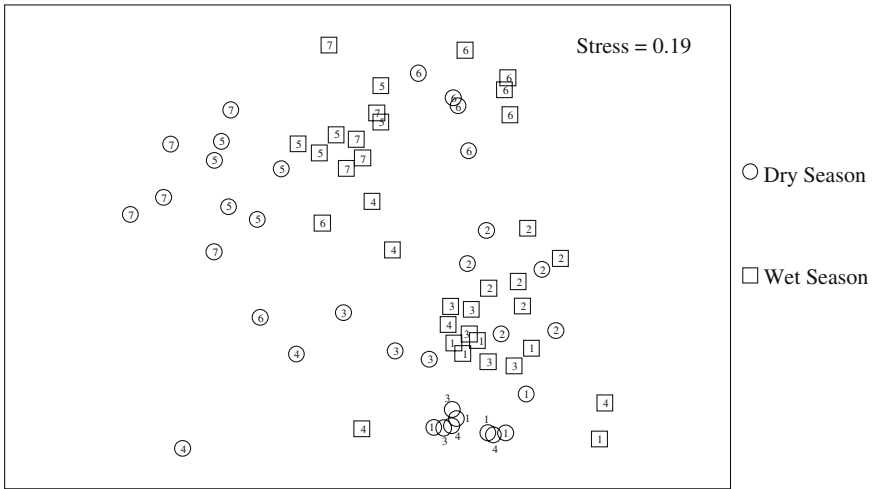


Fig. 6 Nonmetric multidimensional scaling (nMDS) plot of *D. pumilio* alkaloid profiles for individual frogs in seven populations on Isla Bastimentos between the dry and wet season. nMDS plot is based on the presence/absence of alkaloids. Each symbol represents an individual frog from a specific population and season. Site numbers correspond to map in Fig. 1

profiles were positively correlated with geographic distance among all seven sites ($R = 0.51$, $P < 0.001$).

Alkaloid Variation—Dry vs. Wet Season

Alkaloid profiles for individual frogs among the seven sites between the dry and wet season were significantly different (Global $R = 0.502$, $P < 0.001$). Differences in alkaloid profiles among sites and between seasons are graphically displayed with nMDS (Fig. 6). Due to low recoveries in the dry season samples, alkaloid profiles for individual frogs among sites and during both seasons were statistically analyzed based on untransformed presence/absence of alkaloids.

Discussion

Alkaloid profiles of *D. pumilio* examined in this study varied significantly among populations and between seasons on Isla Bastimentos, Bocas del Toro, Panama. The seven populations are located in the northwestern region of Isla Bastimentos and no two populations are more than 3.5 km in distance from each other (with most populations being less than 1 km apart; see Fig. 1), demonstrating that chemical defense can vary considerably over a relatively small geographic range. Our results are consistent with a previous study (Daly et al., 2002), which showed that population-level alkaloid profiles varied among five of the same populations. Alkaloid profiles in this study also varied between populations sampled during the dry and wet season, illustrating that chemical defense in *D. pumilio* can vary across short periods of time. Although differences in alkaloid profiles have been documented among

years in certain populations of *D. pumilio* (Daly et al., 1987, unpublished data), changes have never been described for a time period as brief as two seasons.

Variation in alkaloid profiles among populations of *D. pumilio* within a season is related to geographic proximity, and close populations tend to have more similar profiles than distant ones. Myers and Daly (1976) have shown that geographically close populations of *D. histrionicus* share more alkaloids than do distant populations. Myers et al. (1995) reported that sympatric populations of *D. pumilio* and *D. granuliferus* contain more alkaloids in common with each other than with geographically distant populations of *D. granuliferus*. Daly et al. (1992) illustrated that introduced populations of *D. auratus* in Hawaii have alkaloid profiles more similar to each other than those of their ancestral population from Panama. In our study, geographic distance accounted for 51% of the difference in alkaloid profiles among populations of *D. pumilio*, suggesting that the distribution of certain alkaloid-containing arthropods in this region is also related to geographic distance.

Variation in profiles among individuals within populations is less than variation among populations. Daly et al. (1992) stated that there is variation in the amounts of alkaloids found in individuals within populations of *D. auratus*, and suggested that these differences were less than differences among populations. Daly et al. (1994a,b) illustrated that although alkaloid compositions are similar within populations of *D. auratus*, the relative amounts of a given alkaloid are variable. Our study with *D. pumilio* supports these findings. Myers et al. (1995) described individual alkaloid variation within two populations of *D. granuliferus* and one population of *D. pumilio* from Costa Rica, and concluded that there were differences among individual frogs. Clark et al. (2005) recently reported that individual alkaloid profiles varied within populations for three species of *Mantella* from Madagascar. Mebs et al. (2005) recently reported variable levels of alkaloids (particularly the pumiliotoxin **251D**) in individuals of *Melanophryniscus montevidensis* from Uruguay. In our study, individual frogs within a population differed in overall alkaloid profiles (differences in the number of alkaloids present in individual frogs are illustrated in Fig. 7). In some cases, alkaloids that were predominant in one frog were completely absent in another frog from the same population. Clark et al. (2005) suggested that individual variation within populations of *Mantella* represents the possibility that some alkaloid-containing arthropods are rare. Based on the degree of individual alkaloid variation observed here, we agree that certain alkaloid-containing arthropods are rare, and further suggest that in some cases, the distribution of arthropods is confined to small areas and may be the result of localized hatches or migrations (Daly et al., 2002). Differences in alkaloid profiles among *D. pumilio* individuals within a population likely represent local small-scale geographic differences in the availability of certain alkaloid-containing arthropods.

The presence of an alkaloid-based chemical defense in poison frogs (including *D. pumilio*) is a result of the dietary accumulation of alkaloids from a variety of arthropod prey items. Coccinellid beetles contain coccinelline alkaloids as well as some of the structurally related tricyclic alkaloids (Ayer and Browne, 1977), and an oribatid mite contains two coccinelline alkaloids (Takada et al., 2005). Batrachotoxin alkaloids have been identified in melyrid beetles (Dumbacher et al., 2004) and spiropyrrolizidine alkaloids in siphonotid millipedes (Saporito et al., 2003; Clark et al., 2005). Ants appear to be the largest presumed source of alkaloids in poison frogs. In particular, 2,5-disubstituted pyrrolidines, 2,6-disubstituted piperidines, 3,5-disubstituted pyrrolizidines, 3,5-disubstituted indolizidines, 4,6-disubstituted quinoli-

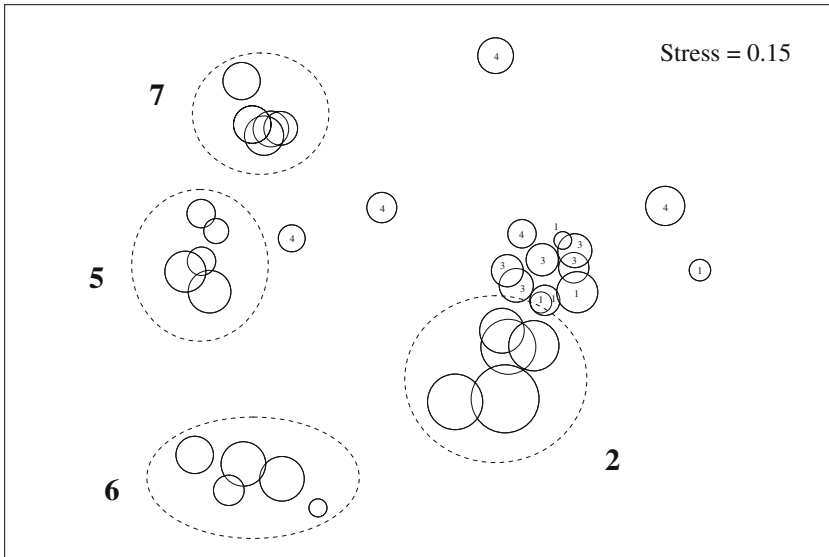


Fig. 7 Nonmetric multidimensional scaling (nMDS) plot of *D. pumilio* alkaloid profiles for individual frogs in seven populations on Isla Bastimentos during the wet season. nMDS plot is based on major, minor, and trace alkaloids. Each symbol represents an individual frog from a specific population. Site numbers correspond to map in Fig. 1. The size of each circle is proportional to the total number of alkaloids in that individual

zidines, and 2,5-disubstituted decahydroquinolines have been identified in ants of the subfamily Myrmicinae (Daly et al., 1994a, 2000; Jones et al., 1999; Clark et al., 2005). These alkaloids contain unbranched carbon skeletons. In addition, 3,5-disubstituted lehmizidines, histrionicotoxins, and gephyrotoxins are alkaloid classes that share certain structural features (i.e., unbranched carbon skeletons) with those of known myrmicine ant alkaloids, and it is expected that they will prove to be of myrmicine ant origin as well (Daly et al., 2005). 5,8-Disubstituted indolizidines have been identified in a mixed collection of leaf-litter arthropods from Isla Bastimentos, Panama (Daly et al., 2002), and recently in ants of the subfamily Myrmicinae from Madagascar (Clark et al., 2005). 2,5-Disubstituted pyrrolidines and 3,5-disubstituted pyrrolizidines were recently reported in ants of the subfamilies Formicinae and Ponerinae from Madagascar (Clark et al., 2005). A 5,6,8-trisubstituted indolizidine has been identified in ants from Panama; however, the sample included several ant species, and the specific ant taxon that contains the compound could not be determined (Saporito et al., 2003). In addition, a 5,6,8-trisubstituted indolizidine and a 1,4-disubstituted quinolizidine have been identified in an oribatid mite (Takada et al., 2005). Pumiliotoxin alkaloids have been identified in ants of the subfamily Formicinae from Panama (Saporito et al., 2004) and more recently in oribatid mites (Takada et al., 2005).

Poison frogs' dependence on arthropods for chemical defense suggests that much of the geographic and temporal variation observed in alkaloid profiles is the result of variation in availability of alkaloid-containing arthropods. In tropical regions, arthropod abundances are known to vary spatially and temporally (Janzen and Schoener, 1968; Janzen, 1973; Lieberman and Dock, 1982; Levings and Windsor,

1984; Levings, 1983), suggesting that the availability of alkaloid-containing arthropods is variable. On Isla Bastimentos, the spiropyrrolizidine alkaloid **236** and the pumiliotoxin alkaloids **307A** and **323A** are major alkaloids in certain populations of *D. pumilio*. Recently, arthropod sources for these two classes of alkaloids have been shown to occur sympatrically with the same populations of *D. pumilio* examined in this study (Saporito et al., 2003, 2004). The spiropyrrolizidine **236** is a major alkaloid in two of the seven populations of *D. pumilio* on Isla Bastimentos; however, in 1972 it was not detected in these populations (Daly et al., 1987, 2003). The appearance of this alkaloid in populations of *D. pumilio* today is most likely due to a recent introduction and current availability of the millipede *Rhinotus purpureus* (Family: Siphonotidae) as a dietary source (Saporito et al., 2003). The presence of **236** in certain populations of *D. pumilio* supports the idea that spatial and temporal differences in arthropod availability plays an important role in alkaloid variation of these dendrobatid frogs. Recently, a variety of different spiropyrrolizidine alkaloids, including **236**, have also been discovered in *R. purpureus* millipedes from Madagascar, which occur sympatrically with alkaloid-containing mantellid frogs (Clark et al., 2005). These findings suggest that variation in alkaloids within the same species of arthropod may also be responsible for alkaloid variation among poison frogs. The pumiliotoxins **307A** and **323A** are present in variable amounts in certain populations of *D. pumilio* on Isla Bastimentos. Dietary sources for these have been identified in two different genera of ants in the Subfamily Formicinae (*Paratrechina* and *Brachymyrmex*) in regions where *D. pumilio* populations are known to contain variable amounts of these alkaloids (Saporito et al., 2004). Interestingly, their presence in *Brachymyrmex* is also variable. The pumiliotoxins **307A** and **323A** were not detected in all *Brachymyrmex* samples collected within a site, and certain ant samples contained only one of the two pumiliotoxins (Saporito et al., 2004). The pumiliotoxins were not detected in ants from the dry season (Saporito et al., 2004). The variable presence of pumiliotoxins in *Brachymyrmex* suggests the possibility of a dietary source or microsymbiont as the ultimate source for these alkaloids in ants (Saporito et al., 2004). Recently, the pumiliotoxins **237A** and **251D** and the deoxypumiliotoxin **193H** were identified in two different species of scheloribatid mites (*Schelorbates azumaensis* and *Schelorbates* sp.), suggesting that mites may be the ultimate source for some of the pumiliotoxin alkaloids (Takada et al., 2005). However, ants in the genus *Solenopsis* are known to be caste-specific in the production of alkaloids (Deslippe and Guo, 2000; Torres et al., 2001), and it remains possible that alkaloid variation in *Brachymyrmex* is attributable to differences among castes. Regardless of the source of alkaloid variation in *Brachymyrmex*, the variable presence of pumiliotoxins in these ants suggests that alkaloid variability of certain arthropod prey items may contribute to alkaloid profile variation in *D. pumilio*.

Other factors may also contribute to alkaloid variation within and among populations of *D. pumilio*. Some poison frogs possess the ability to modify certain dietary pumiliotoxin alkaloids (Smith et al., 2002; Daly et al., 2003). One species in the genus *Pseudophryne* is able to convert the pumiliotoxin **307A** to a significant extent into reduced and/or hydroxylated pumiliotoxins of molecular weights 309, 323, and 325 (Smith et al., 2002). Frogs in the genus *Dendrobates* are able to convert over 70% of the natural dietary (+)-enantiomer of pumiliotoxin **251D** (by stereoselective hydroxylation) into the natural (+)-allopumiliotoxin **267A** (Daly et al., 2003). Frogs of the genus *Dendrobates* did not hydroxylate the unnatural (–)-en-

antimer of pumiliotoxin **251D** (Daly et al., 2003). The presence of such a hydroxylase raises the likelihood that poison frogs are able to convert other pumiliotoxin alkaloids (i.e., side-chain hydroxylated analogs) into allopumiliotoxins. Both of these processes are likely mediated by enzymes and are under some genetic control, and a differential ability to modify certain alkaloids would influence variability in profiles. Pumiliotoxin **251D** is always accompanied by **267A** in populations of *D. pumilio* that have been examined (this study; Daly et al., 1987, 2003, and unpublished data), and, therefore, the data do not suggest a difference in the ability to convert **251D** within and among populations of *D. pumilio*. However, differences among genera of poison frogs in the ability to modify certain pumiliotoxins appear to contribute to alkaloid variation among species (Daly et al., 2003). For instance, frogs of the genera *Phyllobates* and *Epipedobates* did not hydroxylate dietary pumiliotoxin **251D** (Daly et al., 2003). The ability to uptake and sequester certain alkaloids is also likely under genetic control (Myers et al., 1995), and variation in alkaloid profiles of *D. pumilio* may be in part due to genetic variation in the uptake/sequestration process. Although not intended to address genetic differences in alkaloid uptake, Summers et al. (1997) demonstrated that there were low degrees of mitochondrial DNA divergence among populations of *D. pumilio* in the Bocas del Toro region of Panama, including populations on Isla Bastimentos. This study illustrates that there are no prominent genetic differences among populations, at least as revealed by mitochondrial markers, suggesting that genetic factors are not responsible for the variation observed in alkaloid profiles. In addition, genetic factors would not solely explain differences in alkaloid profiles between seasons. Finally, it remains possible that differences in prey selectivity may explain alkaloid variation. If different populations and individuals within populations are differentially utilizing prey, alkaloid profiles could reflect variable prey consumption. In a study of *D. pumilio* diet in Costa Rica, Donnelly (1991) suggested that frogs change their diet over time in response to natural fluctuation in the availability of food. It is likely that differences in diet of *D. pumilio* within and among populations are also attributable to differences in the availability of arthropod prey. Although differences in selectivity may account for some of the alkaloid variation observed among different species of poison frogs, it is unlikely that individuals of *D. pumilio* confined to a small area exhibit such marked geographic and seasonal differences in dietary selectivity. Donnelly (1991) also reported differences in diet between juveniles and adults as well as between sexes of *D. pumilio*. Here, we only examined adults. However, both males and females were examined, and it is possible that some of the individual variation within populations is related to sexual differences in diet. Although males and females were collected from each population in this study, sample sizes were not large enough to statistically examine differences between sexes.

The natural diet of *D. pumilio* consists mainly of ants and mites (Donnelly, 1991; Caldwell, 1996; Saporito et al., unpublished data). In general, dendrobatid frogs of the genera *Dendrobates*, *Epipedobates*, and *Phyllobates* consume a large proportion of ants as part of their diet, and members of the genus *Dendrobates* (including *D. pumilio*) and some members of the genus *Epipedobates* have been referred to as “ant-specialists” (Donnelly, 1991; Toft, 1995; Caldwell, 1996). Frogs of the genera *Mantella*, *Melanophryniscus*, and *Pseudophryne* have also been shown to consume large amounts of ants (Pengilley, 1971; Filipello and Crespo, 1994; Vences et al., 1998). These findings have led many researchers to suggest that myrmecophagy has

played a major role in the evolution of alkaloid sequestration in poison frogs (Toft, 1995; Caldwell, 1996; Vences et al., 1998, 2003; Summers and Clough, 2001; Daly et al., 2002; Santos et al., 2003; Saporito et al., 2004; Clark et al., 2005; Darst et al., 2005). Interestingly, although myobatrachid frogs of the genus *Pseudophryne* consume a large proportion of ants, they do not appear to accumulate myrmicine ant alkaloids (see Smith et al., 2002). Instead, these frogs synthesize unique indolic pseudophrynamine alkaloids (Smith et al., 2002). Therefore, their uptake system may be designed to retain the pseudophrynamines and also to accept certain dietary pumiliotoxins for uptake and storage (see Smith et al., 2002). In our study, many of the alkaloids are from classes known or suspected to be of ant origin, suggesting that myrmecophagy plays a dominant role in chemical defense in these frogs. However, certain tricyclic, indolizidine, quinolizidine, spiropyrrolizidine, and pumiliotoxin alkaloids were recently detected in mites (Takada et al., 2005; Saporito et al., unpublished data), suggesting that mites represent another major source of alkaloids in poison frogs.

In summary, individual alkaloid profiles of *D. pumilio* are shown to vary among populations and between the dry and wet season on Isla Bastimentos, Bocas del Toro, Panama. Variation in profiles among populations is related to geographic proximity, and it is likely that the availability of alkaloid-containing arthropods in this region share a similar distribution. Individual frogs within a population have alkaloid profiles more similar to each other than to frogs from different populations. Individual variation within a population is attributed to small geographic differences in the distribution of alkaloid-containing prey. Overall, alkaloid variation in *D. pumilio* is likely the result of spatial and temporal variation in the availability of certain alkaloid-containing arthropods and the presence of alkaloids in these arthropods. Many of the alkaloids found in *D. pumilio* appear to be from ant sources, further indicating the importance of ants in chemical defense among poison frogs. Recent findings that certain frog skin alkaloids are also known in mites, suggests that mites may also play an important role in the chemical defense of poison frogs.

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Chemical Defenses Promote Persistence of the Aquatic Plant *Micranthemum umbrosum*

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Abstract Five of the most common macrophytes from an aquaculture facility with high densities of the herbivorous Asian grass carp (*Ctenopharyngodon idella*) were commonly unpalatable to three generalist consumers—grass carp and the native North American crayfishes *Procambarus spiculifer* and *P. acutus*. The rooted vascular plant *Micranthemum umbrosum* comprised 89% of the total aboveground plant biomass and was unpalatable to all three consumers as fresh tissues, as homogenized pellets, and as crude extracts. Bioassay-guided fractionation of the crude extract from *M. umbrosum* led to four previously known compounds that each deterred feeding by at least one consumer: 3,4,5-trimethoxyallylbenzene (**1**) and three lignoids: β -apocropodophyllin (**2**); (–)-(3*S*,4*R*,6*S*)-3-(3',4'-methylenedioxy- α -hydroxybenzyl)-4-(3'',4''-dimethoxybenzyl)butyrolactone (**3**); and (–)-hibalactone (**4**). None of the remaining four macrophytes produced a chemically deterrent extract. A 16-mo manipulative experiment showed that the aboveground biomass of *M. umbrosum* was unchanged when consumers were absent, but the biomass of *Ludwigia repens*, a plant that grass carp preferentially consumed over *M. umbrosum*, increased over 300-fold. Thus, selective feeding by grass carp effectively eliminates most palatable plants from this community and promotes the persistence of the chemically defended *M. umbrosum*, suggesting that plant defenses play critical yet understudied roles in the structure of freshwater plant communities.

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Introduction

Herbivores were historically thought to have little impact on the ecology and evolution of freshwater plant communities (e.g., Shelford, 1918; Hutchinson, 1975). However, quantitative reviews show that herbivore impacts in freshwater systems rival those of marine and terrestrial systems, and aquatic herbivores often reduce the standing stock and alter the species composition of freshwater plant communities (Lodge, 1991; Newman, 1991; Cyr and Pace, 1993; Lodge et al., 1998). Additionally, aquatic herbivores exhibit selective avoidance of chemically or structurally defended plants (Newman et al., 1996; Bolser et al., 1998; Cronin, 1998; Cronin et al., 2002); yet there is surprisingly little direct evidence linking consumer feeding preferences to particular plant traits, or ultimately to shifts in plant community structure. For example, the introduced crayfish *Orconectes virilis* selectively consumes the filamentous green alga *Cladophora* over the blue-green alga *Gleotrichia*, and instigates a shift from green to blue-green algae in experimental ponds (Dorn and Wojdak, 2004), but the mechanisms conferring resistance to herbivores in *Gleotrichia* were not investigated.

A number of studies show that freshwater macrophytes are frequently unpalatable and contain a variety of secondary metabolites that could function as herbivore deterrents (Ostrowsky and Zettler, 1986; Cronin et al., 2002). More than one half of the crude extracts from 21 species of aquatic macrophytes that Prusak et al. (2005) surveyed, for example, deterred feeding by an omnivorous crayfish, although they did not identify the metabolites responsible for feeding deterrence. In fact, we know of only three freshwater plants with identified compounds that deter herbivores—watercress, *Rorippa nasturtium-aquaticum* (L.) Hayek (Newman et al., 1996), the waterspider bog orchid, *Habenaria repens* Nutt. (Bolser et al., 1998; Wilson et al., 1999), and lizard's tail, *Saururus cernuus* L. (Kubanek et al., 2000, 2001)—with a total of nine described secondary metabolites that influence herbivore feeding. In contrast, hundreds to thousands of secondary metabolites that deter consumers have been described from marine and terrestrial primary producers (Seigler, 1998; Faulkner, 2002 and references therein). These molecules can have strong cascading impacts on the ecology and evolution of plant–herbivore interactions in these systems (Hay and Fenical, 1988, 1996; Hay, 1996), suggesting that plant chemical defenses may play similar, but relatively uninvestigated, roles in freshwater systems.

Here, we examined the feeding preferences of three generalist consumers among five species of macrophytes collected from an aquaculture facility stocked with high densities of the herbivorous Asian grass carp *Ctenopharyngodon idella*. To determine the traits promoting macrophyte persistence under intense herbivory, we assessed the palatability of (1) whole plants, (2) plant tissues ground and imbedded in a gel-like matrix to retain most of the chemical and nutritional traits but with normal morphological traits removed, (3) plant crude extracts, and (4) specific metabolites isolated using bioassay-guided fractionation. We also conducted

a manipulative field experiment excluding herbivorous fishes. We then assessed the changes in the littoral plant community after 16 mo to determine whether well-defended species were disadvantaged in the absence of herbivores.

Methods and Materials

Study Organisms

We collected macrophytes from two 91 × 61 m wide, 1.3 m deep earthen ponds at the Owens and Williams fish hatchery in Hawkinsville, GA, USA. Each pond was stocked with >100,000 juvenile, triploid Asian grass carp, *C. idella*, an exotic herbivorous fish introduced throughout the United States to reduce aquatic plant abundance (USGS, 2005). On one occasion, we observed one turtle (pond slider—*Trachemys scripta* Wied) and evidence of crayfish (i.e., a single crayfish moult) in one of the ponds; these omnivores also consume macrophytes and, if common, could have further enhanced herbivore impact (Lodge et al., 1998). However, their effects were likely small relative to the large numbers of grass carp in each pond. The rooted, vascular plant *Micranthemum umbrosum* appeared to be the predominant plant species in one pond, while the floating green alga *Spirogyra* sp. appeared to be the predominant plant species in the other. The hatchery owner informed us that grass carp would frequently bite *M. umbrosum* but then forcibly reject it, and that *Spirogyra* sp. often persisted until all other macrophyte species had been consumed (P. Williams, personal communication). Based on these observations, the high density of herbivores in these ponds, and the acrid taste of *M. umbrosum* (J.P., personal observation), we hypothesized that these macrophytes possessed defensive traits promoting their persistence under intense herbivory.

On April 26, 2004, we determined the abundance of macrophytes in each pond by randomly locating five 0.25-m² quadrats on the littoral fringe (<1 m depth) of one side of each pond and determining the identity of macrophytes located beneath 36 points in each quadrat (we did not sample the remaining sides because they were disturbed by seine netting to capture fish). The five most common macrophytes (the green alga *Spirogyra* sp., and the vascular plants *M. umbrosum*, *Ludwigia repens*, *Juncus repens* Michx., and *J. effusus* L.)—were collected, transported to the laboratory in a chilled cooler, and fed to three consumer species within 24 hr of collection. We fed macrophytes to juvenile grass carp and to the native North American crayfishes *Procambarus spiculifer* and *P. acutus*. Both crayfishes have ranges across the southeastern United States (Hobbs, 1981). We used crayfish as a bioassay organism because they can have strong impacts on aquatic macrophyte communities (Lodge and Lorman, 1987; Creed, 1994; Lodge et al., 1994; Dorn and Wojdak, 2004), they are diverse and abundant foragers in aquatic habitats throughout North America (Lodge et al., 2000), they feed well in the laboratory (Bolser et al., 1998; Parker and Hay, 2005), and we observed evidence of crayfish in one of the ponds.

P. spiculifer were collected from the Chattahoochee River, Atlanta, GA (33°54'N, 84°27'W); *P. acutus* were collected from an adjacent wetland. We housed each crayfish in a separate 12 × 12 × 10 cm cubicle with perforated walls that received recirculating, filtered water. All animals were fed a maintenance diet of Bio-Blend Herbivore food (Marineland Labs) 3–4 times wk. Grass carp would not

feed when kept individually, so we housed them in small groups of 3–6 animals in 3.5-l buckets with recirculating water.

Feeding Assays

We determined the relative palatability of all five macrophyte species by offering 12–15 individuals of each consumer species a bite-sized portion of each macrophyte and recording whether each portion was eaten or rejected. If rejected, we fed consumers a piece of palatable aquatic macrophyte (*Egeria densa* Planch.) to ensure that they were not satiated. If the palatable macrophyte was rejected, that replicate animal was not included in the assay because it appeared satiated. Because grass carp were kept in small groups, we report results from the first fish that fed in each bucket as a replicate ($N = 12$ –15 separate buckets). Order of macrophyte presentation was randomized separately for each replicate consumer. We then analyzed (using a Fisher's exact test) the proportion of animals that were willing to feed on each individual macrophyte species relative to the palatable control.

Low palatability of fresh macrophyte tissues could result from structural, morphological, nutritional, chemical, or other characteristics. To determine whether macrophyte morphology could account for feeding preferences, we destroyed morphological traits by incorporating freeze-dried and finely ground macrophyte tissues into gel-based foods constructed with 30% sodium alginate by dry mass (Hay et al., 1998). We added enough macrophyte powder to the paste to approximate the same dry mass per volume of macrophyte found in tissues from each species of macrophyte being assayed (see [Macrophyte Traits](#)). The gel was then coated onto the interior wall of a glass Petri dish and immersed in a hardening solution of 0.25 M calcium chloride. After approximately 1 min, the gel was removed, rinsed in water, and cut into bite-sized portions. This method resulted in reconstituted macrophytes with similar morphologies and a soft, fleshy texture not unlike cooked pasta. Nutritional values and chemical defenses should have remained similar to those of intact macrophytes (however, freeze-drying can alter the structure and the activity of some metabolites; Cronin et al., 1995). These artificially softened macrophytes were then assayed against a palatable control food—a 1:1 mixture of freeze-dried and powdered broccoli and lettuce (“broc-let”) that herbivores readily accept as food (Bolser et al., 1998). Broc-let content matched the dry mass per volume of each macrophyte being assayed. Feeding on pellets was recorded as the frequency of acceptance or rejection of treatment or control pellets, with these pellets being offered alternately. We analyzed (via Fisher's exact test) the proportion of animals feeding on each individual reconstituted macrophyte species relative to the palatable control.

If gel-based treatments were unpalatable, this suggested a chemical basis for feeding rejection. We tested for chemical defenses by conducting feeding assays with crude extracts from each macrophyte incorporated into broc-let based sodium-alginate pellets as above (see above and Hay et al., 1998 for a general review). Extracts were acquired by macerating fresh macrophyte tissues in a 1:1 mixture of water and methanol overnight, then successively extracting the macrophyte material for at least 2 hr in 1:1 and 1:2 methanol/dichloromethane. The extracts were combined, and solvents were removed under vacuum to yield a crude extract. For food preparation, each crude extract was dissolved in acetone, incorporated into broc-let powder and sodium alginate, and the solvent was evaporated by vigorous stirring in a fume hood. Control foods were treated identically (including addition of

acetone) but without the addition of crude extracts. The dry mass content of treatment and control pellets matched the dry mass per volume content of each macrophyte being assayed. Pellets were fed to animals and the results were statistically analyzed as described above.

Macrophyte Traits

We measured macrophyte traits that are thought to be indicative of macrophyte nutritional quality or availability as a food, including: toughness, dry mass/volume, ash-free dry mass/volume, soluble protein/volume, and soluble protein/dry mass. Dry mass/volume was determined by drying 3–8 replicate samples of known volume at 60°C for at least 2 d; ash-free dry mass/volume was determined by combusting these same samples at 450°C for at least 6 hr. Toughness was estimated by using a penetrometer (see Duffy and Hay, 1991) to determine the mass required to pierce a leaf with a needle. Two of the five macrophytes could not be adequately tested with this approach. The rush *J. effusus* was too tough to pierce with our penetrometer; the strands of the green alga *Spirogyra* were too thin to accept the needle.

Soluble protein content was estimated with the Bradford (1976) method. Triplicate composite samples of ground macrophyte material from each species (~5 mg) were digested in 1 ml sodium hydroxide (1 mol/l) for 24 hr at 2.5°C, centrifuged, and 100- μ l aliquots of the supernatant were added to 5-ml samples of Bradford reagent. After 10–15 min, absorbance of each sample at 595 nm was measured using a Spectronic 21D spectrophotometer against bovine serum albumin (BSA) standards.

Results were analyzed with ANOVA followed by Tukey multiple comparison tests, with transformations ($\log + 1$) to correct heteroscedastic variances when necessary. Protein analyses were conducted on pooled samples of tissues from many individual plants. These data were not statistically analyzed because variances associated with the means were methodological rather than associated with differences among individual, replicate plants.

Bioassay-Guided Fractionation

M. umbrosum was the only macrophyte species with consistent evidence for a strong chemical defense. To separate and identify the defensive compounds, we used bioassay-guided fractionation of the total crude extract by assessing the feeding response of the crayfish *P. spiculifer*. We used crayfish rather than grass carp for these assays because grass carp had not yet acclimated to feeding in the laboratory when we began this fractionation. We did, however, use all three consumer species to test the deterrence of each isolated metabolite and also of the remaining crude extract minus these compounds. Extracts were initially tested at twice their extracted concentrations (by volume) to offset loss due to inefficient extractions and/or compound decomposition. Chromatographic fractions and pure compounds were tested by offering crayfish broc-let based pellets incorporated with fractions or compounds vs. control foods; results were statistically analyzed with Fisher's exact tests.

Fresh tissues of *M. umbrosum* were extracted successively with dichloromethane, acetone, and methanol, and these extracts were combined to produce a crude extract. The deterrent crude extract was fractionated by using silica gel flash chromatography (40–63 μ m Aldrich silica gel eluting with increasing concentration

of ethyl acetate in petroleum ether). The resulting 36 fractions were grouped by similar thin layer chromatography (TLC) properties into seven fractions, of which two were deterrent. The deterrent component within the less polar deterrent fraction was purified by repeated flash chromatography, as described above, followed by silica gel high-performance liquid chromatography (HPLC) with hexane/ethyl acetate as the mobile phase. The more polar deterrent fraction yielded two deterrent pure compounds via: (1) repeated silica gel flash chromatography eluting with toluene/ethyl acetate or toluene/petroleum ether/ethyl acetate; (2) recrystallization from hexanes/methanol/toluene (3:2:1); and (3) silica gel HPLC eluted with hexane/ethyl acetate, using a Zorbax RX-SIL HPLC column (9.4 × 250 mm; 5 μm) attached to a Waters Breeze HPLC system consisting of Waters 515 pump and Waters 2487 UV detector recording at 210 and 254 nm. A third fraction from the initial flash column separation did not initially deter crayfish feeding, but unusual ^1H NMR signals motivated the purification of a fourth compound by flash column chromatography and HPLC as described above.

Pure compounds from each fraction were identified on the basis of ^1H , ^{13}C , and 2D NMR spectroscopy, and comparisons of NMR, IR, and mass spectral data with literature data. Optical rotations were obtained using a Jasco P-1010 polarimeter. IR data were acquired on a Nicolet 520 FTIR spectrophotometer with thin films on NaCl plates. ^1H , ^{13}C , and 2D NMR spectral data were obtained on a Bruker Avance DRX 500 MHz spectrometer using CDCl_3 referenced to residual CHCl_3 (δ 7.28).

Quantification of Isolated Compounds

To determine whether our isolated yields were comparable to the natural concentrations in plant tissues, we quantified the concentrations of each of the four compounds from five separate individuals of *M. umbrosum* collected at the same time and under the same conditions as the bulk material used in this study. Frozen plants were individually extracted with a 1:1 mixture of water and methanol, then successively for at least 2 hr in 1:1 and 1:2 methanol/dichloromethane.

Quantification of natural products was achieved by LC-MS/MS using a Micromass Quattro triple quadrupole mass spectrometer in conjunction with an Agilent 1100 HPLC. A reversed-phase Zorbax eclipse XDB-C8 column (1.0 × 150 mm, 3.5 μm) was used to separate the natural products with a gradient system of water/acetonitrile (0.1% formic acid) 95:5 (v:v) to 5:95 (v:v) over 31 min. Three or four standard solutions (0.0001–0.10 mg/ml) of each of the four pure compounds were used to measure sample concentration by integration of the peaks areas for monitored transitions arising from dissociation of $[\text{M} + \text{H}]^+$ precursor ions to a structure-specific fragment ion for each compound. These data were used to establish standard curves ($R^2 > 0.99$ for each compound) for the quantification of compounds in crude extracts of the five macrophyte samples. Once natural concentrations were known, we tested the effects of each compound at its natural concentration and at its isolated yield with each of our three consumers species.

Experimental Exclusion of Herbivores and Tests of Herbivore Preference among Plants

To determine whether the chemically defended *M. umbrosum* was disadvantaged relative to less-defended plants in the absence of herbivory, we excluded grass carp

and other potential consumers from caged areas of the pond for 16 mo and measured the abundance of plants in caged vs. control areas. On April 26, 2004, we established five blocks in the pond with three treatments in each block: (1) an uncaged treatment allowing full herbivore access, (2) a three-sided cage control allowing herbivore access but controlling for cage artifacts, and (3) 2 four-sided cages excluding herbivores. Each block had 2 four-sided cages because we had originally intended to establish another treatment in one of the cages. We never imposed this treatment, thus, both cages were considered replicates in the same block to calculate the cage effect. Each treatment area was $0.9 \times 0.9 \times 0.9$ m, with the cage control and cage areas marked by 1.0-m-tall steel rebar posts. Cages were constructed of 3.0-mm plastic mesh affixed to the rebar posts with cable ties. A 10- to 15-cm skirt was anchored around each cage to prevent consumers from burrowing under the mesh walls. On only one occasion did we encounter grass carp in the cages; both fishes were removed and were likely too small (<2 cm in length) to have begun feeding on macrophytes given that grass carp typically do not become herbivorous on macrophytes until they reach approximately 3 cm in length (Hickling, 1966). Treatment blocks were established in linear arrays separated from each other by at least 4.0 m along the shoreline that was not used to seine fish. Treatments were randomly assigned to each position in the block, with the restriction that the open treatment was on either end of the block. Watermarks on the cage walls suggested that the average treatment depth was approximately 15 cm, but we observed that cages were occasionally dry or up to 30 cm deep, consistent with the variability of water depth that we observed in natural ponds in the area. Poor water clarity, however, prevented monitoring of plant cover when water depth exceeded approximately 10 cm.

We estimated initial plant cover in the treatments by determining the identity of macrophytes located beneath 36 points in a 0.25-m^2 quadrat placed directly in the center of each cage or open treatment. We analyzed the initial total plant cover and the initial cover of the two species (*M. umbrosum* and *L. repens*) that were most abundant at the end of the experiment with a blocked one-way ANOVA, transforming ($\log + 1$) to correct heteroscedastic variances (determined via Cochran's tests) when necessary.

On August 29, 2005, we harvested all of the aboveground plant material from each cage and weighed it to the nearest gram. We analyzed the total aboveground biomass and the biomass of the two most common plant species in our treatments with a blocked one-way ANOVA, transforming ($\log + 1$) to correct heteroscedastic variances (determined via Cochran's tests) when necessary. Significant ANOVA results were followed by multiple comparisons (Tukey tests) among treatment means.

Although our earlier laboratory feeding assays allowed us to determine which plant species were unpalatable, they were unsuitable for determining preference hierarchies among plant species. Thus, we conducted choice-feeding assays comparing grass carp preference for *M. umbrosum*, the plant that dominated cover in one of the grass carp ponds, with *L. repens*, a formerly rare plant that heavily recruited into our exclusion cage treatments (see Results). We also compared grass carp preference for *M. umbrosum* vs. *Najas guadalupensis*, and *L. repens* vs. *N. guadalupensis*; we chose *N. guadalupensis* because it dominated (>80% cover, $N = 20$ quadrats) the cover of a nearby (~300 m distant) pond of natural origin that did not have grass carp. We hypothesized that the dominant plants from the grass

carp pond (*M. umbrosum* and *L. repens*) would be of lower preference than the dominant plant (*N. guadalupensis*) from a habitat that lacked grass carp, and that *M. umbrosum* would be of lower preference than a plant that recruited only to cages where we excluded grass carp.

Each replicate assay consisted of placing a binder clip with a 2.0-cm portion of each of two plant species into 18 buckets containing 2–5 grass carp. Each replicate was periodically checked to determine which plant had been eaten first, with all treatments harvested the following morning. We did not retain the replicates where both plants had been eaten, as we could not determine which plant had been eaten first. We analyzed the number of occasions in which each species was eaten first with Fisher's exact tests.

Results

Macrophyte Abundance

In late April 2004, the littoral fringe of both grass carp ponds was largely unvegetated (mean ± 1 SE of bare space; Pond 1 = $76.7 \pm 10.6\%$, Pond 2 = $80.6 \pm 10.3\%$, both $N = 5$), but macrophyte cover in each of the ponds was dominated by a single species (Pond 1: *M. umbrosum* = $86.5 \pm 6.8\%$ of total plant cover; Pond 2: *Spirogyra* = $94.4 \pm 5.6\%$ of total plant cover). Of the remaining four macrophyte species, *J. effusus* represented $9.8 \pm 5.3\%$ of total plant cover in Pond 1 and $2.8 \pm 2.8\%$ in Pond 2, *J. repens* comprised $2.8 \pm 2.8\%$ in Pond 2, and there were trace amounts of *L. repens* in Pond 1. A single individual of the sedge *Carex* sp. occurred in Pond 1; because this was only one individual, we did not include this species in our feeding assays. No other aquatic macrophytes were observed in the ponds.

Feeding Assays

When offered as fresh macrophyte tissues, each of the five macrophyte species assayed was unpalatable relative to a control food to at least two of the three consumer species tested (Fig. 1A). Of the two most common macrophytes, *M. umbrosum* was significantly less palatable than control food (*E. densa*) to all three consumers, whereas *Spirogyra* was significantly less palatable to *Procambarus acutus* and *C. idella*, but not to *P. spiculifer*. Although relatively uncommon in the ponds, *L. repens* also was significantly less palatable to all three consumers than was the control food. After we destroyed plant morphological traits, palatability increased for some macrophytes, but feeding on *M. umbrosum*, *Spirogyra*, and the rush *J. repens* remained similar to that on intact plants (Fig. 1B). When the crude extracts from macrophytes were incorporated into a palatable control food, only *M. umbrosum* remained unpalatable—suggesting a strong chemical deterrent to feeding by all three consumers (Fig. 1C).

Macrophyte Traits

Table 1 shows toughness, dry mass, ash-free dry mass, and protein content of the macrophytes examined. *M. umbrosum* was the softest macrophyte that we tested with the penetrometer, was of intermediate rank in dry mass and in soluble protein

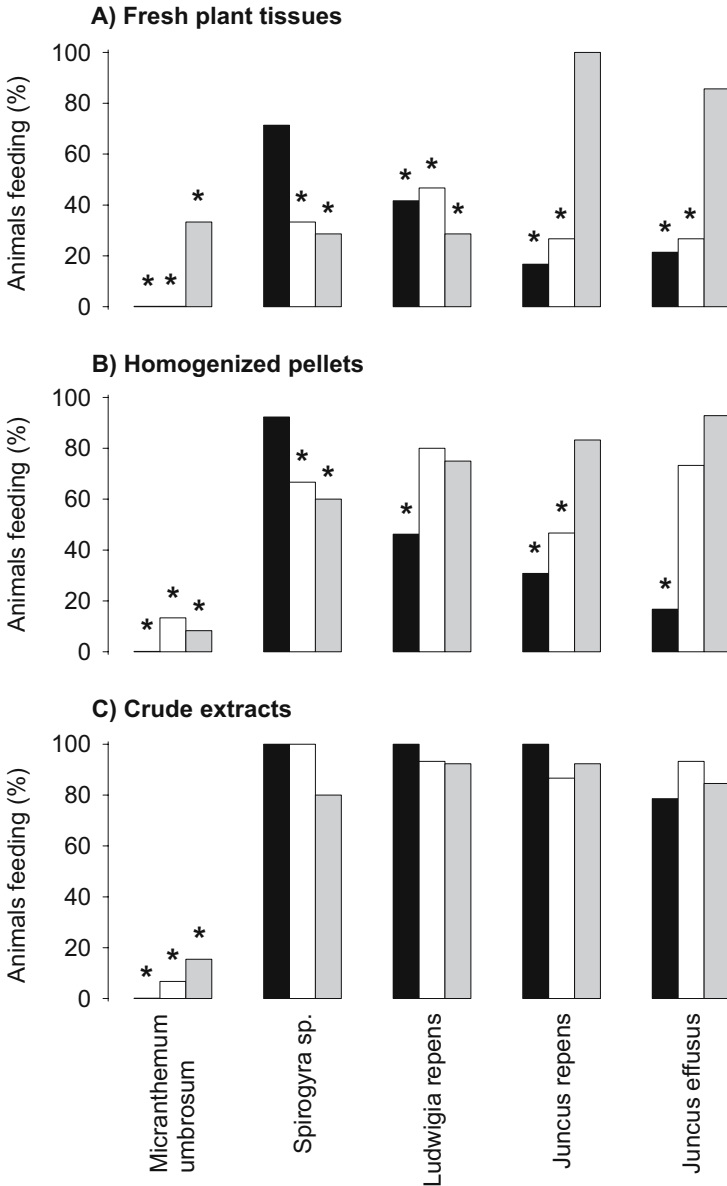
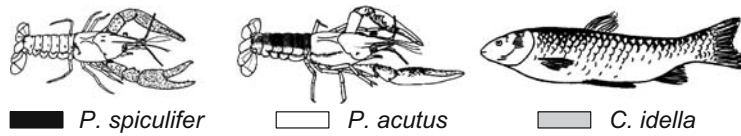


Fig. 1 Percentage of 12–15 individual *Procamburus spiculifer* (filled bars), *P. acutus* (open bars), and *Ctenopharyngodon idella* (gray bars) feeding on (A) fresh macrophyte tissues, (B) homogenized macrophyte pellets at natural dry mass content, and (C) crude extracts from five aquatic macrophyte species. Asterisks denote statistically significant ($P < 0.05$) reductions in feeding relative to a palatable control (*Egeria densa*) for each consumer species (Fisher’s exact tests)

Table 1 Mean (\pm SE) and sample sizes (in parentheses) for each analysis of selected macrophyte traits*

Macrophyte	Mass to pierce (mg)	Dry mass/vol. (mg/ml)	Ash-free dry mass/vol. (mg/ml)	Soluble protein (mg/ml)	Soluble protein (% dry mass)
<i>Micranthemum umbrosum</i>	5.40 \pm 0.768 (5)a	88.0 \pm 7.57 (8)b	25.6 \pm 5.30 (8)b	4.83	5.49
<i>Spirogyra</i> sp.	Too thin to test	45.2 \pm 4.90 (4)a	7.26 \pm 1.95 (4)a	3.06	6.77
<i>Ludwigia repens</i>	9.19 \pm 1.31 (5)a	73.3 \pm 10.1 (5)ab	13.7 \pm 2.44 (5)ab	4.77	6.51
<i>Juncus repens</i>	13.3 \pm 1.04 (5)b	142 \pm 14.3 (5)c	24.4 \pm 4.46 (5)b	6.96	4.90
<i>Juncus effusus</i>	Too hard to test	80.3 \pm 9.97 (5)b	10.5 \pm 2.54 (5)ab	4.81	5.99
Broc-let control	N/A	55.2 \pm 1.41 (3)	6.27 \pm 0.475 (3)	4.04	7.32
ANOVA <i>P</i> values	0.001	0.001	0.004	N/A (Composite samples)	N/A (Composite samples)

*Species that share a letter within a column are not significantly different from one another in unplanned comparisons following ANOVA; broc-let not included in analyses.

per volume of plant, but had the highest ash-free dry mass per volume of macrophyte. *Spirogyra* could not be tested with the penetrometer because of its filamentous morphology, but it has no obvious structural barriers to grazing. *Spirogyra* was generally nutritionally poor relative to the other plants; it ranked lowest in dry mass, ash-free dry mass, and protein content per volume of plant (Table 1). *L. repens* was relatively soft and of intermediate to low rankings in mass and protein content. The prostrate rush *J. repens* was the toughest macrophyte that we could test, and it had the highest dry mass, second highest ash-free dry mass, and protein content when measured volumetrically, but the lowest protein content when expressed as a % of dry mass. The emergent rush *J. effusus* was too tough to test with the penetrometer, and had intermediate dry mass per volume, relatively low ash-free dry mass per volume, and moderately low protein content. Our palatable control food, a 1:1 mixture of powdered broccoli and lettuce (broc-let), had relatively low dry mass, ash-free dry mass, and protein content per volume of plant, but it had the highest protein content of all the foods when expressed as a % of dry mass (Table 1).

Bioassay-Guided Fractionation

The crude extract of *M. umbrosum* strongly deterred feeding by *P. spiculifer* (Fig. 2). Two of the initial seven fractions from this extract strongly reduced crayfish feeding (fractions B and E, Fig. 2). Purification of the active component in fraction B via three silica gel chromatographic columns revealed 3,4,5-trimethoxyallylbenzene (elemicin) (**1**) as the bioactive metabolite (Fig. 2). Similar bioassay-guided separation of fraction E, followed by HPLC purification, led to identification of the deterrent compounds β -apocipopodophyllin (**2**) and (-)-(3*S*,4*R*,6*S*)-3-(3',4'-meth-

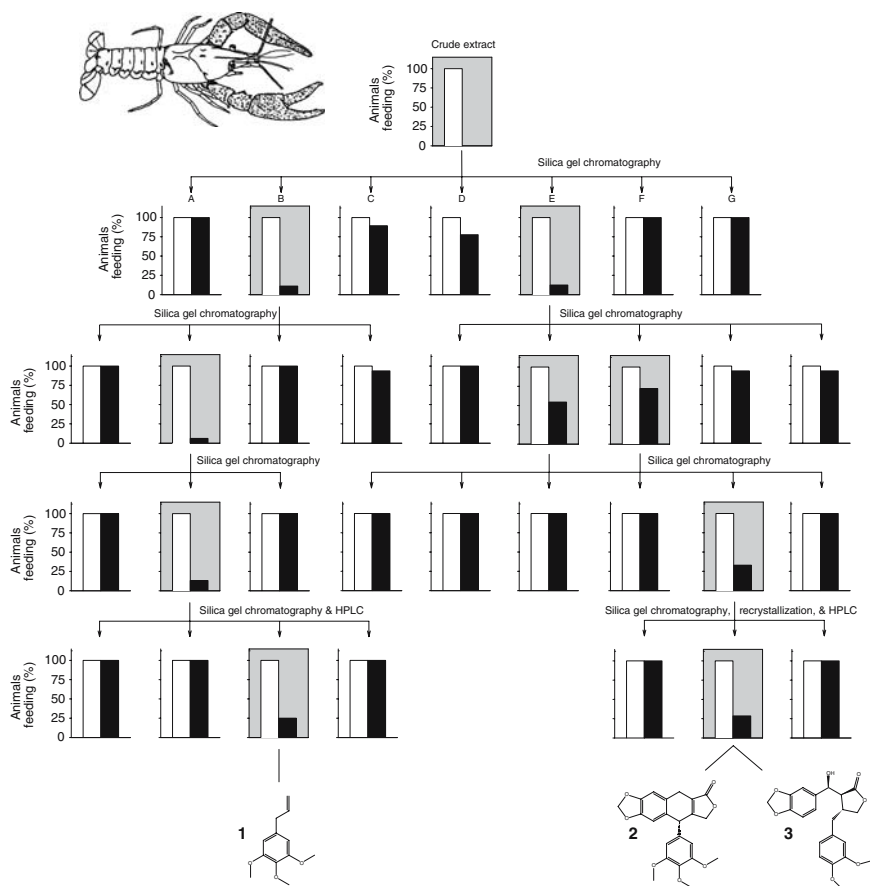


Fig. 2 Bioassay-guided fractionation of crude extracts from *Micranthemum umbrosum*. Each graph shows the percentage of 12–15 individual *P. spiculifer* feeding on a solvent-only control food (open bar) vs. control food containing macrophyte extracts. Shaded graph panels denote statistically significant ($P < 0.05$) feeding reductions relative to the palatable control (Fisher's exact tests). See [Methods and Materials](#) for mobile phases and chromatographic details

ylenedioxy- α -hydroxybenzyl)-4-(3'',4''-dimethoxybenzyl)butyrolactone (**3**) (Fig. 2). Although fraction C did not initially deter crayfish feeding (Fig. 2), unusual ^1H NMR signals motivated the purification of (–)-hibalactone (**4**), also known as (–)-savinin, from this fraction (Fig. 3).

Quantification of Compounds 1–4

We initially tested the deterrent fractions at twice their yield (by volume) to make up for assumed losses during purification, but compound quantification by LC-MS/MS showed that even doubling the presumed natural concentration did not approach the actual concentration occurring in the crude extract for each of the four compounds assayed (Table 2). The isolated yields of compounds **1** and **2** were 19% and 16% of their natural concentrations, respectively, while compounds **3** and **4** were isolated at only 1% and 8% of their natural concentrations, respectively.

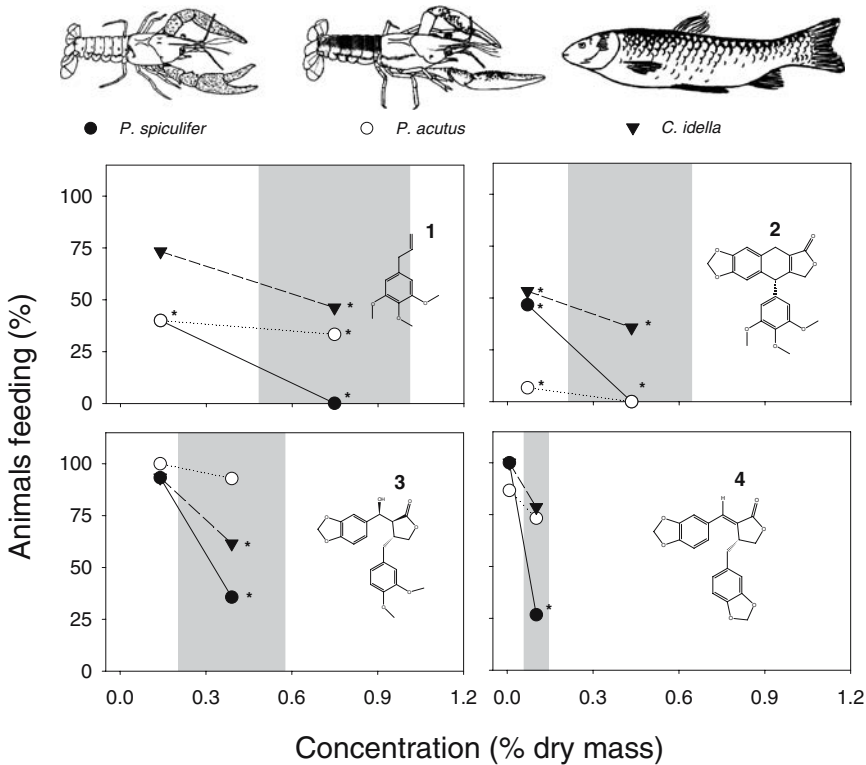


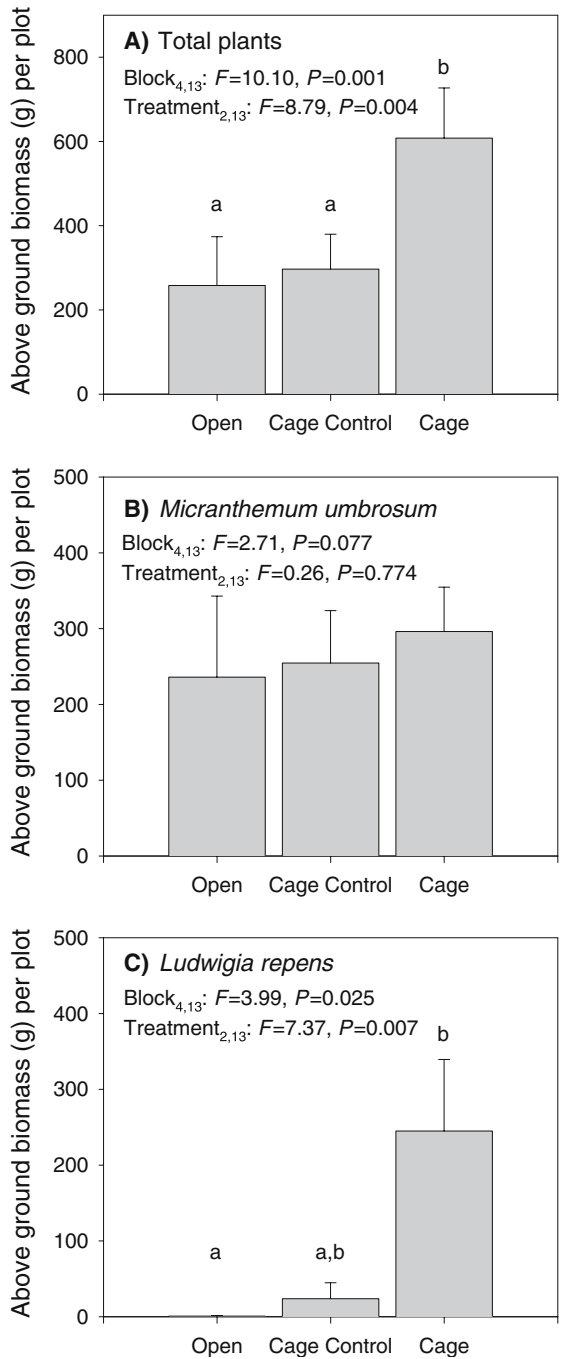
Fig. 3 Percentage of 12–15 individual *P. spiculifer* (filled circles), *P. acutus* (open circles), and *Ctenopharyngodon idella* (filled triangles) feeding on pellets containing (A) 3,4,5-trimethoxyallylbenzene (**1**); (B) β -apopropodophyllin (**2**); (C) (–)-(3*S*,4*R*,6*S*)-3-(3',4'-methylenedioxy- α -hydroxybenzyl)-4-(3',4'-dimethoxybenzyl)butyrolactone (**3**); and (D) (–)-hibalactone (**4**). Asterisks denote statistically significant reductions in feeding relative to a palatable control for each consumer species ($P < 0.05$; Fisher's exact tests). The shaded area is the quantified natural concentration (by dry mass) ± 1 standard deviation (see Table 2); feeding assays to the left of this shaded area were conducted at the isolated yield of each metabolite

When elevated to their natural concentrations, compounds **1** and **2** were both deterrent to all three consumers (Fig. 3). In contrast, when we elevated compounds **3** and **4** to their natural concentrations, compound **3** was deterrent to *P. spiculifer* and to grass carp, but not to *P. acutus*, whereas compound **4** was deterrent to *P. spiculifer* but not to the other consumers (Fig. 3). For three of the four

Table 2 Isolated yield and quantitatively determined dry mass concentrations (% of dry mass \pm SD) of four deterrent compounds isolated from the crude extract of *Micranthemum umbrosum*

Quantification method	Concentration of compound (% of macrophyte dry mass)			
	1	2	3	4
Isolated yield ($N = 1$)	0.14	0.070	0.0045	0.0078
LC-MS/MS ($N = 5$) quantification	0.75 ± 0.26	0.43 ± 0.22	0.39 ± 0.19	0.10 ± 0.04

Fig. 4 Final aboveground biomass per plot of (A) all plants, (B) *M. umbrosum*, and (C) *Ludwigia repens* in open, cage control, and cage treatments after 16 months. Statistical results are from one-way blocked ANOVAs. Bars that share the same letter were not statistically different ($P < 0.05$) from one another in Tukey's tests



compounds tested, the magnitude of feeding depression appeared stronger for *P. spiculifer* than for the other consumers (Fig. 3).

We also tested whether we had isolated all of the strongly deterrent compounds by assaying the crude extract minus the fractions containing the four isolated compounds (i.e., we used TLC to group fractions from the first silica gel column that lacked compounds 1–4). None of our three test consumers were significantly deterred by this “crude minus deterrent fractions” extract ($N = 13$ – 15 for each consumer species, % acceptance $\geq 86.7\%$, $P \geq 0.50$). However, given the significant compound degradation and/or inefficient yields that we observed (Table 2), it is possible that unknown, but potentially deterrent, compounds within this crude extract were tested at concentrations significantly lower than their natural levels.

Experimental Exclusion of Herbivores

At the initiation of the experiment, there was no difference in total plant cover ($P = 0.833$), the cover of *M. umbrosum* ($P = 0.089$), or the cover of *L. repens* ($P = 0.641$) among the open, cage control, and cage treatments (data not shown). After 16 mo of excluding grass carp and other potential herbivores (e.g., crayfish, turtles), there was 2.4-fold more total plant biomass ($P = 0.004$, Fig. 4A) and over 300-fold more *L. repens* ($P = 0.007$, Fig. 4C) in the cage vs. open treatments. Biomass of the unpalatable macrophyte *M. umbrosum* was unaffected ($P = 0.774$, Fig. 4B). Thus, herbivore exclusion allowed other species to increase in abundance but did not alter the abundance of the chemically defended *M. umbrosum*.

When offered a choice between two plant species, grass carp preferred *L. repens* over *M. umbrosum* by 14 to zero ($P < 0.001$), *N. guadalupensis* over *M. umbrosum* by 11 to zero ($P < 0.001$), and *N. guadalupensis* over *L. repens* by 14 to zero ($P < 0.001$). The striking differences in preference for all contrasts clearly establishes a preference hierarchy of *N. guadalupensis* > *L. repens* > *M. umbrosum*.

Discussion

It is a common pattern in marine and terrestrial habitats for selective feeding by herbivores to shift plant species composition toward chemically or structurally defended plants (Hay and Fenical, 1988; Rosenthal and Berenbaum, 1992; Hay, 1997). In contrast, although aquatic herbivores commonly reduce plant standing stock and alter species composition (Lodge, 1991; Newman, 1991; Cyr and Pace, 1993; Lodge et al., 1998), experimental investigations linking herbivore feeding preferences to particular plant traits and ultimately to shifts in plant community structure are rare. Here, we show that five of the most common macrophytes collected from an aquaculture facility for herbivorous Asian grass carp, *C. idella*, were commonly unpalatable to three generalist consumers—nonnative grass carp and the native North American crayfishes *P. spiculifer* and *P. acutus*. The most common macrophytes—*M. umbrosum* and *Spirogyra* sp.—comprised 87% and 94%, respectively, of the total macrophyte cover in two grass carp ponds, and both were unpalatable to grass carp (Fig. 1). *Spirogyra* appeared nutritionally inadequate to these consumers, and *M. umbrosum* was chemically defended by at least four secondary metabolites (Figs. 2 and 3). When we excluded grass carp and other potential herbivores from experimental portions of one of the ponds, a plant that

was preferred over *M. umbrosum*—*L. repens*—increased over 300-fold in the herbivore exclusion treatment. A nearby natural pond that lacked grass carp was dominated by *N. guadalupensis*, a plant that grass carp preferentially consumed over both *M. umbrosum* and *L. repens*. Thus, selective feeding by grass carp effectively eliminates most palatable plants from this community and promotes the persistence of less palatable, chemically defended or nutritionally inadequate plants.

Grass carp and most crayfish species are generalist consumers that will eat a variety of plants (Parker and Hay, 2005) but still selectively feed among species based on their structural, nutritional, and chemical traits (Cronin et al., 2002). However, knowledge of traits alone may not be predictive of feeding preferences among different consumer species (e.g., Hay et al., 1987; Hay and Fenical, 1996). For example, both the grass carp and crayfish *Procambarus acutus* rejected the filamentous green alga *Spirogyra* (Fig. 1). The crayfish *P. spiculifer*, however, readily consumed *Spirogyra* (Fig. 1), and in another study the crayfish *Pacifastacus leniusculus* preferred it over other aquatic plants (Warner and Green, 1995). Among the five plant species that we tested, *Spirogyra* had the lowest protein content per volume of plant (Table 1), suggesting that nutritional inadequacy may explain its low palatability to *P. acutus* and to grass carp, but the variation among consumers (Fig. 1; Warner and Green, 1995) suggests that palatability depends on the palate of the consumer, or on considerable intraspecific variance in the defensive traits of the plants studied (e.g., Taylor et al., 2003). Moreover, *Spirogyra* and other filamentous algae reportedly persist in these and other ponds only until submersed macrophytes have been selectively removed by grass carp (P. Williams, personal communication; Van Dyke et al., 1984). This suggests that plants can delay or reduce herbivory by being nutritionally poor, but they may be unlikely to escape consumption once higher preference plants have been removed.

It is uncertain why *L. repens* was rejected in the fresh tissue assays (Fig. 1). It was readily eaten over *M. umbrosum* in choice assays with fresh tissue, was readily eaten by all but one consumer as homogenized pellets (Fig. 1B), and was eaten by all consumers when extracts were incorporated into a palatable control food (Fig. 1C). It could be that our methodology of offering bite-sized pieces rather than whole plants altered the acceptability of this or other plant species. The prostrate rush *J. repens* and the emergent rush *J. effusus* were both tough plants that may have been structurally defended from consumption by crayfishes (Table 1, Fig. 1). However, at least one crayfish species rejected the softened, homogenized pellets of each species (Fig. 1B), although no consumers rejected the chemical extracts (Fig. 1C). Thus, it appears that both rushes could be structurally defended, but we cannot exclude the possibility that deterrent compounds in the softened foods were lost during the extraction process. Alternatively, the higher protein content of broc-let powder (Table 1) may have provided extra feeding incentives that counterbalanced deterrent chemistry. Other investigations have shown that consumers are more likely to feed on chemically defended foods if they are nutritionally rich (Duffy and Paul, 1992; Cruz-Rivera and Hay, 2003).

Despite these ambiguities for some consumer and macrophyte species, *M. umbrosum* was clearly chemically distasteful to all three consumers (Fig. 1), and we isolated four natural products that serve as chemical defenses against herbivory (Figs. 2 and 3). Each of the four compounds has previously been described, but this is the first study to report on their ecological function as defenses against herbivory. Compound **1** is an essential oil commonly found in aromatic plants including nutmeg

and parsley (De Vincenzi et al., 2004). It has been implicated as an antimicrobial compound (Marston et al., 1995), a growth inhibitor of green algae (Della-Greca et al., 1992), and as an insecticide (Miyazawa et al., 1992). β -Apopicropodophyllin (**2**) has previously been isolated from the Mexican medicinal plant *Hyptis verticillata* “bushmint,” and is from a class of lignoids active against several cancer cell lines (e.g., Novelo et al., 1993). Compound **3** has been synthesized but was not previously known as a natural product (Pelter et al., 1988). Lignan **4** occurs in juniper and several woody plant species (e.g., Hartwell et al., 1953); it inhibits prostaglandin E₂ production (Ban et al., 2002), tumor necrosis factor- α production and T-cell proliferation (Cho et al., 2001), and is a synergist for insecticides (Matsubara, 1972). We lost from 81% to 99% of the natural concentrations of these four molecules during isolation procedures (Table 2). Given this poor yield, it is possible that additional deterrents were present but recovered at concentrations too low to be biologically active.

Prior to this study, there were only three freshwater plants with described herbivore feeding deterrents—watercress, *R. nasturtium-aquaticum* (L.) Hayek (Newman et al., 1990, 1996); the waterspider bog orchid, *H. repens* Nutt. (Bolser et al., 1998; Wilson et al., 1999); and lizard’s tail, *S. cernuus* L. (Kubanek et al., 2000, 2001)—with a total of nine described secondary metabolites demonstrated to influence herbivore feeding. Our study brings the new total of described herbivore antifeedants in freshwater plants to 13. Of these 13, 10 are lignoids, including three in this study and seven compounds isolated from *S. cernuus* (Kubanek et al., 2001). This general, though still preliminary, pattern suggests that lignoids—of which several thousand have been described from numerous plant taxa (Seigler, 1998; Ward, 1999)—are common, but often overlooked, defensive compounds warranting additional study.

Plant defense theory predicts that chemically defended plants have fewer resources for growth and will be competitively displaced by less defended plants when herbivore pressure is lessened (Herms and Mattson, 1992). To test this, we excluded grass carp for 16 mo and documented a 300-fold increase in the abundance of *L. repens* (Fig. 4), a plant that was preferred over *M. umbrosum* in a choice feeding assay. However, we did not observe a decrease in the abundance of *M. umbrosum* in the cage treatments (Fig. 4). Thus, although chemical defenses in *M. umbrosum* appear to promote its persistence in the face of intense herbivory, we saw little evidence to suggest competitive displacement of *M. umbrosum* by *L. repens* in the absence of herbivores. There are several potential explanations. Our experiments ran through two growing seasons, but the long history (>20 yr) of grass carp herbivory in this habitat may have consistently excluded other species and reduced the potential pool of new colonists exhibiting high-growth, low-defense strategies. In support of this hypothesis, *M. umbrosum* typically takes at least 4 yr to recruit into new ponds in this system, after which it persists indefinitely (P. Williams, personal communication). Additionally, the only species that showed a large increase in abundance—*L. repens*—is also relatively unpalatable (Fig. 1), and may not be a much better competitor than *M. umbrosum*. Moreover, despite the long-standing view that constructing and storing defensive compounds is physiologically costly and detracts from growth and reproduction, empirical evidence is conflicting (Koricheva, 2002), suggesting that investment in chemical defense need not necessarily restrain growth and competitive ability (Cronin, 2001). Finally, grass carp will repeatedly sample foods even if they do not ingest the plants (P. Williams,

personal communication); this chronic sampling may have depressed *M. umbrosum* abundance in the open and cage control treatments and obscured competitive effects in the cage treatments. Nevertheless, on a percentage basis, excluding herbivores led to dramatic increases in *L. repens* that reduced the relative abundance of *M. umbrosum* from 89% to 54% of the total plant community, indicative of chemical defenses promoting the relative dominance of *M. umbrosum* in this community.

Herbivory in freshwater systems is more important than previously thought (Lodge and Lorman, 1987; Newman, 1991; Cyr and Pace, 1993; Lodge et al., 1994, 1998; McKnight and Hepp, 1995), and freshwater plants are frequently chemically or structurally defended from consumers (Newman et al., 1996; Bolser et al., 1998; Cronin, 1998; Kubanek et al., 2001; Cronin et al., 2002; Prusak et al., 2005). Rarely, however, have the mechanisms of deterrence (e.g., structural or chemical defenses) been linked to the broader context of community structure. Here, we show that selective herbivory by grass carp shifts the species composition of freshwater plant communities toward plants that are distasteful, structurally defended, or nutritionally inadequate (Figs. 1 and 4), suggesting that plant defenses can play critical yet understudied roles in the structure of freshwater plant communities.

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Quantitative GC-ECD Analysis of Halogenated Metabolites: Determination of Surface and Within-Thallus Elatol of *Laurencia obtusa*

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Abstract Information on natural concentrations or variability of secondary metabolites in marine organisms may be important both to ecological/evolutionary and applied approaches. A gas chromatographic procedure with an electron capture detector (GC-ECD) was developed to quantify the sesquiterpenoid elatol at the surface and within-thalli of 70 specimens of the red seaweed *Laurencia obtusa*. The concentration of elatol was highest within-thalli [9.89 mg g^{-1} of *L. obtusa*, dry weight (d.w.)], compared to lower values found at the surface [0.006 mg g^{-1} of *L. obtusa* (d.w.), or $0.5\text{--}10.0 \text{ ng cm}^{-2}$]. This method provides a rapid and inexpensive quantification of small quantities of elatol, and probably may also be used to quantify other halogenated compounds usually found in red seaweeds.

Keywords Red seaweed · Secondary metabolites · Sesquiterpene · Elatol · Quantification · Halogenated secondary metabolites · GC-ECD method

Introduction

Marine secondary metabolites are undoubtedly important in mediating interactions at the surface of many host marine organisms. Knowledge of both the amount and variation of secondary metabolites of marine organisms is an essential element for assessing studies in chemical ecology, and more significant if put into an ecological

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and evolutionary background (Schmitt et al., 1995; De Nys et al., 1995, 1998; Hay et al., 1998). However, one of the major problems faced by researchers in marine chemical ecology is determining natural secondary metabolite concentrations and, consequently, the reproduction of realistic concentrations that can be applied in bioassays. In general, measurements have been made for entire plants, with few results being related to variability in parts thereof. For instance, variations between surface and within-thallus concentrations of seaweed metabolites are rarely studied (e.g., De Nys et al., 1998).

Determination of near or at the surface antifouling metabolites of host organisms is essential because an antifouling function cannot be inferred without demonstrating the secondary metabolites concentrations *in situ* (De Nys et al., 1998). Only investigations that quantify the amounts of compounds released from organisms, or use known concentration of metabolites that are ecologically realistic should be used in assays (Hay et al., 1998).

Until the 1970s, the scarcity of reports was largely determined by technological limitations (Schmitt et al., 1995), i.e., difficulty in identifying, quantifying, and testing chemical cues for colonization *in situ* (Steinberg and De Nys, 2002). Current analyses have been carried out with modern technologies such as high-performance liquid chromatography (HPLC) (e.g., Gerwick et al., 1985; Paul and Fenical, 1986; Hay et al., 1987; Paul and Van Alstyne, 1992; Schmitt et al., 1995; Cronin and Hay, 1996) or gas chromatography coupled to mass spectrometry (GC-MS) (e.g., Mynderse and Faulkner, 1978; De Nys et al., 1998). However, few reports explain the method applied (see Phillips and Towers, 1982; De Nys et al., 1996a, 1998; Taylor et al., 2003, for exceptions). Therefore, when marine chemical ecology studies are evaluated, it often remains unclear if concentrations, methods, and organisms applied in marine bioassays constitute ecologically realistic conditions. This study quantified a marine halogenated secondary metabolite based on a gas chromatographic technique coupled to electron-capture detection (GC-ECD).

Methods and Materials

GC-ECD was used to quantify elatol in specimens of the seaweed *Laurencia obtusa* (Ceramiales, Rhodophyta) from Cabo Frio Island, in the state of Rio de Janeiro, Brazil. Elatol, the major secondary metabolite in Brazilian specimens of *L. obtusa* from Cabo Frio Island, is known as an effective chemical defense against herbivores and fouling organisms (Da Gama et al., 2003; Pereira et al., 2003). Specimens ($N = 70$) were collected and maintained in individual plastic bags and transported to the laboratory. Under these conditions, there is no stress that might lead to release of elatol (Sudatti et al., unpublished data). At the laboratory, fronds were weighed wet (mg), their surface area (cm^2) was estimated, and they were submitted to extraction procedures. All weights were obtained from wet seaweed, from which excess water was removed by using a “salad spinner.”

To determine the surface concentration of elatol, surface areas of pieces of *L. obtusa* were calculated by using a wet weight (mg) to surface area (cm^2) conversion factor as proposed by De Nys et al. (1998). Additionally, the levels of within-thallus elatol were estimated by dry weight for each individual specimen (mg g^{-1}). Superficial area/weight was measured via two methods. In the first

approach, 13 branches of the *L. obtusa* frond were separated. Each was weighed to a precision of 0.01 mg, and the area of a cylinder, defined by the mean diameter (taken at three heights) and the height of the frond, were calculated. The mean value of all measurements was determined. In the second method, a total of 21 subunits of the branches were weighed, and the areas of the cylinders, defined by their diameter and height, were calculated. The mean value of all measurements was determined. Difference among the values obtained by these methods was considered significant when $P < 0.05$ ($\alpha = 5\%$), evaluated by *t* test.

Extraction Procedure for Surface Metabolites

Each specimen of *L. obtusa* was held in hexane according to the protocol proposed by De Nys et al. (1998). These authors dipped the algae in different solvents and for different time periods for extraction. After each period, a piece of alga was directly observed and quantitatively assessed for the presence of lysed cells by using epifluorescence microscopy. Of the five solvents tested, all except hexane caused lysis of surface cells for *L. obtusa* at all tested times. There was a significant increase in the concentration of total metabolites between extraction times of 50 and 60 sec, corresponding to an increase in the mean number of cells lysed. More stringent extraction procedures with other solvents, or with longer extraction times in hexane (>50 sec) caused significant quantifiable cell damage. In addition, dipping thalli for 30 sec also caused no cell lysis in eight other seaweeds, including brown and green species. Thus: 1) this method may be used for quantitative extraction of nonpolar natural products from the surface of seaweeds without disrupting cells; 2) the time period for extraction is about 40 sec. Based on this study, our procedure for extraction of surface metabolites consisted in dipping the frond into a solvent (hexane, 85%) for 40 sec. Each of the 70 fronds was individually immersed in 100 ml solvent, then air-dried at room temperature, and transferred, with hexane, to 2-ml vials through consecutive washes. These fronds of *L. obtusa* were individually reserved for further extraction of within-thallus metabolites.

Extraction Procedure for Within-Thallus Metabolites

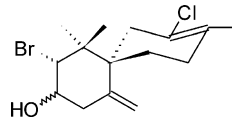
Extractions of total secondary metabolites were obtained by exhaustive extraction of each specimen in hexane, following standard procedures for marine natural products. The crude extract was transferred, with hexane, to 2-ml vials through consecutive washes.

Both extraction procedures sought to verify surface and within-thallus metabolites. Although extractions promote total secondary metabolites removal, only the major compound elatol was quantified. Both extracts obtained (from the surface and within-thalli) were weighed and dissolved in hexane for quantification.

Extraction of Blank

In order to evaluate any solvent interference in the above extraction processes, 100 ml of hexane were processed and dried at room temperature, then transferred, with hexane, to 2-ml vials through consecutive washes.

Fig. 1 Elatol, the major secondary metabolite from Brazilian specimens of *Laurencia obtusa*



Elatol Standard

Additional fronds of *L. obtusa* were submitted to exhaustive extraction in hexane or dichloromethane. The resulting extract containing elatol was identified by using thin layer chromatography (TLC) (Merck Al TLC 20 × 20 cm silica gel 60F₂₅₄) and ¹H NMR and by matching the results found in the literature (Konig and Wright, 1997). Elatol isolation was carried out on precoated TLC plates, and quantification was performed by using a GC-ECD by the external standardization method.

GC-ECD Analysis

Chromatographic analyses were performed with a Varian CP-3800 and processed by Star Chromatography Workstation 6.0 version software. The GC was fitted with a WCOT CP sil 8 CB capillary column (30.0 m × 0.25 mm, 5% phenyl, 95% dimethylpolysiloxane; Chrompack). The oven temperature program was as follows: 80°C (1 min), 10°C min⁻¹ to 250°C (16 min) then 15°C min⁻¹ to 80°C. Nitrogen (N₂) 99.999% (Aga or White Martins) was used as carrier (28 cm sec⁻¹), makeup (35 m sec⁻¹), and purge (15 ml sec⁻¹) gas. Detector temperature was 320°C and injection pressure was 120 kPa. Splitless cold injections were carried out (0.5–1 μl).

Purified elatol was used as an external standard, and quantifications were made through analytical curves calculated from four to six different concentrations

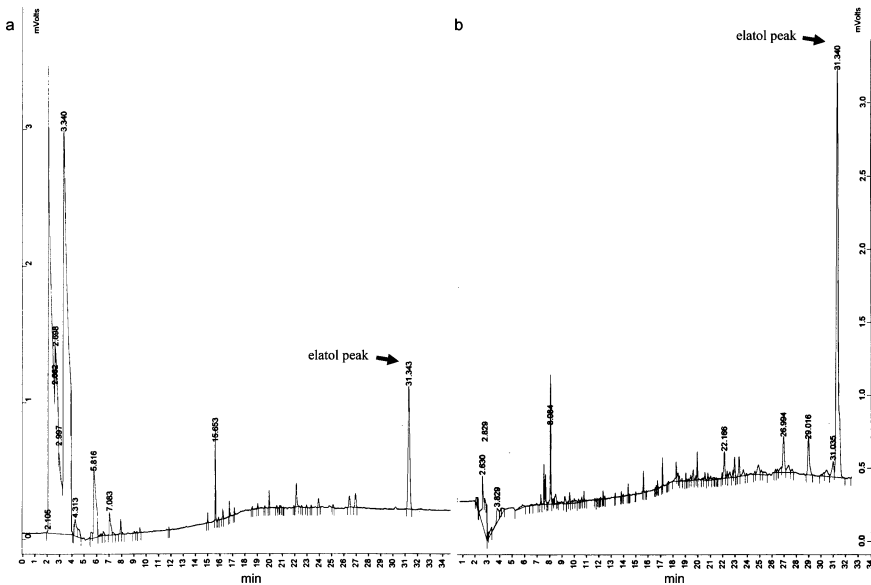


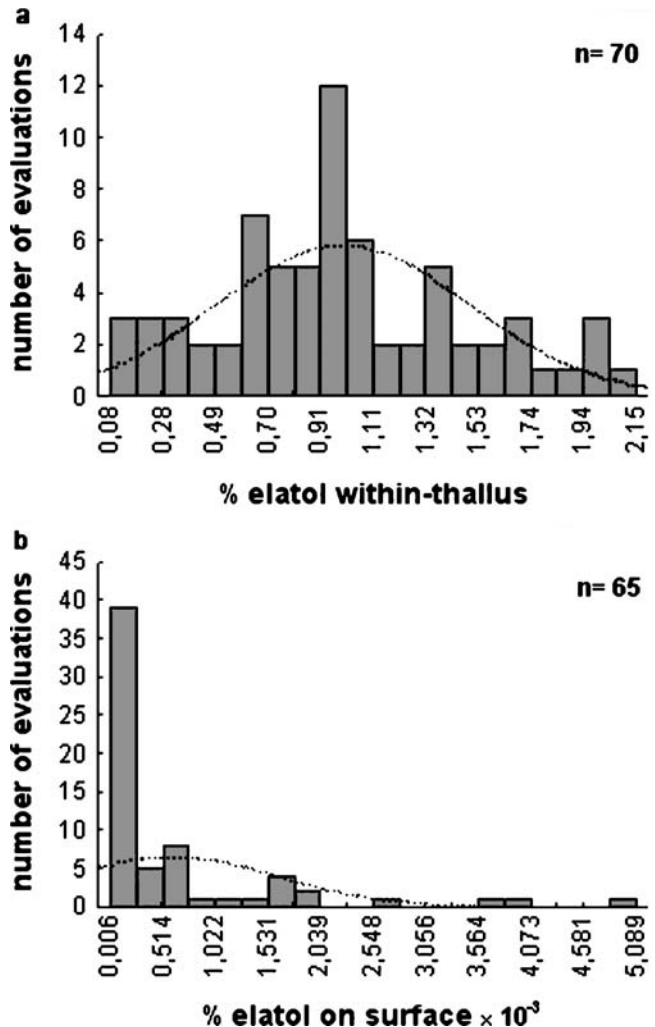
Fig. 2 Chromatograms: (a) standard, (b) surface sample

through standard solutions in hexane. New analytical curves were constructed whenever the values obtained for the standards exceeded the previous series by more than 15%. Detection and quantification limits were determined according to U.S. EPA provisions; an analytical curve in a concentration range almost 5 times the noise of the baseline was obtained. Then, the concentration of seven replicates of a standard was determined from this curve, and its standard deviation(s) was calculated. The limits of detection and quantification were defined as 3 and 7 times the standard deviation, respectively.

Results

The halogenated sesquiterpene elatol (Fig. 1) is a transparent and viscous purple oil easily recognizable via TLC ($r_f = 0.45$) and ^1H NMR analyses. Pure elatol was

Fig. 3 Amount of elatol found within-thallus (a) and on the surface (b) of *L. obtusa*. Values are expressed in percentage (elatol weight/dry weight of the frond)



obtained by removing and filtering the silica band corresponding to its rf region. The pure compound was used as a standard. After chromatographic conditions were optimized, it was eluted at 250°C, and the average retention time was 32.5 min. Detection and quantification limits were established at 0.048 and 0.112 mg elatol l⁻¹ solution, corresponding to 48 and 112 pg, respectively, in 1.0 µl injected. Elatol was detected on the surface and within-thalli of *L. obtusa* specimens. Figure 2 shows the chromatograms of the standard (Fig. 2a) and the surface sample (Fig. 2b).

Artifacts or contaminants derived from the solvent or the extraction method had no interference in the analyses: the chromatograms of pure hexane and the method blank showed no peak at 32.5 min. Two analytical curves with different concentration ranges were established due to the widely different levels observed on the surface (4 standards from 0.06 to 1 ppm, $R^2 = 0.999$) and within-thallus samples (6 standards, from 1 to 20 ppm, $R^2 = 0.991$) of *L. obtusa*.

The effectiveness of this method allowed the quantification of low levels of elatol, especially those found at the surface of *L. obtusa*. Among the 140 quantitative analyses performed, corresponding to 70 specimens collected (70 surface samples and 70 within-thallus samples), only two were under the detection limit, and another three were under the quantification limit. The mean within-thallus elatol concentration was 9.89 mg g⁻¹, d.w., varying from 0.1 to 2.2% d.w. (Fig. 3a). Conversely, the mean surface elatol contents reached much lower values (0.006 mg g⁻¹ d.w.) varying from 5×10^{-6} to 5×10^{-3} % d.w. (Fig. 3b). The ratios between surface area and wet weight of the fronds, obtained by the two methods described, were 71.69 and 65.01, respectively. Since there was no significant difference between these two values ($P > 0.05$, *t* test), the amount of elatol per surface area of *L. obtusa* was estimated to vary from 0.5 to 10 ng cm⁻² for 52% of the analyzed specimens.

Discussion

To our knowledge, the application of a GC-ECD method to quantify a seaweed secondary metabolite is a new initiative. Research on seaweed secondary metabolite quantification is usually carried out via colorimetric techniques (e.g., Ragan and Glombitza, 1986; Tugwell and Branch, 1989; Denton et al., 1990; Pereira and Yoneshigue-Valentin, 1999), HPLC (e.g., Phillips and Towers, 1982; Gerwick et al., 1985; De Nys et al., 1996b), or GC-MS (e.g., Mynderse and Faulkner, 1978; De Nys et al., 1996a, 1998; Dworjanyn et al., 1999). The literature on quantification of marine secondary metabolites usually lacks details, making the possible application or necessary comparisons between methods difficult.

Colorimetric techniques do not allow for the identification of compounds, limiting use to that of assessing specific classes of compounds such as polyphenols from brown seaweeds (Ragan and Glombitza, 1986; Denton et al., 1990). Some studies have applied this kind of polyphenolic quantitative analyses to evaluate chemical defenses against herbivores in a marine environment (see Targett and Arnold, 1998).

HPLC is widely applied to quantify marine secondary metabolites (De Nys et al., 1995, 1996b); however, there are sensitivity limitations. It is usually applied to measure total levels (e.g., Phillips and Towers, 1982; Taylor et al., 2003). Once the molecular weight of a molecular structure is known, the GC-MS technique has high

sensitivity, and the GC-ECD technique can be superior for halogenated compounds (Weinberg et al., 2002).

Ordinarily, such methods allow one to quantify metabolites localized in parts of thalli or in the whole seaweed. In both cases, detectable levels may vary from 0.1 to 5.0% d.w., particularly for aldehydes, acetogenins, terpenoids, or other more complex molecules (Paul and Fenical, 1986), and from 5.0 to 20.0% d.w. for polyphenols found in brown seaweeds (Tugwell and Branch, 1989; Denton et al., 1990).

Quantification of surface metabolites from seaweeds has only been described twice (De Nys et al., 1998; Wright et al., 2000) by GC-MS. Unlike other techniques, the GC-ECD method applied here was successfully used in measuring very low levels, revealing detection and quantification limits of 28 and 60 pg of elatol, respectively. Of the 140 analyses performed, only two were under the detection limit and three were under quantification limits. The lower levels of elatol varied from 5×10^{-6} to 5×10^{-3} % d.w. Similar very low levels of surface metabolites were described by De Nys et al. (1998): the mean total surface concentration of natural products from *L. obtusa* was $<1 \text{ ng cm}^{-2}$, while much higher values ($7 \text{ } \mu\text{g mg}^{-1}$) were found for the whole plant. Low concentrations of secondary metabolites on surface of seaweeds, compared to those of whole plants, were also found via a method in which surface material was sampled with a cotton wool swab (Schmitt et al., 1995).

Natural concentrations of elatol, similar to those found within-thalli, exhibit some biological or ecological activities. For example, this sesquiterpenoid inhibited the establishment of the mussel *Perna perna* (Da Gama et al., 2003), and its consumption by the sea urchin *Lytechinus variegatus* (Pereira et al., 2003), the crab *Pachygrapsus transversus* (Pereira et al., 2003), and certain fish (Hay et al., 1988). In addition, it had an effect against larval settlement of the barnacle *Balanus amphitrite* and the bryozoan *Bugula neritina* at concentrations ranging from 0.01 to $10.0 \text{ } \mu\text{g cm}^2$ (De Nys et al., 1996b). On the other hand, it had less or no effect on bacterial growth or on the germination and settlement of the green alga *Ulva lactuca*. Recent work has verified that low concentrations of elatol on the surface of *L. obtusa* have no inhibitory effects on feeding by sea urchin, or against the establishment of mussel and barnacle larvae (the authors, unpublished). The indirect evidence that low concentrations of compounds at the surface of *L. obtusa* inhibit fouling settlement in concentrations that are 2 to 3 orders of magnitude greater than those naturally found at the surface of this seaweed from Australia (Steinberg et al., 1998) also supports our argument. Differing or conflicting results reveal the importance of realistic assays that pay careful attention to concentration scales and target organisms, because a simple compound can vary in its concentration, its effects at different levels, or its action in a species-specific way.

Besides the sesquiterpene elatol, many *Laurencia* species and other red seaweed species produce halogenated metabolites (Hay and Fenical, 1988). Since the GC-ECD technique is sensitive to halogens, especially to bromine, the methodology developed in this study could be applied to other typical halogenated compounds from red seaweeds.

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Comparison of Glass Vessels and Plastic Bags for Enclosing Living Plant Parts for Headspace Analysis

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Abstract Plants release volatile chemicals into their surrounding air space that can affect the physiology of neighboring plants and influence the behavior of insects. In studying these interactions, it is desirable to collect volatiles from plants that have not been excised and are growing under as natural conditions as possible. We compared a vessel of borosilicate glass and Nylon-6 or polyester [poly(ethylene terephthalate) or PET] cooking bags for enclosing plants during collection of volatiles. A push–pull airflow system was used, and volatiles were trapped on Tenax TA and analyzed by gas chromatography after thermal desorption. Low levels of impurities were found for the glass vessel and polyester bags. Nylon bags contained higher levels and more impurities. Recoveries of standards of 10 plant volatiles were measured in static and dynamic systems. In a static air system, there was good recovery only from the glass vessel. In a dynamic system, there was generally good recovery from both the glass vessel and polyester bags. Recoveries of α -pinene and (*Z*)-jasmone were poor throughout. The former was shown to have a very low breakthrough volume on the Tenax TA adsorbent, and the latter may be strongly adsorbed on glass. All three materials were essentially transparent in the IR and visible (photosynthetic) range but with significantly different absorptions in the UV range. In a simulated dynamic entrainment in full sunlight, internal vessel temperatures were higher than ambient by up to 9.5°C in the glass vessel and 7.5°C in the polyester bag. Lower increases in temperature relative to ambient (<1°C) were recorded when entrainments were conducted in the shade. In a field trial, the profiles of volatiles collected from an apple tree infested with rosy apple aphid using a glass vessel and a polyester bag were similar. Polyester bags are recommended as more convenient than glass vessels for the enclosure of plants during the collection of volatiles.

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Introduction

Plants release chemicals into their surrounding air space that can affect the physiology of neighboring plants as well as influencing the behavior of insects. In recent years, there has been considerable interest in identifying such volatiles and understanding their effects in plant–plant systems (Bruin and Dicke, 2001; Pickett and Poppy, 2001; Engelberth et al., 2004), plant–herbivore systems (Visser, 1986; Bernasconi et al., 1998; Blackmer et al., 2004), and tritrophic systems (De Moraes et al., 1998; Powell et al., 1998; Smid et al., 2002; Birkett et al., 2003).

A diverse range of sampling methods has been employed for the collection of plant volatiles, including microwave-assisted extraction, sampling from warmed leaves using a gas syringe, or entraining over macerated plant, chopped foliage, whole leaves cut from the plant, whole branches/stems including leaves, uprooted plants in water, and whole plants including pot and soil (respective examples: Töllsten and Bergström, 1988; Smith et al., 1996; Geervliet et al., 1997; Landon et al., 1997; Du et al., 1998; Rojas, 1999; Choh et al., 2004; De Boer et al., 2004). Whereas these approaches have increased our understanding of plant semiochemicals, there are drawbacks to physically wounding the plant. Workers try to limit sample contamination from the wound by placing cut parts in vials of water, or wrapping them in wet cotton, Parafilm, or Teflon (respective examples: Mumm et al., 2003; Choh et al., 2004; Russell et al., 2004; Van Den Boom et al., 2004). However, wounding a plant not only causes the localized release of volatiles, but it also generates electrical, hydraulic, and/or chemical signals that act systemically. These signals stimulate the release of new volatiles far from the site of damage and also cause quantitative and qualitative changes in the volatiles relative to those from undetached parts (Galliard, 1978; Davies, 1987; Mookherjee et al., 1990; Rhodes et al., 1999; Backman et al., 2001; de Bruxelles and Roberts, 2001; Schmelz et al., 2001). Furthermore, detection by the plant of C_6 volatiles associated with physical damage can again trigger rapid systemic responses in which volatiles are released (Farang and Paré, 2002).

To minimize damage to a plant during sampling of volatiles, a common approach has been the use of a guillotine-style enclosure vessel. This isolates part of or a whole plant, and clean air is blown into the enclosing vessel at a rate greater than that at which headspace is sampled through a trapping adsorbent (see Heath and Manukian, 1994). Typically, the main body of such vessels is made of glass and the guillotine of Teflon or aluminum (Heath and Manukian, 1994; Röse et al., 1996; Paré and Tumlinson, 1997; Agelopoulos et al., 2000; Mattiacci et al., 2001; Rodriguez-Saona et al., 2003). However, these heavy, bulky glass vessels require considerable support and are inconvenient for sampling plants growing naturally.

Translucent plastic oven bags offer an easily transportable and disposable alternative to glass vessels, that would also facilitate replicated sampling from plants in the field. Reports in which different types of bags have been used are listed in Table 1. Although studies have compared the effects of gas syringe sampling, solid-phase microextraction (SPME), different trapping adsorbents, elution solvents,

solvent desorption vs. thermal desorption, and pump flow rates in the collection and analysis of plant volatiles (Agelopoulos and Pickett, 1998; Raguso and Pellmyr, 1998), there has been no study that critically assesses the merits and limitations of glass vs. plastic bag enclosure methods.

Two principal considerations for any enclosure material are chemical contamination and chemical adsorption. The effects of enclosing on the plant itself are often overlooked. Enclosure will qualitatively and quantitatively affect light wavelengths reaching the plant and may also affect both temperature and humidity. In this paper, we compare a glass chamber, Nylon-6 oven bags, and polyester (PET) oven bags. Using 10 functionally and structurally diverse chemicals produced by a wide range of plants, we assessed recoveries of headspace injected into static air sealed in the vessels for 30 min and recoveries when headspace was added to air drawn through the different vessels. We also investigated light absorption properties of the materials by spectrophotometry and measured the internal temperatures of the vessels vs. ambient temperatures. Finally, the glass chamber and the polyester bag were tested in the field by sampling volatiles from aphid-infested leaves from an apple tree growing in a commercial orchard.

Methods and Materials

Headspace Mixtures

Standards of (*E*)-2-hexenal, α -pinene, 6-methyl-5-hepten-2-one, (*Z*)-3-hexenyl acetate, limonene, linalool, citronellal, methyl salicylate, (*Z*)-jasmone, and (*E*)-caryophyllene were obtained from commercial suppliers (Sigma-Aldrich Company Ltd., Gillingham, Dorset, UK; Fluka, Buchs, Switzerland; Avocado, Heysham, Lancashire, UK). All chemicals were of $\geq 98\%$ purity except for citronellal, (*Z*)-jasmone, and (*E*)-caryophyllene that were of 87.5, 93.7, and 86.8% purity, respectively. Headspace mixtures were prepared in glass vessels (2 l) fitted with Teflon-backed silicone septa. To one vessel, (*E*)-2-hexenal, α -pinene, 6-methyl-5-hepten-2-one, (*Z*)-3-hexenyl acetate, and limonene (5 μ l of each) were added before sealing. Linalool, citronellal, methyl salicylate, (*E*)-caryophyllene (5 μ l of each), and (*Z*)-jasmone (100 μ l) were added to the other vessel before sealing, and these are referred to as mixture 1 and mixture 2. Both vessels containing headspace mixtures were kept in a dark incubator (20°C, Sanyo MIR 152, Watford, Hertfordshire, UK), and the vessels were equilibrated for 24 hr before any samples were taken.

Vessels Used as Enclosures

A cylindrical borosilicate glass jar (30.8 cm \times 11.1 cm \times 3.5 mm thick) with a ground glass flange at one end was selected as an aeration chamber. The jar had threaded fittings on the top end (outlet) and on the side near the flange (inlet). The base was sealed using an aluminum plate bound tight using PTFE tape and clamped onto the flange. The inlet near the flange was sealed using a Teflon-backed silicone septum, and the outlet on the top of the vessel was sealed using two bands of PTFE tape onto which a prebored (5 mm), Teflon-backed silicone septum was tightened.

Table 1 Examples of plastic bags used for the sampling of volatile compounds originating from plants and a fungus

Bag description	Sampling system	Sampling from	Location	Reference
“Plastic bag”	Pull; filter inlet	Tree branch with leaves	Field	Quiroz et al., 1999
“Small plastic bag”	Push	Flowers	Field	Tan et al., 2002
“Polyethylene bag”	Push	Magnolia leaves	Field	Azuma et al., 1997
“Plastic frying bag [polyethylene terephthalate (PET)]”	Push	Beech leaves	Field	Töllsten and Müller, 1996
“Plastic bag made of thermostable polyester film”	Push	Flowers	Controlled	Karin and Karlson, 1990
“Polyester cooking bag (Meny Toppits, Terinex Ltd., England, 35 × 43 cm)”	Pull; filter inlet	Tree branches with leaves	Field	Zhang et al., 1999
“Polyethylene terephthalate (Look, Meny)”	Pull; inlet open to atmosphere; also SPME	Fungi	Field	Fäldt et al., 1999
“Nalophan [®] bag (Kalle Nalo, Wiesbaden, Germany)”	Push > pull	Maize plant	Controlled	Bernasconi et al., 1998
“PET cooking bags (Nalophan)”	Pull; inlet open to atmosphere	Flowers	Field	Huber et al., 2005
“PET cooking bags (Nalophan) (1 m × 22.5 cm diam.)”	Push > pull	Maize plant	Controlled	Turlings et al., 1998a
“Odorless polyethylene terephthalate (PET) Nalophan cooking bags”	Push > pull	Whole plants	Controlled	Kalberer et al., 2001
“PET (Nalophan) bag (Kalle Nalto, Würsthüllen, Germany)”	Push > pull	Palm leaves	Controlled	Dufa et al., 2004

Table 1 (continued)

Bag description	Sampling system	Sampling from	Location	Reference
“Polyacetate bag”	Pull; inlet open to atmosphere	Flowers	Field	Pellmyr et al., 1990
“Polyacetate bag (35 × 43 cm or 25 × 38 cm)”	Push	Flowers	Field	Knudsen et al., 2004
“Polyacetate oven bags (Reynolds Inc.)”	Pull; frame	Cut walnuts	Controlled	Henneman et al., 2002
“Polyacetate bag (Reynolds oven bags)”	Pull; internal frame and filter inlet	Flowers	Field and controlled	Levin et al., 2001
“Polyvinylacetate bags (Reynolds, Inc.: 500 or 1000 ml)”	Pull; internal frame and filter inlet	Flowers and chemical standards	Controlled	Raguso and Pellmyr, 1998
“Polyacetate bags (Pingvin frying bags, Art. no. 352, Kalle Nalo GmbH, D-65203 Wiesbaden, Germany)”	Push	Flowers	Field	Andersson and Dobson, 2003
“Reynolds, Inc. (Nylon resin) oven bags”	Push	Flowers	Controlled	Raguso et al., 2003

Unless specified, plants are intact and have not been excised; table ordered according to bag description.

Two different plastic bags were used: Turkey Roasting Bags (Nylon-6, volume 10 liters, ~20- μ m thickness, max. 200°C, Baco Consumer Products Ltd., Amersham, UK) and Multi-Purpose Cooking Bags [poly(ethyleneterephthalate) or PET, volume 3.2 liters, ~12.5- μ m thickness, max. 200°C, Sainsbury’s Supermarkets Ltd., London, UK]. Bags were fitted with threaded glass tube inserts (5 cm long, 8 mm i.d.) fixed using UNEX tubing clips (Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK). The inlet was fitted to the middle of the open end of the bag, and the outlet was fitted to a corner of the bag after cutting off with scissors. Inlet and outlet inserts were closed with Teflon-backed septa as above.

The glass jar, aluminum plate, and threaded glass inserts were cleaned using detergent, acetone, and distilled water before baking overnight in an oven (200°C). Soon after purchase, bags were always removed from packaging and nylon tags discarded (included in both bag types). Bags were then stored in the laboratory until

required. To clean, the nylon bags were opened and baked in an oven (120°C, 4 hr) before fitting the glass inserts and stored overnight in an incubator (50°C; Sanyo MIR 152) while inflated and with activated charcoal filtered air flowing through (1 l/min). The polyester bags were relatively clean and were opened and baked in an oven (120°C) for 2 hr. Before experiments, both bags were inflated with clean air and then deflated three times to remove any residual contamination.

Headspace Recovery

All headspace samples were trapped on Tenax TA (50 mg, mesh 60–80, Supelco, Bellefonte, PA, USA) held in injector liners (81 mm long, o.d. 5 mm, i.d. 3.2 mm) by plugs of silanized glass wool. Before use, filled liners were washed with redistilled diethyl ether (5 ml) and thermally desorbed under a slow flow of purified nitrogen (225°C, 2 hr). Recoveries of chemicals from the glass vessel or plastic bags were always paired to a control sample from the headspace source that was taken and analyzed about an hour previous to that replicate. A closed loop system was used, consisting of a diaphragm pump (12 V, KNF Neuberger, Germany), flow meter, activated charcoal filter, and glass tube, connected together with Teflon tubing (i.d. 1.5 mm). The glass tube was fitted with an inlet, an outlet with screw adapter holding a Tenax-filled liner, and sidearm with Teflon-backed septum allowing injection of materials onto the neck of the liner. For controls, headspace samples of mixture 1 (100 µl) and mixture 2 (1 ml) were injected onto the liner with gas-tight syringes such that the mixtures were immediately pulled onto the Tenax (1 l/min), and airflow was maintained for 30 sec.

For static headspace sampling, the glass jar or bags were first flushed (three times vessel volume) and inflated with clean air by means of a Pasteur pipette pushed through the intact septum fitted on the vessel inlet and connected by Teflon tubing to the pump and activated charcoal filter. The Pasteur pipette was then removed, and headspace samples of mixtures 1 and 2 were injected through the same septum (100 µl and 1 ml, respectively). After 30 min, a Tenax-filled liner was pushed through the cored septum at the outlet and connected to the pump. The Pasteur pipette was then pushed through the inlet septum, thus completing the closed system. The pump was turned on (1 l/min), and the vessel was flushed through 10 times for the glass jar (30 min) or 5 times for the bags (10-l nylon, 50 min; 3-l polyester, 15 min). The bags were also collapsed by removing the Pasteur pipette, thus pulling the remaining air volume in the bag onto the Tenax.

For dynamic sampling, the glass jar or bag was incorporated in a closed loop system with pump, flowmeter, and charcoal filter. A three-way glass tube, as used for the preparation of control samples, was also connected to the inlet of the vessel. Thus, headspace samples could be added to the air entering the vessel by injecting through the septum in the sidearm of the inlet tube. A Tenax-filled liner was fitted to the outlet of the vessel and connected to the pump (circulating airflow of 1 l/min). After injection of headspace samples of mixtures 1 and 2 (100 µl and 1 ml, respectively) into the inflowing air, the experiment was run such that each vessel was flushed through five times (glass jar, 15 min; nylon bag, 50 min; polyester bag, 15 min).

Following observation of low recoveries of α -pinene, a breakthrough test was performed with two Tenax-filled liners held in series. Headspace mixtures were injected onto the neck of the first liner as above, and in different trials, airflow was thereafter maintained for 0.5, 2.5, 5, 10, or 20 min before analysis of the trapped volatiles.

Spectrophotometric Analyses

A sample of borosilicate glass was cut from the same source that was used to make the glass vessel. Samples were also taken from the nylon and polyester bags (after the cleaning process). Absorption (200–800 nm) was quantified on a spectrophotometer (U-2000, Hitachi, Maidenhead, Berkshire, UK) at 2-nm intervals.

Measurement of Vessel Internal Temperature

Glass and polyester vessels only were compared in this experiment because these were to be used in the field trial. Two experiments were performed: one in an exposed location and the other in the shadow of a large bush. The glass vessel was held horizontally, and aluminum base plates with a hole through the middle were clipped on as used in field experiments. Similarly, the polyester bag was inflated and held horizontally. A thermometer was inserted horizontally into each such that the thermometer bulbs were in the center of the vessels, and a third thermometer was held horizontally by a tripod to measure ambient air temperature. For both experiments, the temperatures of air, glass, and polyester vessels were recorded at the start of the experiment and every 10 min for 3 hr. Notes of weather conditions in the preceding 10 min were also taken. To reproduce the conditions of a real collection of volatiles from a plant, clean air was blown into the vessels (1.2 l/min) and drawn out onto a Tenax-filled liner (1 l/min), and this airflow was maintained throughout the experiments. For the experiment performed in the shadow of a large bush, at the end of the experiment, the vessels were brought into full sun, and their internal temperatures were recorded after 10 min to confirm that differences observed were not a day artifact.

Field Collections

Field entrainments were conducted in orchards belonging to the Leckford Fruit Farm, Leckford Estate, Hampshire, UK. Apples were Cox's Orange Pippins on MM106 rootstocks, and the tree entrained was infested by rosy apple aphids *Dysaphis plantaginea* (Passerini) (Homoptera: Aphididae). Entrainments were performed to compare the polyester and glass vessels only. A branch with aphid-infested leaves on the shaded side of the tree was selected and enclosed in a vessel. Air cleaned by activated charcoal was blown into the vessel (1.2 l/min), and headspace was drawn out through Tenax-filled liners at the outlet (1 l/min). The pumps were driven by using a car battery (12 V). Control sample was taken from an empty conainer before sampling from the branch to check for system/filter contamination (2 hr), and then the branch was sampled by using the polyester bag and then the glass vessel (2 hr each). For the polyester bag, the inlet was a glass tube (6 mm) that ran parallel to the branch and was held in place by a gardening twist-tie that also closed the bag around the plant. The outlet was a Tenax-filled glass liner fitted into a corner of the bag. The glass vessel was held in place by a tripod and was enclosed around the plant with two semicircular aluminum plates with a hole in the middle that allowed the branch to pass through (similar to Agelopoulos et al., 2000).

Analysis by Gas Chromatography

Entrainment samples were desorbed using an Optic 2 programmable injector (Anatune, Cambridge, UK) fitted to an Agilent 6890N gas chromatograph (GC) with a flame ionization detector. Injector conditions were equilibrated (1 min) then ramped from 30 to 220°C at 16°C/sec while operated in splitless mode. An HP-1 column (50 m × 0.32 mm i.d., 0.32- μ m film) was used. The carrier gas was helium (constant flow 30 cm/sec), and the oven temperature program was 30°C/2 min, 5°C/min to 150°C, then 10°C/min to 250°C and held for 6 min; detector temperature was 300°C. Data were processed by using ChemStation Plus software (Rev. A09.03). Compounds from the field entrainment were tentatively identified with Kovats indices and by thermal desorption on an Optic 2 injector fitted to a Hewlett-Packard 5890 series II GC linked to an HP 5972 mass selective detector using similar conditions to those described above.

Data Analyses

For the two recovery experiments, five replicates were carried out, and chemical recoveries were tested against respective controls by one-sample *t* tests. A two-way analysis of variance was used to determine whether differences and interactions existed between bags and headspace chemicals, and the Fisher least significant difference test was used to identify where such differences existed. UVC, B, A, visible, and infrared absorption chroma (% absorption within band) of spectrophotometric data were characterized, and absorption cutoff points (upper points of inflection) were calculated. Differences in spectrophotometric properties of the material were tested using a Freidman test. Internal temperature experiments were also analyzed by using a Freidman test. All data were analyzed on Minitab 13.1 (Minitab Inc., State College, PA, USA), and differences were considered significant if the probability of their occurrence by chance was less than 5%.

Results

Contamination

After cleaning, the glass chamber gave a low background, but it was difficult to completely eliminate minor contaminant peaks. When a polyester bag was taken directly from its cardboard packaging and sampled (15 min), significant background contamination was detected. The main contaminant was tentatively identified as isopropyl dodecanoate, and low levels of *n*-alkanes (C14–18) were identified, as were traces of some branched alkanes. Bags that had been baked (120°C, 2 hr) and inflated/deflated three times before sampling (1 hr at 1 l/min) were almost contamination free (at most, 2 ng when compared to detector responses for *n*-alkanes). No dimethyl terephthalate or ethylene glycol was detected in polyester bags. Greater contamination problems were associated with the Nylon-6 bags. Before baking, significant quantities of caprolactam (hexahydro-2*H*-azepin-2-one) could be collected, and this gave a broad fronting peak just after methyl salicylate. After baking and cleaning as above (see [Methods and Materials](#)), caprolactam was much reduced and occasionally absent. Other unidentified peaks (including two

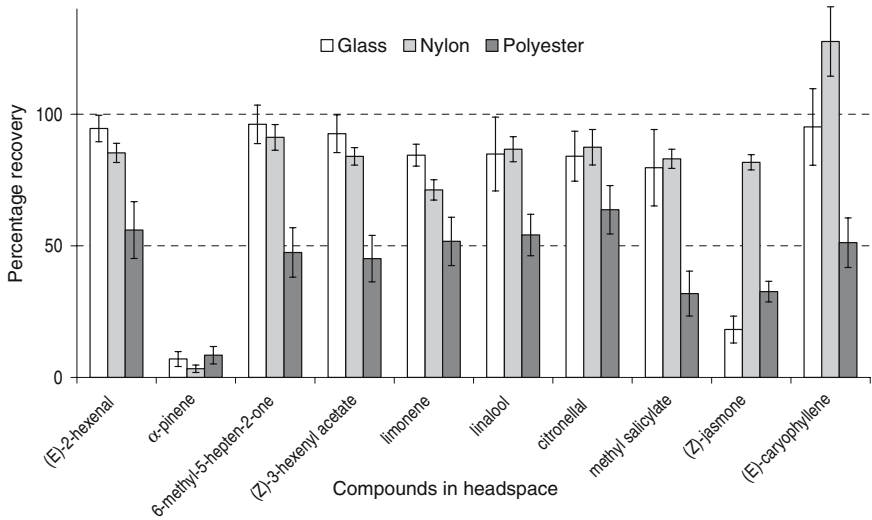


Fig. 1 Recoveries of headspace injected into a glass vessel, inflated Nylon-6, and inflated polyester (PET) bags after allowing 30 min for chemicals to interact with the internal surfaces ($N = 5$)

phthalates) associated with bag contamination were observed, but their presence and abundance were quite variable.

Headspace Recoveries

In recoveries from static headspace, vessel material, headspace chemical, and interactive effects were all significant factors contributing toward the variation in

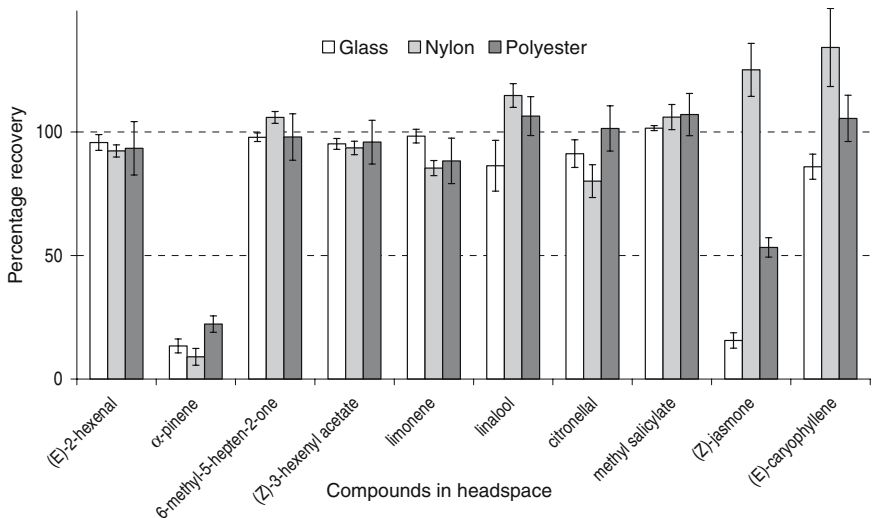


Fig. 2 Recoveries of headspace chemicals when injected into the air flowing through a glass vessel, inflated Nylon-6 bag, and an inflated polyester (PET) bag ($N = 5$)

Table 2 Summary of spectrophotometric data for vessel materials

Material	Absorption chroma bands (% total absorption in band)					Absorption cutoff ^a (nm)
	UVC, 10–280 nm	UVB, 280–320 nm	UVA, 320–400 nm	Visible, 400–700 nm	Infrared, 700–800 nm	
Glass	97.8	39.1	3.2	1.3	1.3	276
Nylon	50.7	10.0	6.9	2.2	2.2	228
Polyester	99.3	76.0	4.2	2.1	2.1	306

^a Midway between points of inflection.

recoveries in comparison to controls (Fig. 1; $P \leq 0.001$ for all), with less material recovered from the polyester bag than from the glass vessel or nylon bag ($P \leq 0.001$). For glass, by comparison to the controls, there were losses in recovery of α -pinene, limonene, and (*Z*)-jasmone ($P \leq 0.001$, $P = 0.02$, and $P \leq 0.001$, respectively). From nylon bags, there were losses of (*E*)-2-hexenal, α -pinene, (*Z*)-3-hexenyl acetate, limonene, linalool, methyl salicylate, and (*Z*)-jasmone when compared to the controls ($P = 0.016$, $P \leq 0.001$, $P = 0.009$, $P = 0.002$, $P = 0.049$, $P = 0.01$, and $P = 0.003$, respectively). For polyester bags, there were losses in recovery for all compounds when compared to the controls (all $P < 0.05$).

Fisher least significant difference tests were performed to see how material affected the ratios of headspace chemicals recovered. For glass, all chemicals were recovered equally except for α -pinene and (*Z*)-jasmone, for which recoveries were lower but no different from each other ($P \leq 0.001$). For nylon, all chemicals were recovered equally except for α -pinene that was lower than others and (*E*)-caryo-

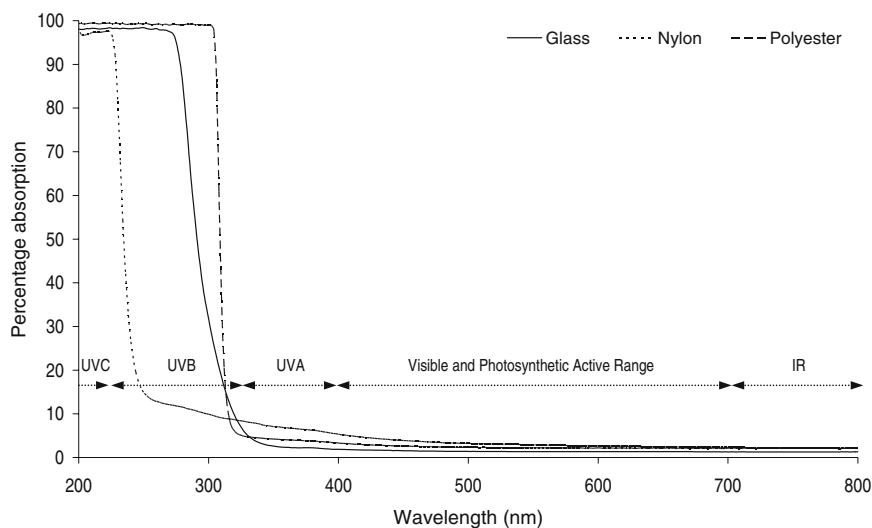


Fig. 3 Percentage absorption of different wavelengths by the glass, Nylon-6, and polyester (PET) materials when tested over the 200- to 800-nm range. UVC (10–280 nm), B (280–320 nm), A (320–400 nm), visible (400–700 nm), and infrared (700–800 nm)

phyllene that was higher ($P \leq 0.001$). The latter observation was probably caused by an unidentified minor contaminant found in collections from empty nylon bags that coeluted with (*E*)-caryophyllene. For polyester bags, all chemicals were recovered equally except for α -pinene that was lower than all others ($P = 0.002$). Recoveries of (*Z*)-jasmone from all materials were different ($P \leq 0.001$), with recovery from nylon bags the highest, polyester intermediate, and glass the lowest.

As with recoveries from static air, for headspace recoveries from flowing air, vessel material, headspace chemical, and interactive effects were all factors contributing to variation (Fig. 2; $P \leq 0.001$ for all). α -Pinene and (*Z*)-jasmone were lost in the glass and polyester vessels (glass: both $P \leq 0.001$; polyester: $P = 0.002$ and $P = 0.008$, respectively), but for all other chemicals tested in these vessels, there were no changes in recovery in comparison to the controls. In the nylon vessel, there were losses of (*E*)-2-hexenal, α -pinene, limonene, citronellal ($P = 0.036$, $P \leq 0.001$, $P = 0.009$, $P = 0.04$, and $P \leq 0.001$, respectively), and an increase in linalool peak area ($P = 0.038$) when compared to the controls. The slight increase in linalool peak area was caused by an unidentified coeluting minor contaminant from the nylon bag. Fisher least significant difference tests for the glass vessel and polyester bag confirmed that all chemicals were recovered equally with the exception of α -pinene

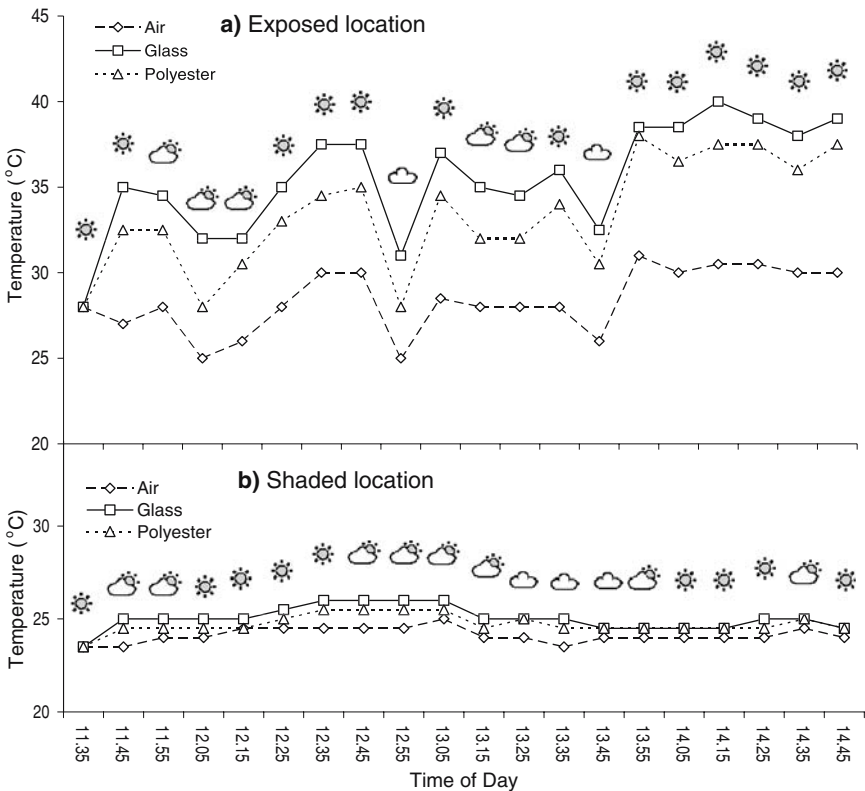


Fig. 4 Ambient temperature and internal temperatures of the glass vessel and polyester (PET) bag in a replicated dynamic entrainment conducted in (a) an exposed location and (b) the shadow of a large bush. Symbols indicate weather conditions in the 10 min preceding the temperature reading

and (*Z*)-jasmone, and hence ratios were preserved. For glass, recoveries of α -pinene and (*Z*)-jasmone were not significantly different, but for polyester, recovery of (*Z*)-jasmone was significantly higher than that of α -pinene.

Breakthrough

The breakthrough test showed that low recoveries of α -pinene in the above experiments were a result of breakthrough on the adsorbent. Within 30 sec, traces of α -pinene (<1 ng) were detected on the second liner held in series, and after 5 min, there was more α -pinene on the second liner than on the first. No breakthrough was detected for any of the other chemicals tested in the headspace mix.

Spectrophotometric Analyses

Although all the materials tested were essentially transparent to the human eye, there were differences in the light absorption properties of all these materials ($P \leq 0.001$), particularly in the UV range (Table 2; Fig. 3). Both glass and polyester had similar transmission properties in the IR, visible, and UVA ranges, allowing over 95% of the light through. Nylon absorbed slightly more light of these wavelengths, but the difference was small. The main differences in transmission were in the UVB

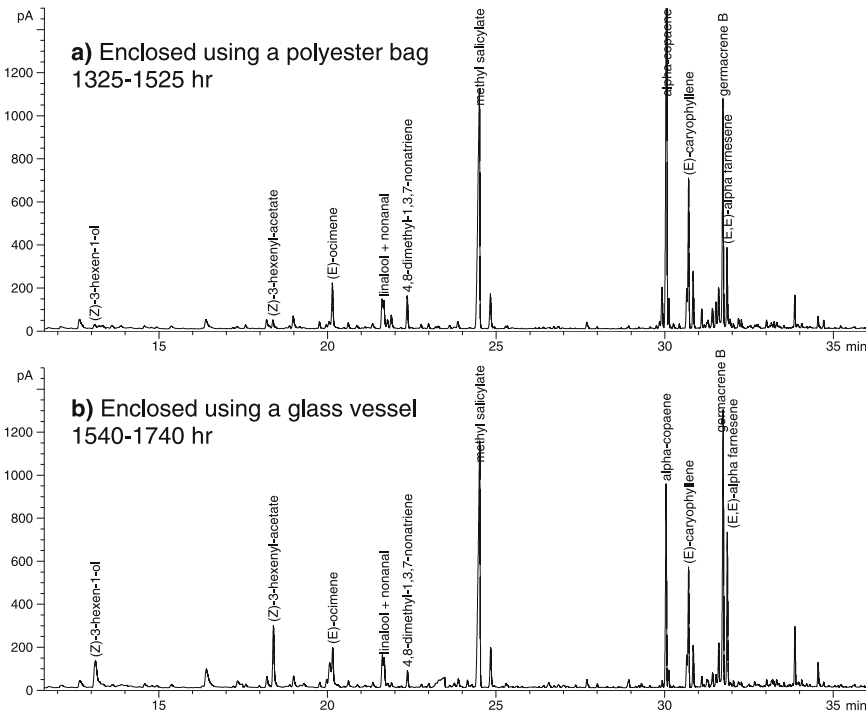


Fig. 5 Example of field entrainments from an apple tree infested with rosy apple aphids. Samples were captured on Tenax TA in a dynamic entrainment system, and plant parts were enclosed using (a) a polyester (PET) bag and (b) a glass vessel. Time of day during which samples were collected are indicated for both

range. Polyester absorbed most light in the UVB range (76%), glass about 40%, and nylon 10%. Both glass and polyester blocked all UVC, and nylon allowed about 50% transmission.

Measurement of Vessel Internal Temperature

In simulated dynamic entrainments conducted in full sun, the internal temperatures of both the glass and polyester vessels were higher than the ambient air ($P \leq 0.001$; Fig. 4a). On average, the glass vessel was 7.5°C hotter and the polyester 5.2°C hotter. Maximum differences relative to ambient were 9.5 and 7.5°C, respectively. When simulated dynamic entrainments were conducted in the shade, the glass vessel was, on average, only 0.9°C hotter, and the polyester 0.6°C hotter than ambient, but these differences were significant ($P \leq 0.001$; Fig. 4b). Although vessels were always marginally hotter than ambient when shaded, the presence/absence of cloud did not cause rapid fluctuations in temperature as observed for exposed vessels (Fig. 4a). At the end of the shaded experiment, when the vessels were taken from the shade and placed in full sun, after 10 min, ambient air, glass, and polyester temperatures were recorded as 26.0, 33.5, and 29.5°C, respectively.

Field Entrainments

The glass vessel and polyester bags performed similarly when tested in field entrainments (Fig. 5). No significant contamination was detected from the bag, and the small differences seen between the enclosures might be attributable to time-of-day effects.

Discussion

Chemical ecologists trying to identify plant volatile blends accurately and/or understand their roles in plant–plant or plant–insect systems need to be able to sample from unexcised plants, ideally both in the laboratory and in the natural environment. Indeed, for rare plants or tree species, sampling in the natural environment might be the only option.

Previous reports and the work described here show that enclosing a plant in a glass vessel with guillotine-type seal and push–pull airflow gives good results in terms of low levels of contamination and good recovery for most volatile compounds. However, much of this equipment is not readily available and is expensive to make. As a result, sample replication in the field is difficult because collections are limited by the number of vessels available and the need for these to be cleaned between collections. Furthermore, the bulk and fragility of glass apparatus makes it inconvenient for carrying to the field and, once in the field, requires substantial devices to hold it in position. The inflexibility of the chamber can also lead to damage of the enclosed plant or plant part. Enclosure of plants in disposable plastic bags can avoid many of these difficulties. When used with the push–pull airflow system, the bag will remain inflated and, to some degree, self-supported. In the field, additional support can be easily improvised to avoid plant injury. From a survey of the literature, bags made of polyester seem to be most commonly used for collection of plant/leaf volatiles, whereas bags made from poly(vinyl acetate) seem to be the most common

material used in floral collections (see Table 1). There is no obvious reason for this, and some have questioned whether bag materials are optimal or simply used because of lack of evidence to the contrary (Raguso and Pellmyr, 1998). One source of confusion we encountered was in the bag terminology (see Table 1). More precise and standardized descriptions are desirable, such as common name, chemical name, supplier, and dimensions. Similarly, descriptions of the actual entrainment systems were generally vague. Better descriptions of experimental methodology would help other workers with their collection methods, and also support meaningful comparative work, e.g., across taxa.

Contamination

Clean gas sampling bags made from Tedlar or Teflon are available (e.g., Alltech UK Ltd., Stamford, Lincolnshire, UK), and these come with centrally positioned molded fittings to attach inlet and outlet sources. Although these bags could be cut open and used for field entrainments, a bag of similar size to the polyester bags used in experiments costs about 200 times more. As a result, cost makes these bags unsuitable for repeated field sampling.

Chemical contamination was a particular problem with the Nylon-6 oven bags, and we tentatively identified the principal contaminant as caprolactam, the starting monomer that is used to manufacture Nylon-6 by ring opening polymerization. Numerous other contaminants were observed, and the amounts of these were variable. Another problem we found with the Nylon-6 bags was that they could contaminate glassware in the oven if baked together, so we developed our two-stage cleaning method. This solution was not really practical nor was it consistently satisfactory. Other cleaning methods, such as baking in a vacuum oven, might further reduce the level of impurities and make such bags usable. In contrast, it was easy to purge contaminants from polyester oven bags. High-temperature baking, however, was detrimental to these bags because seams became brittle and cracked, causing leakage. Nevertheless, other workers have tailor-made bags from polyester tubing by heat-sealing an end (Kalberer et al., 2001). Reports from the food industry indicate that polyester heated up to 270°C (melting) does not release artifacts or other volatiles (Freire et al., 1999), and such tailor making of bags to fit particular plant shapes offers great versatility. Unlike Nylon-6 bags, we did not detect the monomers used in the manufacture of polyester, dimethyl terephthalate, or ethylene glycol. Contamination that was detected might have come from the printed packaging or the machinery used during manufacture. Despite low levels of contamination in polyester bags and glass vessels, it is always good practice to take control blank entrainments before use in experiments.

Recovery of Volatiles

In our experiments, there were consistently low recoveries of two chemicals, α -pinene and (*Z*)-jasnone. For α -pinene, this was caused by breakthrough on the Tenax TA adsorbent. Because control samples were prepared by pulling headspace onto the Tenax TA for 30 sec, breakthrough would not have affected their preparation. Although we have not conclusively explained the behavior of (*Z*)-jasnone, our experiments provide circumstantial evidence for strong absorption onto glass. We

checked that loss was not to the aluminum base plate by running trials in which a sheet of glass replaced the aluminum and still obtained poor recoveries of (*Z*)-jasmane (data not shown). In preparation of the headspace mixtures in glass vessels, 100 μl of (*Z*)-jasmane headspace were required to give a headspace concentration comparable to those of the other compounds for which only 5 μl were required. Furthermore, in both recovery experiments, loss of (*Z*)-jasmane was greatest in the glass vessel. We also recorded similar behavior for methyl jasmonate with glass (data not shown). Adsorption of other volatiles onto glass because of interactions between hydroxyl groups or the π electrons of a double bond has been reported, and double-bond interactions were strongest when bonds were in the (*Z*)-configuration (Shani and Clearwater, 1997). Glass surfaces should, therefore, be deactivated, but, with few exceptions (e.g., Zini et al., 2003), this is rarely carried out.

The static headspace experiments were designed to test the different materials rigorously for chemical porosity and absorbence. Apart from α -pinene and (*Z*)-jasmane, there were good headspace recoveries from the glass vessel, and the ratios between compounds were well preserved. There were greater losses of some chemicals in the nylon bag, suggesting that some had absorbed or diffused out, and a slight distortion of ratios. Interestingly, (*Z*)-jasmane was recovered well in the headspace from this material, reinforcing ideas of selective absorption onto glass. Recoveries were poor for all compounds when sealed in the polyester bag for 30 min. The data suggest that this bag material, which was very thin (about 12.5- μm thickness), might be chemically porous, and that significant outward diffusion takes place relatively quickly. Although there was significant quantitative loss, the polyester was qualitatively better than the nylon. The polyester bags that we used are, therefore, not effective for the sampling of headspace in sealed systems, e.g., by SPME. Nylon bags would be better, although there would be some loss/distortion, and our experiment only sealed headspace for 30 min. Nylon bags have been used for the creation of enriched floral headspace (15 min) and subsequent SPME sampling (15 min; Raguso et al., 2003). Overall, we found the glass vessel to be most suitable for the sealing of headspace and later sampling.

The dynamic headspace experiments were designed to test the suitability of the different materials for continuous headspace sampling. By adding chemicals to the air blowing/being-pulled through the vessel, we tried to replicate the way chemicals might be pulled away from plants as they are released. With the exception of α -pinene and (*Z*)-jasmane, there were excellent recoveries and preservation of ratios for both the glass and polyester materials, indicating that both would be suitable for dynamic headspace entrainments. As used in our experiments, we would recommend relatively high flow rates to vessel volume (e.g. 1:3). The moderate loss of (*Z*)-jasmane in the polyester system was puzzling and may have occurred because a glass insert was used at both the inlet and outlet ends of the bag. The inclusion of the insert was necessary at the inlet for both static and dynamic recovery experiments but would have only been necessary at the outlet for the static recoveries experiment. However, for experimental consistency, we also included the glass insert at the outlet for the second laboratory experiment as well as for the field entrainment. In future field entrainments, it would be better not to include a glass insert and to fix the liner into the cut corner of the bag using several twists of wire. Nylon performed badly in the dynamic headspace experiment, and significant distortion of relative ratios occurred. This may have been caused by the relatively

voluminous nature of the bag as well as by the presence of a number of low-level background contaminants.

Spectrophotometric Properties

Photosynthetic available radiation roughly corresponds to what humans describe as visible light (400–700 nm), so if a material is transparent, it should not interfere with photosynthetic processes within a plant. The spectrophotometric study confirmed that little visible light was absorbed, and also showed that IR and UVA absorption was slight for all materials. UVA is not absorbed by the ozone layer and reaches the earth's surface, so translucence in this bandwidth by all materials was important. UVB is only partially absorbed by the ozone layer, and so would also reach plants growing naturally *in situ*. Glass (5-mm thickness) did absorb some UVB (39%), whereas the polyester bag (~12.5 µm thick) was an effective UVB screen (76%) due to the benzene rings in the polymer. However, such reduction of UVB is unlikely to unnaturally stress the plants because they grow well in greenhouses (reduced UVB) and are exposed to natural daily variations in UVB (e.g., clouds). UVC does not penetrate the atmosphere and does not reach the surface of the earth; therefore, strong absorption in this band range by all the materials is irrelevant. Overall, the polyester was optically more similar to the glass than was the nylon.

Temperature Effects

It was expected that enclosure would raise internal vessel temperatures by a “greenhouse effect.” When tested in an exposed area, internal temperatures of both the glass and polyester vessels increased, even when there was cloud cover. For glass and polyester, average increases of 7.5 and 5.2°C were calculated, and maximum differences above ambient were 9.5 and 7.5°C. From a temperature perspective, this smaller increase makes polyester marginally better than glass. The temperature effect was almost immediate such that within 10 min, the temperatures were elevated to levels that were typical for within the enclosure. These recorded internal temperatures, however, may have been lower than would occur during an actual headspace collection from plants because the vessels did not include the dark foliage of plants or water vapor resulting from evapotranspiration. Both these factors might further contribute to a warming effect. Indeed, under natural light in a greenhouse, workers have reported a glass vessel internal temperature to reach 38°C (Paré and Tumlinson, 1997). In contrast, under artificial lighting using bags, only 1°C increases have been reported (Zhang et al., 1999). This small increase in temperature was similar to the increases we recorded from outdoor entrainments in the shade (average increase of 0.9 and 0.6°C for glass and polyester, respectively). Whereas elevated temperatures might be advantageous in the short term due to increased release rates of volatiles from the plant, extended periods at elevated temperatures during entrainment might also initiate the release of stress-related compounds. Monoterpene emission rates have been reported to increase exponentially with temperature from a number of tree species (Dement et al., 1975; Kamiyama et al., 1978; Guenther et al., 1991; Tingey et al., 1991). However, others have reported a plateauing effect for all volatiles with little increase in emissions above 24–32°C, possibly caused by the closing of stomata (Zhang et al., 1999), and even a decrease in induced emissions beyond 27°C (Gouinguene and Turlings, 2002). Although field

entrainments are desirable, conducting them in full sun clearly creates problems. Higher flow rates relative to vessel volume may help, but we suggest shading with netting or an umbrella. Although artificial, this should avoid excessive heating of a plant. Such overheating problems should not affect entrainments conducted under forest canopy or in shadow.

Field Sampling

When volatiles were collected from aphid-infested leaves on an apple tree in the field, samples collected using polyester were not significantly different from those collected using the glass vessel. Although use of cut plants in the laboratory is a convenient way to isolate electrophysiologically/behaviorally active chemicals, it is important to confirm that such compounds are also released from unexcised plants growing in their natural habitat. Indeed, some authors have voiced concern over the commonplace extrapolation from controlled laboratory experiments, often on seedling crop plants, into wider ecological generalizations occurring in the field (van der Meijden and Klinkhamer, 2000). Furthermore, it is known that for a number of species, there is a great genotypic variation in the release of tritrophic defense volatiles (Loughrin et al., 1994; Turlings et al., 1998b), and some “silent” genotypes that do not release such volatiles have been identified (Halitschke et al., 2000). Examples reiterate the importance of field sampling, where the role of plant volatiles can be properly assessed in fitness terms and in the appropriate ecological/habitat context. Both qualitative and quantitative assessments under field conditions are required to determine whether, at these realistic levels, volatiles have significant plant fitness effects. Quantitation is particularly important because plants in the field may be stressed by a multitude of abiotic and biotic factors that may draw antagonistically on the same resource pool.

In summary, of the materials tested, both in the laboratory and in the field, we found polyester bags to perform well in dynamic headspace entrainments. Under dynamic entrainment conditions in the laboratory, polyester bags actually performed better than the borosilicate glass because of problems of adsorption onto the glass. Furthermore, polyester is optically similar to glass, but might be better than glass in the field because it created less of a greenhouse effect. The bags are cheap, easy to set up and use, and well suited for use in push–pull entrainment systems. We hope our results will encourage more widespread use of polyester bags for enclosing intact plants or plant parts during headspace sampling in the field. Such replicated *de vivo* and *in situ* sampling will contribute to the task of increasing our understanding of plant volatile chemistry and ecological function.

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Identification of Sex Pheromone Components of a New Zealand Geometrid Moth, the Common Forest Looper *Pseudocoremia suavis*, Reveals a Possible Species Complex

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Abstract Gas chromatography–electroantennographic detection analysis of sex pheromone gland extracts of the common forest looper *Pseudocoremia suavis* (Lepidoptera: Geometridae), a polyphagous defoliator of introduced Pinaceae and many New Zealand trees, revealed four compounds that elicited antennal responses. The two major active compounds (6Z)-*cis*-9,10-epoxynonadec-6-ene and (3Z,6Z)-*cis*-9,10-epoxynonadeca-3,6-diene were identified by comparison with known standards. Of the two minor active compounds, one was tentatively identified as (3Z,6Z)-*cis*-9,10-epoxyhenicosa-3,6-diene, whereas the other could not be identified because of insufficient amounts in extracts. (6Z)-*cis*-9,10-Epoxynonadec-6-ene, (3Z,6Z)-*cis*-9,10-epoxynonadeca-3,6-diene, and (3Z,6Z)-*cis*-9,10-epoxyhenicosa-3,6-diene were present in *P. suavis* gland extracts from Eyrewell Forest, a *Pinus radiata* plantation in the South Island of New Zealand, in a ratio of 35:65:5, respectively. Trapping trials in Eyrewell Forest established that (6Z)-*cis*-9,10-epoxynonadec-6-ene attracted male *P. suavis*. However, addition of (3Z,6Z)-*cis*-9,10-epoxyhenicosa-3,6-diene to the lure at <10% of (6Z)-*cis*-9,10-epoxynonadec-6-

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ene reduced capture of male moths, suggesting that one of its enantiomers was acting as a behavioral antagonist. During January–March of 2005, a blend trial involving single, binary, and ternary mixtures of the three components at Eyrewell Forest and at three other sites (two in the South Island and one in the North Island) revealed the existence of a second taxon of *P. suavis* at the three additional sites that was attracted to lures containing (3Z,6Z)-*cis*-9,10-epoxynonadeca-3,6-diene, either singly or in binary and ternary mixtures with (6Z)-*cis*-9,10-epoxynonadec-6-ene and (3Z,6Z)-*cis*-9,10-epoxyhenicosa-3,6-diene. This second taxon was not attracted to lures loaded solely with (6Z)-*cis*-9,10-epoxynonadec-6-ene.

Keywords Common forest looper · Species complex · *Pseudocoremia suavis* · *Pseudocoremia fenerata* · *Pseudocoremia leucelaea* · Geometridae · Ennominae · *Pinus radiata* · (6Z)-*cis*-9,10-epoxynonadec-6-ene · (3Z,6Z)-*cis*-9,10-epoxynonadeca-3,6-diene · (3Z,6Z)-*cis*-9,10-epoxyhenicosa-3,6-diene · Behavioral antagonist

Introduction

The common forest looper *Pseudocoremia suavis* (Butler) (Lepidoptera: Geometridae) is a polyphagous moth endemic to New Zealand, the larvae of which eat the leaves of a wide variety of native and exotic trees and shrubs, including southern beech (*Nothofagus* spp., Nothofagaceae), Podocarpaceae, and introduced Pinaceae (Dugdale, 1958; Stephens, 2001). In the 1950s and 1960s, *P. suavis* was responsible for major defoliation in *Pinus radiata* (D. Don) plantations at Eyrewell Forest (North Canterbury, South Island, NZ; White, 1974), and in the 1970s, it also caused considerable damage in Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] stands in Kaingaroa Forest (Bay of Plenty, North Island, NZ; Alma, 1977).

There are over 30 species of *Pseudocoremia* in New Zealand (Stephens, 2001). The genus is endemic to New Zealand and Norfolk Island, but has close affinities to geometrids from the Western Pacific (Dugdale, 1989). Both males and females of *P. suavis* are known to demonstrate developmental polymorphism, and the species appears not to have clearly synchronized generations (Berndt et al., 2004).

Our objective was to identify the sex pheromone of *P. suavis* as a tool for further study of its ecology and phenology, and for detection and delimitation surveys. A sex pheromone may also be useful for controlling future outbreaks by using mating disruption, lure and kill, or mass trapping technologies (e.g., Suckling and Karg, 2000).

To date, sex pheromones or attractants have been identified for more than 120 geometrids but none from the genus *Pseudocoremia* (El-Sayed, 2006). Typically, geometrids have sex pheromones consisting of single or multicomponent blends of unsaturated hydrocarbons and/or epoxides, with enantiomeric specificity often serving as a species-isolating mechanism (Mori, 1998; Millar, 2000; Ando et al., 2004). These types of compounds have also been identified from the pheromone glands of lymantriids, arctiids, noctuids, pyralids, and crambids (Ando et al., 2004; El-Sayed, 2006).

In this study, we describe the identification of two compounds in pheromone gland extracts of *P. suavis* from Eyrewell Forest, by gas chromatography–electro-

antennographic detection (GC-EAD) and gas chromatography–mass spectrometry (GC-MS) analysis, and confirm their biological activity with field bioassays. We also establish that *P. suavis* is likely to be a species complex with two distinct pheromone taxa that so far are only distinguishable by differential attraction to pheromone components and by geographic location. This is also the first identification of a sex pheromone of a New Zealand geometrid.

Methods and Materials

Insects

A colony of *P. suavis* was established at Forest Research (now Ensis), Christchurch, with material gathered from Eyrewell Forest, a pine plantation in North Canterbury (43.4170°S, 172.4235°E; see Berndt et al., 2004). Pupae were placed individually in plastic containers (Lily[®] 35-ml portion cups with lids, Huhtamaki Henderson Ltd., Auckland, NZ) at HortResearch, Lincoln, under a 12-hr light/12-hr dark reverse photophase lighting regime, at 22°C and 50% RH. Newly emerged female *P. suavis* were transferred to a humidified, 350-mm³ clear polycarbonate plastic box and provided with 10% sugar solution.

Pheromone Gland Extracts

The pheromone glands of 24- to 48-hr-old female moths were excised and placed into 20–30 µl of *n*-hexane (BDH Laboratory Supplies, Poole, UK), contained within a liquid-nitrogen-cooled 0.5-ml conical vial (Wheaton, Millville, NJ, USA). Gland extracts were taken from females 2–3 hr into the scotophase, when moths were actively calling. After all glands had been excised, the vial and its contents were brought to room temperature, and the liquid phase was transferred to a 1.1-ml conical glass vial (Alltech, Deerfield, IL, USA). The volume of extract was then reduced to 10 µl by using a stream of argon and was then stored at –18°C.

Gas Chromatography–Electroantennographic Detection

Pheromone gland extracts of *P. suavis* females were analyzed by GC-EAD with a Varian 3800 GC equipped with two columns of differing polarity, coupled to an EAD Recording Unit (Syntech, Hilversum, The Netherlands). Extracts were run on DB-5 (30 m × 0.25 mm ID × 0.5 µm film; Agilent Technologies, Palo Alto, CA, USA) and DB-Wax columns (30 m × 0.25 mm ID × 0.5 µm film; Agilent) with 1:1 split outlets. Helium was used as carrier gas (1 ml/min), and injections were in splitless mode. Injector and detector temperatures were 220 and 300°C, respectively, and the GC oven was programmed from 80°C/min to 240°C at 10°C/min, hold 35 min. An excised male *P. suavis* antenna was positioned between two glass electrodes containing BE Ringer's solution with 10% polyvinylpyrrolidone (molecular weight 360,000; Sigma Chemical Co., North Parramatta, NSW, Australia). Each glass electrode held a length of 1-mm silver wire to connect the preparation to the recording unit. The EAD exit port temperature was maintained at 200°C, and the antennal preparation was placed in a charcoal-filtered and humidified airstream (400 ml/min). Kováts retention indexes (KIs; Kováts, 1965; Marques et al., 2000) were

calculated for the compounds that elicited strong antennal responses to compare relative retention times.

Gas Chromatography–Mass Spectrometry

The retention times and mass spectra of the active compounds in a *P. suavis* pheromone gland extract [1 μ l = 5.8 FE (female equivalents/aliquot)] were compared with those of known standards on BPX-70 (30 m \times 0.25 mm ID \times 0.25 μ m film; SGE, Victoria, Australia) and VF-5ms (30 m \times 0.25 mm ID \times 0.25 μ m film; Varian Inc., Walnut Creek, CA, USA) columns, by using a Varian 3800 GC in splitless mode, coupled to a Varian 2200 MS. Helium was used as carrier gas (1 ml/min) with the transfer line at 250°C and the ion trap temperature at 200°C. The GC injector temperature was set at 220°C, and the oven was programmed from 80°C/min to 140°C at 10°C/min, hold 5 min, then to 200°C at 5°C/min, hold 25 min (BPX-70 column), or 80°C/min to 240°C at 10°C/min, hold 35 min (VF-5ms column).

Chemicals

(6*Z*)-*cis*-9,10-Epoxynonadec-6-ene (6*Z*-*cis*-9,10-epo-19Hy) and (3*Z*,6*Z*)-*cis*-9,10-epoxynonadeca-3,6-diene (3*Z*,6*Z*-*cis*-9,10-epo-19Hy) were synthesized at HortResearch, Palmerston North (see below). Samples of (3*Z*,6*Z*)-*cis*-9,10-epoxyhenicosa-3,6-diene (3*Z*,6*Z*-*cis*-9,10-epo-21Hy), (3*Z*,6*Z*)-9*S*,10*R*-epoxyhenicosa-3,6-diene, and (3*Z*,6*Z*)-9*R*,10*S*-epoxyhenicosa-3,6-diene were supplied by T. Ando, Tokyo University of Agriculture and Technology. (6*Z*)-9*S*,10*R*-Epoxyhenicos-6-ene (6*Z*-9*S*,10*R*-epo-21Hy) was available from previous work (El-Sayed et al., 2005).

Synthesis of Mono- and Diunsaturated Racemic Epoxides

6Z-cis-9,10-Epo-19Hy. 6*Z*-*cis*-9,10-Epo-19Hy was synthesized by the methods of Zhang et al. (1999) and Soulie and Lallemand (1995). The final step (reduction of the triple bond with Lindlar's catalyst) was carried out at 0°C, and the final product contained less than 2% of the *E* isomer. A small quantity of *E*-isomer-enriched (25%) final product was also produced for GC analysis by carrying out the same reaction at room temperature. 6*Z*-*cis*-9,10-epo-19Hy: ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 5.50 (1H, m), 5.38 (1H, m), 2.90 (2H, m), 2.35 (1H, dt, *J* = 13.4 Hz, 6.4 Hz), 2.15 (1H, dt, *J* = 13.4 Hz, 6.7 Hz), 2.01 (2H, q, *J* = 6.9 Hz), 1.50 (4H, m), 1.24 (18H, m), 0.86 (3H, t, *J* = 6.9 Hz), 0.85 (3H, t, *J* = 6.9 Hz). ¹³C NMR (CDCl₃, 100 MHz): δ (ppm) 132.71, 123.81, 57.24, 56.58, 31.90, 31.50, 29.58, 29.58, 29.53, 29.31, 29.25, 27.79, 27.42, 26.61, 26.22, 22.68, 22.57, 14.21, 14.06.

3*Z*,6*Z*-*cis*-9,10-Epo-19Hy

3*Z*,6*Z*-*cis*-9,10-Epo-19Hy and its regioisomers (3*Z*,9*Z*)-*cis*-6,7-epoxynonadeca-3,9-diene (3*Z*,9*Z*-*cis*-6,7-epo-19Hy) and (6*Z*,9*Z*)-*cis*-3,4-epoxynonadeca-6,9-diene (6*Z*,9*Z*-*cis*-3,4-epo-19Hy) were synthesized by the method of Ando et al. (1993). 3*Z*,6*Z*-*cis*-9,10-epo-19Hy: ¹H NMR (CDCl₃, 400 MHz): 5.43 (3H, m), 5.27 (1H, m), 2.91 (2H, m), 2.77 (2H, t, *J* = 7.0 Hz), 2.37 (1H, m), 2.19 (1H, m), 2.04 (2H, pent, *J* = 7.3 Hz), 1.5 (4H, m), 1.24 (12H, m), 0.94 (3H, t, *J* = 7.5 Hz), 0.85 (3H, t, *J* = 6.7 Hz).

^{13}C NMR (CDCl_3 , 100 MHz): 132.65, 131.18, 127.08, 124.63, 57.65, 56.86, 32.30, 29.98, 29.98, 29.94, 29.72, 28.19, 27.02, 26.65, 26.13, 23.10, 20.99, 14.67, 14.54.

Field Bioassays—Eyrewell Forest Trials 1–5

An initial series of bioassays (Trials 1–5) were carried out in mature stands of *P. radiata* in Eyrewell Forest, during December 2004 and January 2005. In all trials, either green delta traps (Clare et al., 2000) or green Unitrap (International Pheromone Systems Ltd., Cheshire, UK) bucket traps were suspended from rope strung between two pine trees at a height of 2 m, with >20-m spacing between treatments and >100-m spacing between replicates. Trap positions within each replicate were rerandomized, and sticky inserts were replaced at each check for capture of male *P. suavis*. Test compounds were loaded onto red rubber septa (Thomas Scientific Inc., Philadelphia, PA, USA).

- Trial 1. Trial 1 (December 7–14, 2004) investigated the attraction of *P. suavis* males to various combinations of the three candidate racemic epoxides, based on ratios established from analysis of *P. suavis* gland extracts by GC. Delta traps were checked four times at intervals of 1–2 d. Treatments were (1) blank control, (2) 100:0:0, (3) 0:100:0, (4) 35:65:0, and (5) 35:65:5 μg loadings of 6Z-*cis*-9,10-epo-19Hy, 3Z,6Z-*cis*-9,10-epo-19Hy, and 3Z,6Z-*cis*-9,10-epo-21Hy ($N = 5$).
- Trial 2. A dose response trial based on treatment 2 (6Z-*cis*-9,10-epo-19Hy) from trial 1 was set up from December 14 to 20, 2004. Delta traps were checked four times at intervals of 1–3 d. Treatments were (1) blank control, (2) 10, (3) 100, and (4) 1000 μg doses of 6Z-*cis*-9,10-epo-19Hy ($N = 4$).
- Trial 3. A third trial was undertaken from January 26 to February 14, 2005 to investigate whether the addition of 3Z,6Z-*cis*-9,10-epo-19Hy to lures inhibited capture of male *P. suavis*. Delta traps were checked six times at intervals of 3–4 d. Treatments were (1) blank control, (2) 100:0, (3) 99.35:0.65, (4) 93.5:6.5, (5) 35:65, and (6) 35:0 μg loadings of 6Z-*cis*-9,10-epo-19Hy and 3Z,6Z-*cis*-9,10-epo-19Hy ($N = 5$).
- Trial 4. During trials 2 and 3, on several occasions, traps captured >20 male moths in a 3- to 4-d period. Because of the relatively large size of *P. suavis* (wingspan of c. 30 mm), trap saturation was likely to be a problem. Therefore, in trial 4, we investigated nonsaturating bucket traps for capture of *P. suavis* males. We were also interested in establishing an alternative to the use of dichlorvos strips (Agrisense-BCS Ltd., Pontypridd, UK) as the insecticide “knockdown” component of this system because of availability and toxicity concerns. We compared trap catch of *P. suavis* males in delta traps with trap catch in bucket traps containing either (1) dichlorvos strips, (2) diazinon strips [20-mm strips of dog flea collar (Vitapet Corp. Ltd., Gracefield, NZ) with 15% diazinon as the active ingredient], or (3) 200 ml of water plus 5 ml of 200 Fluid 350 cs paraffin oil (Ajax Chemicals, Auckland, NZ).

Trial 4 ran from December 20 to 24, 2004 with traps checked daily between 0800 and 1000 hr ($N = 5$). Septa were loaded with 100 μg of 6Z-

cis-9,10-epo-19Hy. Bucket traps were opened inside a polyethylene bag to avoid the loss of live males. Numbers of live and dead moths in each bucket trap treatment and the delta trap treatment were recorded.

Trial 5. Trial 5 (December 24, 2004 to January 19, 2005) compared trap catch in delta traps and the bucket trap oil-and-water-system over a 4-wk period using lures loaded with 100 µg of 6*Z*-*cis*-9,10-epo-19Hy. Traps were checked every 7–10 d, rotating the positions of each trap type at each count date.

P. suavis Taxa Trial

To discover how widespread *P. suavis* was in exotic forestry blocks in the Canterbury region, we placed a replicate of trial 1 in a small south-facing, hillside forestry block at Cass Bay in Lyttelton Harbor, Banks Peninsula (43.5984°S, 172.6902°E), from December 26, 2004 to January 6, 2005.

A last trapping trial was set up in early 2005 (same treatments as trial 1) to investigate the possibility of *P. suavis* comprising more than one taxon, e.g., cryptic species. It was undertaken at three sites on the South Island in Canterbury: Eyrewell Forest (February 15–March 9, $N = 5$, traps checked five times at intervals of 3–5 d); Burnham Plantation, Kerrs Road, Burnham (43.5793°S, 172.3399°E; February 8–March 8, $N = 5$, traps checked five times at intervals of 3–4 d, except for the last occasion when traps were left for 15 d because of low catches during that period); Cass Bay (January 21–March 7, $N = 3$, traps checked six times at intervals of 5–7 d, except for the last occasion when traps were left for 12 d), and one site in the North Island [Forest Research/Ensis campus, Rotorua (38.1615°S, 176.2669°E); February 17–March 7, $N = 3$, traps checked five times at intervals of 3–4 d]. These trials were run concurrently to avoid temporal differences in response by *P. suavis* males to the candidate pheromone components (e.g., Steck et al., 1982; Szöcs et al., 1993). Green delta traps were used at all sites.

Statistical Analyses

Only treatments that trapped moths were included in the statistical analyses. For each trial, the sum of moths captured per trap was analyzed for treatment effects using analysis of variance (ANOVA; SAS Institute Inc., 1998). Residual plots were used to check the validity of the ANOVA assumptions, and $\log_{10}(x + 1)$ transformations of the summed captures were undertaken when necessary to remove variance heterogeneity. Means were compared by Fisher's protected least significant differences test ($P = 0.05$; SAS Institute Inc., 1998).

Results

Identification of Gland Components Eliciting Antennal Responses

Antennal responses were elicited by four compounds in gland extracts of *P. suavis* from Eyrewell Forest, only two of which (peaks 1 and 2, Fig. 1) gave peaks detectable by GC (KIs of 2052 and 2058, respectively, on the DB-5 column and 2341 and 2405, respectively, on the DB-Wax column). Male antennae were also screened

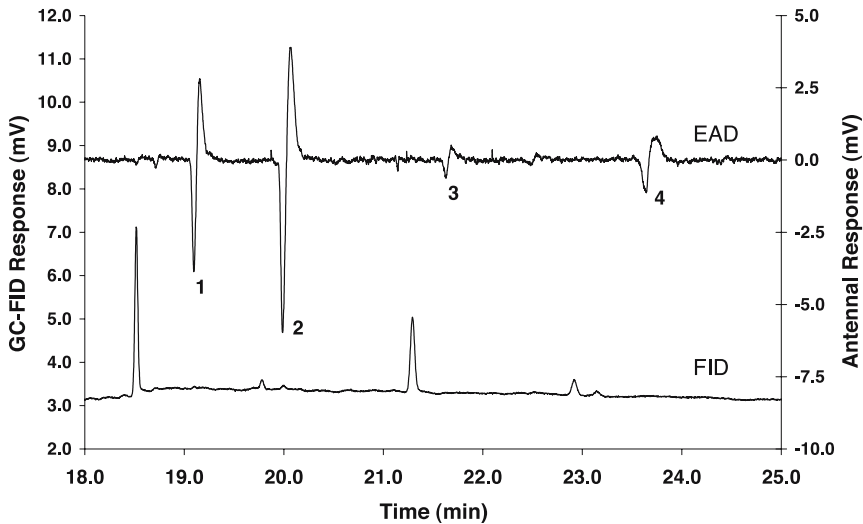


Fig. 1 Coupled gas chromatogram–electroantennogram of a *P. suavis* male antenna responding to compounds (**1**=6*Z*-*cis*-9,10-epo-19Hy, **2**=3*Z*,6*Z*-*cis*-9,10-epo-19Hy, **3**=unknown, and **4**=tentatively identified as 3*Z*,6*Z*-*cis*-9,10-epo-21Hy) in pheromone gland extracts, eluting from a DB-Wax column

with a range of synthetic standards of unsaturated hydrocarbons, epoxides, and ketones using GC-EAD analysis. From the standards tested, the diene monoepoxide 3*Z*,6*Z*-*cis*-9,10-epo-21Hy had retention times indistinguishable from those of compound **4** on both columns.

A search of the literature indicated that C19 trienes and the corresponding diunsaturated monoepoxides occur most frequently as sex attractants in the family Geometridae (El-Sayed, 2006), and comparison of the KIs for peaks **1** and **2** suggested that they might have differing degrees of unsaturation because their retention indices were relatively closer on the DB-5 column than the DB-Wax column. In addition, the relative retention times of compounds **2**, **3**, and **4** (Fig. 1) suggested that they could be a homologous series. Therefore, we hypothesized that compounds **1**–**4** were C19, C20, or C21 diene or triene alkenes, or the corresponding mono- and diunsaturated monoepoxides.

GC-MS analysis of 5.8 FE of *P. suavis* gland extract on the VF-5ms column revealed two closely eluting peaks, the mass spectra of which were consistent with those of a mono- and a diunsaturated monoepoxide, respectively (Ando et al., 1993, 1995; Millar, 2000). The spectrum of compound **1** had a base peak of m/z 67 [C_5H_7]⁺ with diagnostic ions at m/z (relative intensity) 71 (18) [C_5H_{11}]⁺, 81 (80) [C_6H_9]⁺, 97 (31) [C_7H_{13}]⁺, 110 (12) [C_8H_{14}]⁺, 124 (5) [C_9H_{16}]⁺, 153 (7) [$C_{10}H_{17}O$]⁺, 155 (5) [$M-C_9H_{17}$]⁺, 169 (2) [$M-C_8H_{15}$]⁺, 209 (7) [$M-C_5H_{11}$]⁺, 262 (1) [$M-H_2O$]⁺, and 280 (1) [M]⁺, and matched the spectrum of a 6*Z*-*cis*-9,10-epo-19Hy standard. The retention times of **1** and the 6*Z*-*cis*-9,10-epo-19Hy standard were the same on the VF-5ms and BPX-70 columns (KIs of 2054 and 2413, respectively), and 6*Z*-*cis*-9,10-epo-19Hy and 6*E*-*cis*-9,10-epo-19Hy were easily separated on the VF-5ms column (KIs of 2054 and 2067, respectively), confirming compound **1** as 6*Z*-*cis*-9,10-epo-19Hy.

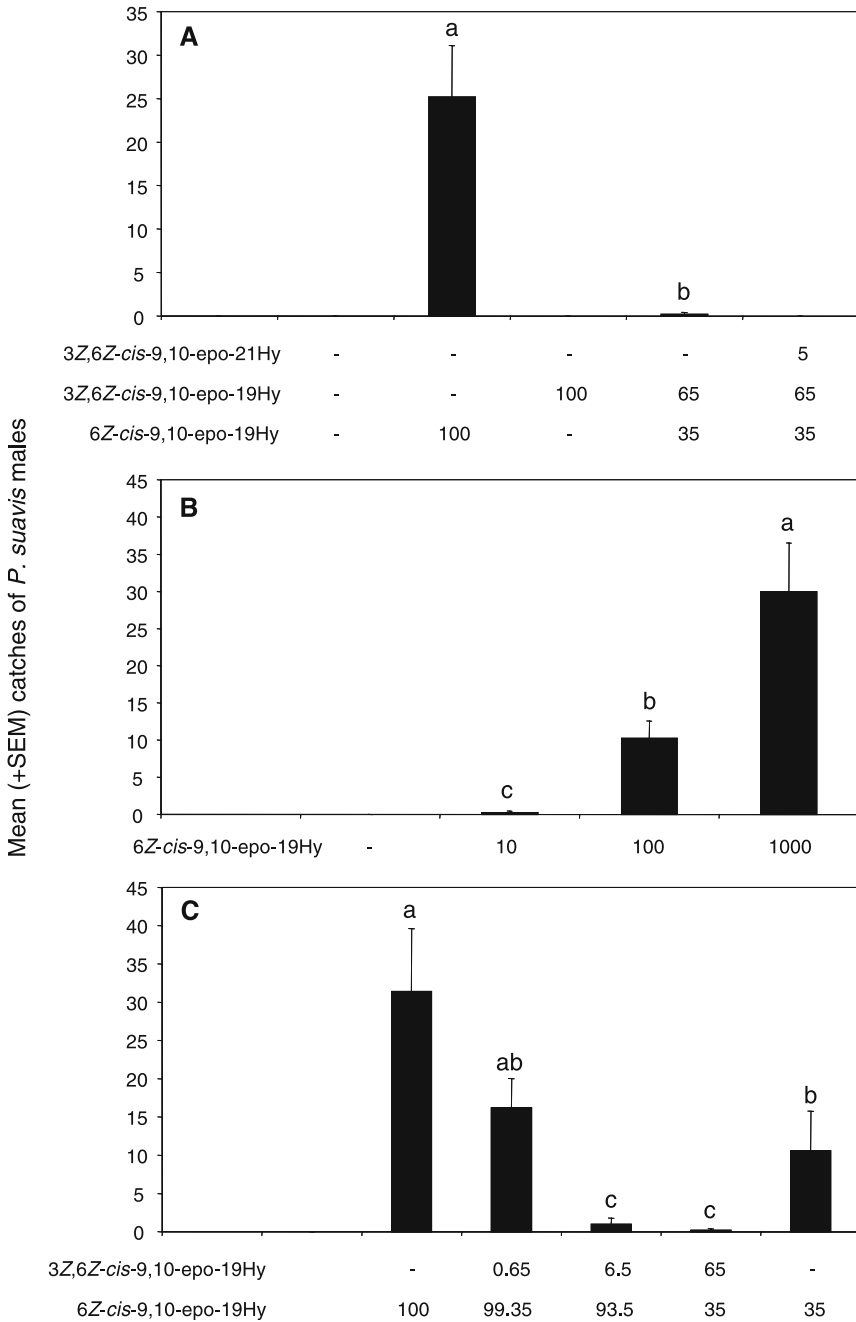


Fig. 2 Attraction of *P. suavis* males to lures consisting of (A) the three GC-EAD active components in *P. suavis* pheromone gland extracts, (B) various doses of 6Z-*cis*-9,10-epo-19Hy, and (C) addition of 3Z,6Z-*cis*-9,10-epo-19Hy to a lure containing 6Z-*cis*-9,10-epo-19Hy. All trials were undertaken at Eyrewell Forest, between December 2004 and January 2005. Amounts on the x-axis are micrograms per lure. Treatments labeled with the same letter are not significantly different ($P > 0.05$)

The spectrum of compound **2** had a base peak of m/z 79 $[C_6H_7]^+$ with diagnostic ions at m/z 108 (21) $[C_8H_{12}]^+$, 122 (9) $[C_9H_{14}]^+$, 209 (2) $[M-C_5H_9]^+$, 249 (4) $[M-C_2H_5]^+$, 260 (3) $[M-H_2O]^+$, and 278 (4) $[M]^+$, and matched the spectrum of authentic 3Z,6Z-*cis*-9,10-epo-19Hy. The retention times of compound **2** and the 3Z,6Z-*cis*-9,10-epo-19Hy standard were the same on the VF-5ms and BPX-70 columns (KIs of 2059 and 2486, respectively). 3Z,6Z-*cis*-9,10-epo-19Hy eluted later than its two regioisomers, 3Z,9Z-*cis*-6,7-epo-19Hy (KI 2050) and 6Z,9Z-*cis*-3,4-epo-19Hy (KI 2056) on the VF-5ms column, confirming compound **2** as 3Z,6Z-*cis*-9,10-epo-19Hy.

Whereas it was not possible to identify minor compound **3** from gland extracts because of the small amount present, we were able to tentatively identify compound **4** as 3Z,6Z-*cis*-9,10-epo-21Hy. The spectrum and retention time (KI 2261) of compound **4** were the same as those of 3Z,6Z-*cis*-9,10-epo-21Hy on the VF-5ms column, with a base peak of m/z 79 $[C_6H_7]^+$, and diagnostic ions at m/z 108 (45) $[C_8H_{12}]^+$, 122 (8) $[C_9H_{14}]^+$, 237 (5) $[M-C_5H_9]^+$, 277 (2) $[M-C_2H_5]^+$, 288 (1) $[M-H_2O]^+$, and 306 (1) $[M]^+$. In addition, 6Z-9S,10R-epo-21Hy eluted well before this peak (KI 2255). However, we were not able to categorically confirm the structure of compound **4** by GC-MS analysis because it coeluted with another compound in gland extracts on the BPX-70 column.

Field Bioassays

A total of 127 male *P. suavis* were captured in trial 1 at Eyrewell Forest over seven nights, and all of these moths, with the exception of 1 in treatment 4, were attracted by the single-component lure loaded with 100 μ g of 6Z-*cis*-9,10-epo-19Hy (Fig. 2A).

In a dose response trial (Fig. 2B), more male *P. suavis* moths were attracted to lures loaded with 1000 μ g of 6Z-*cis*-9,10-epo-19Hy ($P < 0.05$) than to those loaded

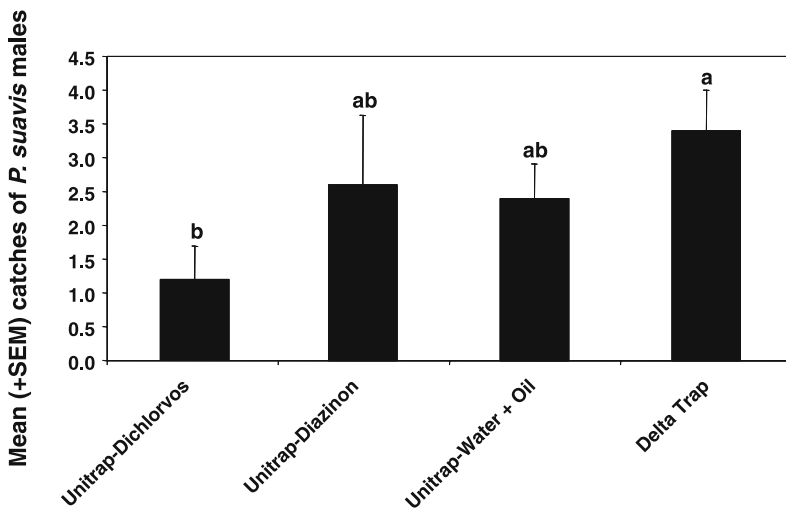
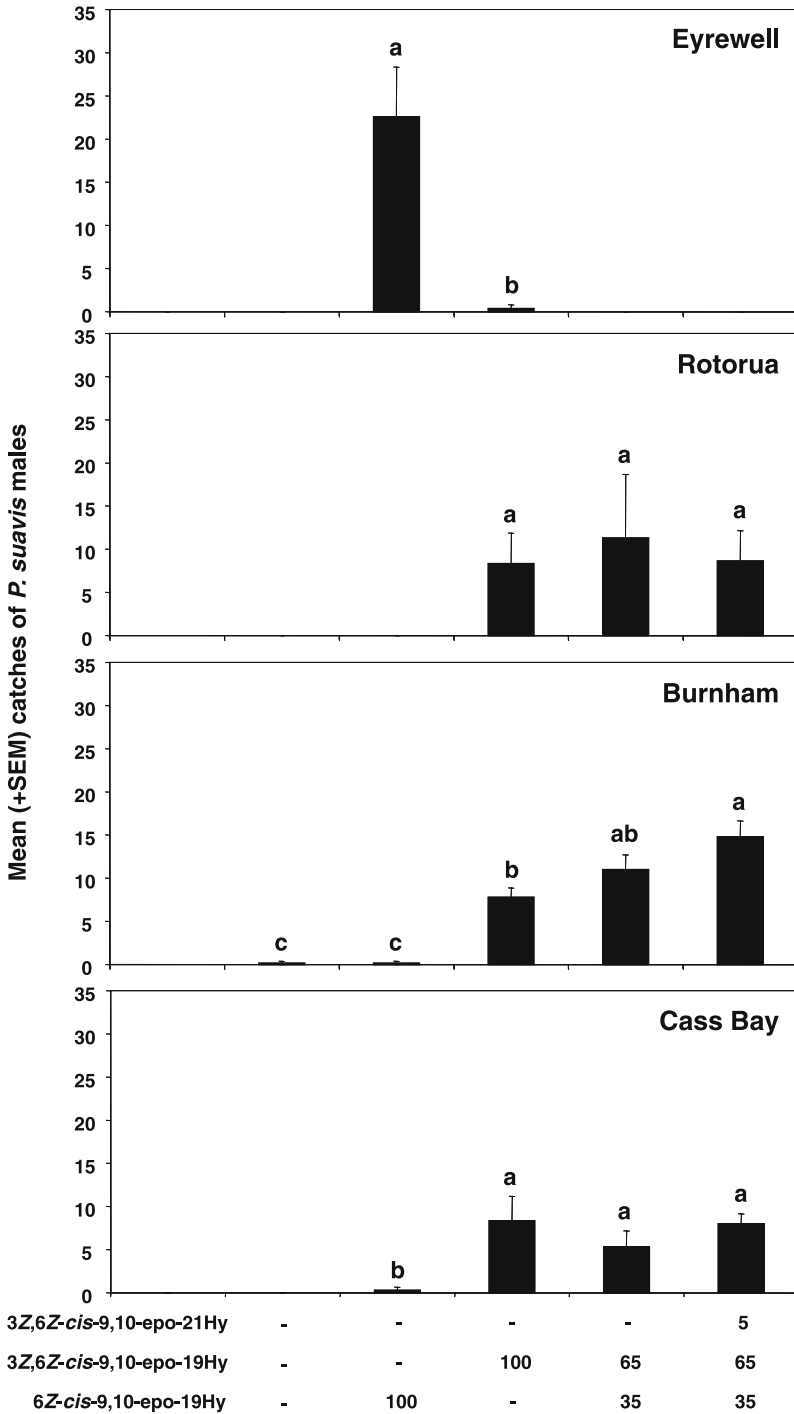


Fig. 3 Mean catches (\pm SEM) of *P. suavis* males in a trapping trial to test an alternative trapping system to delta traps for capturing male *P. suavis*. All treatments used a 100- μ g lure, loaded with 6Z-*cis*-9,10-epo-19Hy. The trial was carried out at Eyrewell Forest in December 2004. Treatments labeled with the same letter are not significantly different ($P > 0.05$)



with either 100 or 10 µg. One hundred sixty-two moths were captured over six nights, with a strong positive correlation between dose and number of moths captured ($r^2 = 0.84$, $df = 14$, $P < 0.001$).

In the third trial (Fig. 2C), we tested the effect of the addition of 3Z,6Z-*cis*-9,10-epo-19Hy to a lure containing 6Z-*cis*-9,10-epo-19Hy. A total of 297 moths were trapped during the 19 nights, and the results indicated that 3Z,6Z-*cis*-9,10-epo-19Hy was inhibitory. Addition of <10% of 3Z,6Z-*cis*-9,10-epo-19Hy significantly reduced trap capture of male moths ($P < 0.05$).

In the fourth and fifth trials at Eyrewell Forest, we tested an alternative trapping system for capturing male *P. suavis* because of the likelihood of saturation of delta traps. Forty-eight moths were captured during the four nights of trial 4, and the results (Fig. 3) indicated that bucket traps, with either a diazinon strip or with water and oil, were as effective as the delta trap ($P > 0.05$). Bucket traps with a dichlorvos pesticide strip trapped fewer male moths ($P < 0.05$) than did the delta traps, suggesting a repellent effect from the dichlorvos strip.

In the bucket-trap diazinon strip trapping system, all moths were alive (13 moths trapped in total) when the traps were cleared the following morning, whereas only 1 moth was alive in the bucket-trap dichlorvos strip system (6 moths trapped), indicative of the relative vapor pressures of these two insecticides (1.4×10^{-4} and 1.2×10^{-2} mm Hg at 20°C, respectively; Windholz, 1983). In the bucket-trap water–oil system, 12 moths were trapped, and all of these were immobilized in the water–oil solution. In trial 5, we trapped 193 moths over a 4-wk period, and there was no difference ($P > 0.05$, data not shown) in mean catches of *P. suavis* males, using either delta traps or the bucket trap water–oil system.

P. suavis Taxa Trial

Trap catches indicated the presence of *P. suavis* in a forestry block in Cass Bay during late December 2004 and early January 2005. However, male moths were not attracted to treatment 2 (100 µg of 6Z-*cis*-9,10-epo-19Hy), but instead to treatments 3–5 (a total of 12 moths trapped over 11 nights). A repeat of trial 1 at four forestry sites (Fig. 4) confirmed the existence of two distinct taxa of *P. suavis*, based on attraction of male moths to the racemates of the two major pheromone components identified from gland extracts of female moths from Eyrewell Forest. A preliminary morphological investigation of the male genitalia of *P. suavis* has revealed no consistent differences between moths from Eyrewell Forest and moths from Rotorua, Burnham, or Cass Bay (Stephens, personal communication).

At the Eyrewell site, only 2 of the moths captured (115 moths trapped in total) were attracted to lures other than 6Z-*cis*-9,10-epo-19Hy (treatment 2), consistent with trial 1. However, at Rotorua (85 moths trapped), Burnham (170 moths trapped), and Cass Bay (66 moths trapped), the majority of *P. suavis* males were attracted to treatments 3–5, with only 3 being attracted to other treatments (1 moth in treatment 2 at Burnham and Cass Bay, and 1 moth in the blank control at Burnham).

Fig. 4 Mean catches (\pm SEM) of *P. suavis* males in a blend trial of the three components in *P. suavis* pheromone gland extracts that elicited EAD responses, at four locations in New Zealand. Trials were undertaken between January 2005 and March 2005. Amounts on the x-axis are micrograms per lure, and treatments labeled with the same letter are not significantly different ($P > 0.05$)

At the Burnham site, the three-component lure of 6*Z*-*cis*-9,10-epo-19Hy, 3*Z*,6*Z*-*cis*-9,10-epo-19Hy, and 3*Z*,6*Z*-*cis*-9,10-epo-21Hy trapped more moths ($P < 0.05$) than the single component 3*Z*,6*Z*-*cis*-9,10-epo-19Hy lure, suggesting that 3*Z*,6*Z*-*cis*-9,10-epo-21Hy might be a component of the sex pheromone of this population of *P. suavis*. However, in an earlier trial at Eyrewell Forest in March of 2003, 100- μ g doses of 3*Z*,6*Z*-*cis*-9,10-epo-21Hy or its enantiomers in single-component lures failed to attract any moths (Brockerhoff, unpublished data), indicating that this diunsaturated monoepoxide is not active as a single component.

We also attracted small numbers of two other closely related *Pseudocoremia* spp. (Stephens, 2001) to 6*Z*-*cis*-9,10-epo-19Hy lures over the course of the trial at the Rotorua site [16 *Pseudocoremia fenerata* (Felder and Rogenhofer) males during February and March], the Burnham site [1 *Pseudocoremia leucelaea* (Meyrick) male in March], and at Cass Bay (3 *P. leucelaea* males in February). Similarly, a trap baited with 6*Z*-*cis*-9,10-epo-19Hy in a *P. radiata* block at Whataroa (43.1167°S, 170.3085°E), South-Westland, in early January 2005 captured 2 *P. leucelaea* males but no *P. suavis*. At Cass Bay, *P. productata* (Walker) adults were observed in the *P. radiata* plantation on numerous occasions during January and February 2005, but no males of this species were ever captured in baited traps.

Discussion

We have identified 6*Z*-*cis*-9,10-epo-19Hy and 3*Z*,6*Z*-*cis*-9,10-epo-19Hy from pheromone gland extracts of the common forest looper *P. suavis*. We have also tentatively identified the homologous diene monoepoxide 3*Z*,6*Z*-*cis*-9,10-epo-21Hy in gland extracts. All of these compounds have previously been found as either sex pheromone components or attractants in other geometrids of the subfamily Ennominae (El-Sayed, 2006).

At Eyrewell Forest, male *P. suavis* moths were attracted to 6*Z*-*cis*-9,10-epo-19Hy as a single component. Because we used the racemate in our trapping trials, the active attractant may be one of the enantiomers or some mixture of the two enantiomers. For example, Millar et al. (1991) found that racemic 6*Z*-*cis*-9,10-epo-19Hy was attractive to the geometrid *Euchlaena madusaria*, and the individual enantiomers failed to attract moths of this species, whereas *Xanthotype sospeta* (a sympatric species) was selectively attracted by 6*Z*-9*S*,10*R*-epo-19Hy and 6*Z*-9*R*,10*S*-epo-19Hy antagonized attraction. Therefore, removal of one of the enantiomers from the blend may enhance, reduce, or eliminate attraction of male *P. suavis* at the Eyrewell site.

Addition of 3*Z*,6*Z*-*cis*-9,10-epo-19Hy to the lure inhibited capture of moths at Eyrewell Forest. Because 3*Z*,6*Z*-*cis*-9,10-epo-19Hy was identified in gland extracts from female moths from Eyrewell Forest, this observed inhibitory effect was possibly due either to one of the enantiomers in the racemic mix or to an incorrect blend of the two enantiomers. Thus, determination of the natural enantiomer of 3*Z*,6*Z*-*cis*-9,10-epo-19Hy or blend from gland extracts may aid in developing a better lure for *P. suavis* males at Eyrewell Forest.

Both 6*Z*-*cis*-9,10-epo-19Hy and 3*Z*,6*Z*-*cis*-9,10-epo-19Hy attracted male moths but not at the same location. Therefore, *P. suavis* is likely to be a species complex comprising two distinct pheromone taxa. This finding will be verified by further trapping trials using the enantiomers of the monoepoxides identified in gland ex-

tracts of *P. suavis* from Eyrewell Forest. In addition, an investigation to determine subtle differences in morphology of genitalia for this species complex and DNA analysis of *P. suavis* from the various locations is underway. There are other examples in the New Zealand Lepidoptera where identification of sex pheromone components has enabled cryptic species to be recognized. For example, Foster et al. (1991) investigated the sex pheromones of distinct geographic populations of various tortricid species in the genera *Planotortrix* and *Ctenopseustis*, and Frérot and Foster (1991) elucidated two distinct pheromone taxa within the noctuid *Graphania mutans*.

A cladistic analysis by Stephens (2001) indicated that *P. suavis* belongs to the clade containing *P. leucelaea*, *P. fenerata*, and *P. monacha*. Our study of the sex pheromone of *P. suavis* supports this finding. We captured both *P. fenerata* and *P. leucelaea* in our field trials, in addition to *P. suavis*. Both *P. suavis* and *P. fenerata* have been recorded as exotic forestry pests (Alma, 1977; Nuttall, 1983), and *P. leucelaea* also feeds on exotic pines (Dugdale, personal communication). Whereas *P. monacha*, like its host genus *Phyllocladus* spp., is restricted in its distribution, being characteristic of upland or montane forest sites (Dugdale, personal communication), the other three species are widespread throughout New Zealand.

At the Rotorua site, *P. fenerata* males were attracted to 6Z-*cis*-9,10-epo-19Hy, and whereas *P. leucelaea* is also present (Alma, unpublished data), we did not trap any of this species during our trial. However, *P. leucelaea* were attracted in low numbers to 6Z-*cis*-9,10-epo-19Hy at three sites in the South Island. Further trapping trials, using enantiomers of the two major pheromone components identified from *P. suavis* gland extracts, could be useful in establishing species-isolating mechanisms among these three sympatric species.

Of the trapping systems tested for capture of *P. suavis*, bucket traps with water and oil were most appropriate for long-term studies. However, it may be possible to further refine the trapping system because *P. suavis* males were caught in reasonable numbers in bucket traps with neither an insecticidal strip nor water and oil (Gibb, unpublished data). Such traps would be useful for collection of live specimens, for example, for GC-EAD or DNA analyses.

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Individual, Sexual, Seasonal, and Temporal Variation in the Amount of Sagebrush Lizard Scent Marks

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Abstract Although many animals deposit scent marks, previous studies have focused almost entirely on rodents or on the chemical structure of the signal. Here, we study the quantity and temporal pattern of chemical deposition by the territorial sagebrush lizard *Sceloporus graciosus*, measuring both femoral pore and fecal deposits. Specifically, we tested whether variation in deposition is a good cue of individual and sexual identity and/or whether it is more closely associated with body size and reproductive state, indicators of physiological condition. The results support the latter hypothesis. We found that although the amount of fluid deposited on a single perch (rarely quantified in mammals) carries little information on individual or sexual identity, it reflects the physiological condition and reproductive state of individual lizards and is replenished on a roughly weekly cycle, potentially providing additional information on the producer's activity level. The amount of deposition may thus provide important information to chemical receivers making mate choice and territorial defense decisions. The results further suggest that seasonal increases in gland production allow lizards to mark more sites rather than to influence the quality of the signal on a single perch.

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Introduction

In many animals, the behavior of scent marking, including spatial and temporal patterns of deposition, can supplement a chemical signal with information about the producer's activity, physiology, or motivational state. Many animals leave chemical signatures passively wherever they go, offering a receiver information about their general activity (see review by Wyatt, 2003). Others modify the quantity and quality of chemical signals by changing their position with respect to the wind, by wing fanning, or by urinating, defecating, or rubbing against a substrate. For example, voles scent-mark in response to chemical signals left by other animals (Wolff et al., 2002; Mech et al., 2003). In rodents, there are considerable individual and seasonal differences in the frequency of scent marking (Nevison et al., 2003; Ferkin et al., 2004), such that the deposition of chemical signals also contains information on the producer's individual identity and reproductive state. Despite major recent advances in determining the structural composition and functional use of chemical signals, little is known about the quantity and temporal pattern of chemical deposits in nonrodent taxa (Wyatt, 2003). In this study, we document patterns of sexual, seasonal, and individual variation in chemical deposition by *Sceloporus graciosus*, the sagebrush lizard, and test hypotheses to determine whether the quantity of deposits carries information on the individual and sexual identity of resident animals, or whether it better reflects physiological condition.

The chemical structures of lizard secretions convey information on the individual, sex, population, and species identity of producing animals (Alberts et al., 1993; Escobar et al., 2001). As reviewed by Mason (1992) and Halpern (1992), most lizards produce chemical secretions in skin or specialized glands and have highly developed chemical discrimination abilities. Many species can use olfaction to distinguish the sex (Labra and Niemeyer, 1999; López and Martín, 2001), social status (Alberts et al., 1994; Molina-Borja et al., 1998), and body size (Aragón et al., 2000) of conspecifics. They also can use chemical cues to identify preferred prey (Cooper and Habegger, 2000) and to avoid predation (Downes, 2002). An impressive diversity of lizard species can distinguish familiar from unfamiliar conspecifics on the basis of chemical cues (Aragón et al., 2001, 2003; Labra et al., 2001; Bull and Lindle, 2002).

The quantity of chemical produced by individual lizards also exhibits intraspecific variation and may be used to transfer information on physiological or reproductive condition as well as identity. As reviewed by Mason (1992), chemical-secreting glands are usually larger and more active in breeding males than in females or juvenile animals. For example, male green iguanas, *Iguana iguana*, maximize production of femoral pore secretions during breeding months, and pore size is associated with plasma levels of testosterone and visual display behavior (Alberts et al., 1992). Testosterone implants cause female whiptail lizards (*Cnemidophorus uniparens*) to exhibit the large, active femoral pores typical of males (Wennstrom and Crews, 1998; Crews et al., 2004), and juvenile tree lizards (*Urosaurus ornatus*) treated with testosterone develop larger femoral pores (Hews et al., 1994).

In contrast, the deposition of lizard chemical signals has received minimal study, making it difficult to determine whether the frequency of scent marking is an

important element of the chemical signal that may also evolve via natural and sexual selection. Here, we monitored lizard scent marks on a primary perch for 35 consecutive days before and 21 d after a hibernation period. We then used these data to determine whether the amount of secretion is better explained by individual and sexual differences, and/or whether they are better explained by general physiological (as reflected by body size and time in captivity) and reproductive condition (as reflected by season). Because these hypotheses are not mutually exclusive, we used multiple regression analyses to compare the relative importance of each factor. We further tested for the importance of individual as opposed to physiological differences by comparing the amount of secretion produced by the same lizards in different seasons. Finally, we examined the time series of chemical deposition to determine how often scent marks were replenished. Although we focused on femoral pore secretion deposits, we also measured fecal spots, which may also carry chemical information (see Mason, 1992).

Methods and Materials

Animals and Housing

Sagebrush lizards are small, insectivorous lizards found throughout the western US. In the field, both males and females are active from mid-March to September. Males are especially territorial in May and June (during the mating season), whereas both sexes defend territories aggressively from July through September (Martins, 1991, 1993). We used 82 males and 49 females that were captured near Wrightwood in southern California (34°23'30"N, 117°42'W) in two separate collections (May 2002 and 2003) and established in separate rooms of our laboratory colony at Indiana University. We used only adult animals. Males were slightly larger than females in both snout-to-vent-length [SVL; male = 5.8 ± 0.04 cm (mean \pm SE); female = 5.7 ± 0.04 cm] and weight (male = 8.8 ± 0.17 g; female = 8.1 ± 0.14 g).

Lizards were kept under standard housing, lighting, feeding, and hibernation conditions. For most of the year, individuals were housed in 5-gal aquaria, visually isolated from their immediate neighbors by opaque cardboard barriers, but able to see animals on other cage racks across the room (2–3 m away). Each aquarium was equipped with sand substrate, a water dish, and a split-level brick perch (5.5 cm wide \times 21.5 cm long \times 6.5/11 cm tall). Each row of cages was illuminated on a 12-hr light cycle by two fluorescent light bulbs stretched across the row and by a single 40-W incandescent bulb hung directly over the highest level of the brick perch on one side of each cage. Although air temperature outside the cages was held at a constant 28°C, the brick perch warmed to as much as 4°C higher than other parts of the cage and was used frequently by active animals. Lizards were watered and misted daily and were fed three times each week with *ad lib* mealworms and vitamin-enriched crickets. Once every 2 wk, large fecal material was removed. From June to September, animals were placed in groups of two to eight animals in 3 \times 3 m outdoor enclosures (prior to experiments). In November–January, animals were allowed to hibernate in individual cloth bags at 11°C (during the experimental period; see next section).

One room housed animals (35 males and 24 females) that had been in captivity for more than 1 yr, having been captured in May 2002 and undergoing one period in the outdoor enclosures and one hibernation period before entering the experiment in

2003. The second room housed animals that were brought into the lab in May 2003 but did not spend time in the outdoor enclosures or undergo hibernation before beginning the experiment. Lizards increase in SVL continuously as adults, such that body size and weight are reasonable indicators of physiological condition. Lizards grew quickly in the outdoor enclosures, such that animals that had been in captivity for longer were also slightly larger (SVL = 5.8 ± 0.04 cm compared to SVL = 5.7 ± 0.04 cm).

Chemical Sampling

Although lizards move and potentially deposit secretions throughout their cages, we measured only secretions deposited on the split-level brick, a preferred basking perch. In the field, *Sceloporus* lizards defend several preferred basking perches, leaving the intervening space undefended such that there is considerable home range overlap between individuals (Sheldahl and Martins, 2000). We limited the current study to deposits on a single preferred perch to ensure that our efforts addressed territorial use of chemical deposits rather than general activity. We used white marking tape to attach white paper towels to the top surface of the single brick in each cage. We changed the paper towels daily so that spots could be counted and measured. Used towels were placed under a black light, which makes the femoral pore secretion spots (FPSs; which are UV-reflective, Alberts, 1993) visible to a human observer. Fecal spots are not UV-reflective, and hence were easily distinguished from femoral pore spots. We counted and measured the diameter (to the nearest 0.5 mm) of each femoral pore and fecal spot, using the radii (r) to estimate total area of spots for each lizard on each day (area = $\sum \pi r^2$, where the sum is across all femoral pore or fecal spots produced by one lizard in 1 d).

Data were collected daily from 13 October to 16 November 2003 (35-d prehibernation sequence) and then again from 26 January to 15 February 2004 (21-d posthibernation sequence). During the intervening period, animals were measured, weighed, and allowed to hibernate. Data were not collected 1 wk before and 1 wk after hibernation. In captivity, sagebrush lizards will remain active for 10 or more months of the year, with females readily laying two successive clutches of eggs. The prehibernation period of our study corresponds with a period of territorial aggression in which animals of both sexes actively exclude conspecifics of both sexes from their preferred feeding and basking sites. In contrast, the posthibernation period corresponds to the early mating season when both males and females were at peak activity. During the mating season, males extend their territories into larger areas, each of which encompasses the smaller home ranges of several females, whereas females reduce aggressive territorial behavior. Territorial aggression is stronger in males than in females, and this sexual difference is more pronounced during the mating season than afterwards (Martins, 1993). Thus, if femoral pore or fecal spots are used in territorial defense, we also might expect them to be larger or more abundant in males than in females, especially during the mating season.

Statistical Analyses

We began by using generalized linear mixed models [GLMMs, implemented using the GLIMMIX procedure of SAS (2005)] to estimate the relative importance of identity (individual and sex) and condition (season, body size, and time in captivity) in explaining variation in femoral pore spot deposition. GLMMs are regression-type

models that rely on a family of exponential distributions instead of the usual normal distribution to describe the response variable (in our case, spot number or size). The SAS GLMMIX procedure chooses from among discrete (e.g., Poisson, negative binomial) and continuous (e.g., normal, gamma) exponential distributions using likelihood statistics to find the best fit to the data.

We fitted two GLMMs (one model for spot number and a second model for spot area) to describe the effects of various factors on variation in femoral pore spot deposition. The factors included individual identity (codes for 128 animals), sex (male or female), body size (SVL and weight in grams, as measured just before hibernation), and time in captivity (0 or 1 to distinguish the two collection periods corresponding to 5 or 17 mo in captivity) as potentially relevant predictors. We also included a factor for fecal deposition (fecal spot number and area) for comparison. We report results from analyses using type III sums of squares, which estimate effects for each factor while taking all other variables into account. Residual analyses confirmed that we did not violate the homoscedasticity assumptions of these models.

As a second test of the same hypothesis, we estimated Pearson product moment correlations between daily averages of spot number and area, calculated for each lizard before and after hibernation. Strong correlations indicate the importance of individual identity in determining deposition quantity. Although the counts and areas were not normally distributed, average spot number for a single lizard across several days was close to normal. For comparison, we did the same calculations for both femoral pore and fecal spots and estimated also correlations between femoral pore and fecal spots.

Finally, we examined the temporal pattern of spot deposition. First, we assumed that lizards were temporally synchronized, averaging spot number across all lizards on each day of our sample. This is a reasonable expectation because all animals were kept on identical environmental, husbandry, and feeding regimes for at least 5 mo prior to and during data collection. Again, although the counts are not normally distributed, the averages across all of our lizards on a single day were well simulated by a normal distribution. We used these averages to fit time series models, forecasting across a single series of 56 d (35 d before hibernation + 21 d afterwards) to maximize our predictive ability.

Results

Although all lizards produced FPS on at least 1 d in the 35-d sequence before hibernation, 10 of 82 males and 5 of 49 females did not contribute to the 21-d posthibernation data set. The number of FPS deposited onto the paper towels ranged between 0 and 11 per day (Fig. 1A). Most lizards deposited spots on the brick perch on fewer than 30% of the days in our study. Femoral pore spots varied considerably in size, with most spots being less than 3 mm² in area, but a few ranging as high as 18 mm² (Fig. 1B). Distributions of fecal spot numbers were similar, but less dispersed, with fewer lizards exhibiting the rare large numbers of spots observed for femoral pore secretions (Fig. 1A). Fecal spots were sometimes larger, ranging up to 40 mm² in area (Fig. 1B).

The number and area of femoral pore spots were explained better by physiological condition than by individual identity or sex. Counts of femoral pore

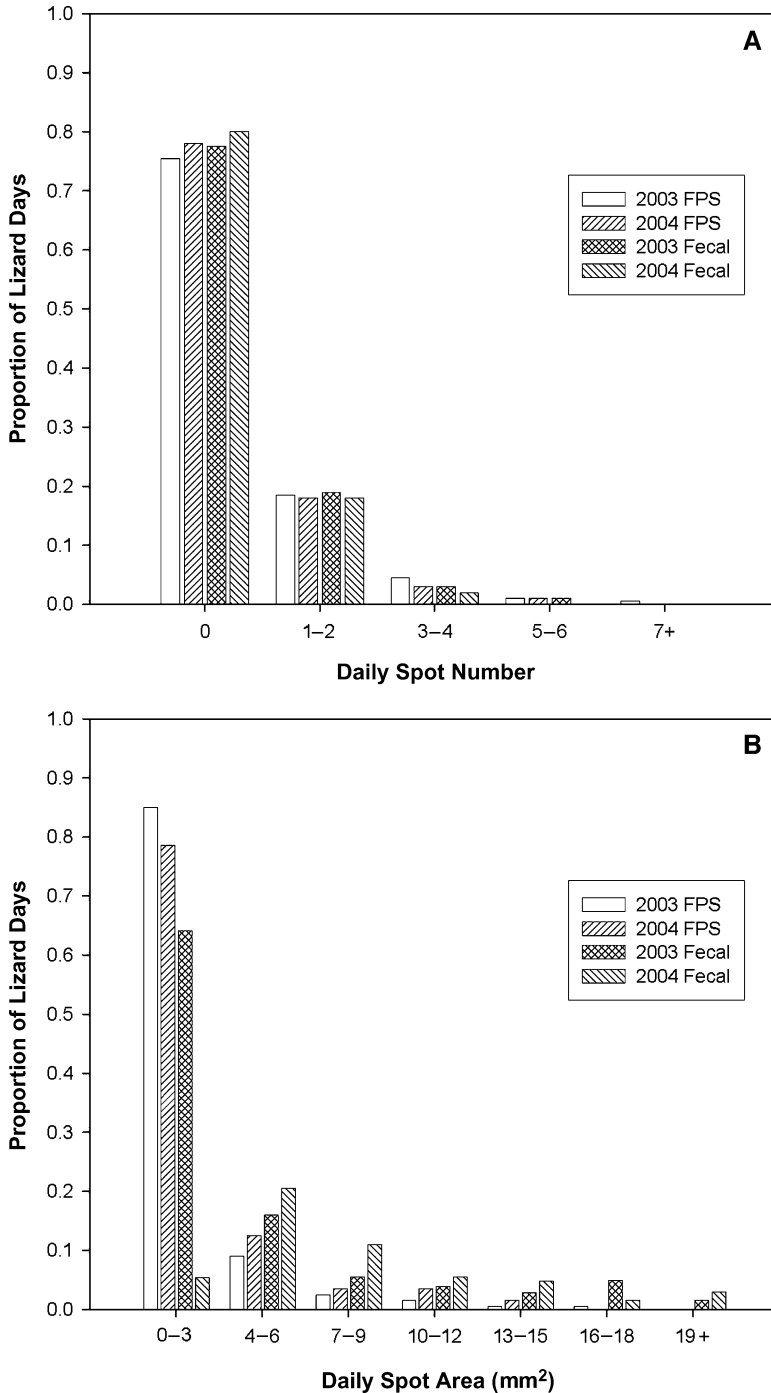


Fig. 1 Distribution of the number (A) and size (B, area in mm²) of spots produced each day, combining data from both sexes and across the full 56 d of our experiment. 2003: Prehibernation, territorial season. 2004: Posthibernation, mating season

Table 1 *F* Values and predicted effect size estimated by general linear mixed models of femoral pore secretion spot (FPS) number and size (area in mm²)

Factor	Number ^a	Predicted effect (%)	FPS size ^b	Predicted effect
Season ^c	16.18*	−20	16.24*	0.16
Sex ^c	0.43	−2	2.00	0.08
Captivity ^c	10.41*	−23	111.15*	0.54
Length (mm)	7.19*	−33	0.40	−0.15
Weight (g)	2.37	7	0.52	0.03
Fecal spot no.	15.75*	14	1.91	0.04
Fecal spot area	5.89	1	0.06	0.00

^a *df* = 6993, distribution = negative binomial, link function = log.

^b *df* = 1643, distribution = lognormal, link function = identity.

^c Prehibernation territorial season = 0, mating season = 1; males = 0, females = 1; 17 mo in captivity = 0, 5 mo in captivity = 1.

**P* < 0.01.

spots were too widely dispersed to be described well by normal or Poisson distributions, but a GLMM with a negative binomial distribution and a log-link function provided a good fit to spot counts. Femoral pore spot area was described better by a lognormal distribution. There was a seasonal effect on both FPS number and area (Table 1). Lizards deposited about 20% fewer FPS during the mating season than during the prehibernation territorial season and increased FPS area by about 0.16 mm². Moreover, lizards with longer snout-vent lengths produced fewer, but not smaller, spots (Table 1). Although weight was not a good predictor of either response variable, time in captivity appeared as a related and highly significant predictor, with animals that had spent more than a year in captivity producing fewer but larger FPS than did animals that had been in captivity for only 5 mo. Lizards that had been in captivity for more than 1 yr were also slightly larger (*F* = 4.1, *df* = 1,121, *P* < 0.05 for SVL, including sex also in the model) and considerably heavier (*F* = 13.6, *df* = 1,121, *P* < 0.001) than animals that had been in the lab for a shorter

Table 2 Pearson product-moment correlations between number and size (mm²) of spots in the same (repeatability) and different years

	FPS size	Fecal no.	Fecal size
2003 (prehibernation territorial period)			
FPS no.	0.30*	0.18**	−0.14
FPS area		0.26*	0.44*
Fecal no.			0.41*
2004 (mating season)			
FPS no.	0.63*	0.58*	0.36*
FPS area		0.34*	0.45*
Fecal no.			0.53*
Repeatability (2003 to 2004)			
FPS no.	0.08		
FPS area	0.16		
Fecal no.	0.08		
Fecal area	0.39*		

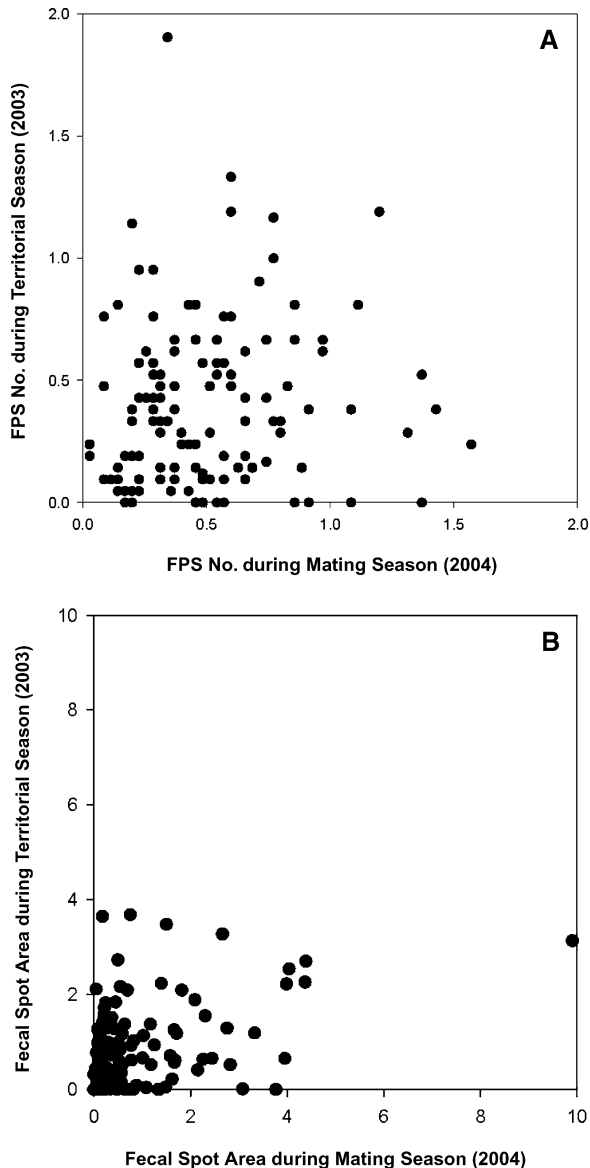
**P* < 0.01.

***P* < 0.05.

period. In using type III SS to estimate GLMM parameters (Table 2), we confirmed that time in captivity was an important factor even after differences in body size had been taken into account. Surprisingly, sexual differences were small and did not contribute to variation in FPS number or area, despite visibly larger femoral pores in males, especially during the mating season. Animals that produced more fecal spots also produced more FPS, but size of fecal spots was not related to FPS number or size.

In our second test, individual lizards that produced more or larger spots during the territorial season did not tend to be the same animals that produced more or

Fig. 2 (A) Relationship between number of femoral pore secretion spots (FPS) in territorial and mating seasons ($r = 0.08$, $P > 0.05$). Size of FPS and number of fecal spots give very similar patterns (Table 2). (B) Relationship between size of fecal spots in the two seasons is statistically significant ($r = 0.4$, $P < 0.05$), but probably because of a single influential outlier (Table 2)



larger spots during the mating season (Table 2, Fig. 2A). Only the area of fecal spots showed a positive relationship between seasons, and this relationship seems to be driven by outliers (Fig. 2B). For the most part, measures of femoral pore and fecal spot deposition were positively associated with each other within but not across seasons (Table 2). The number of FPS was only loosely associated with fecal spots during the

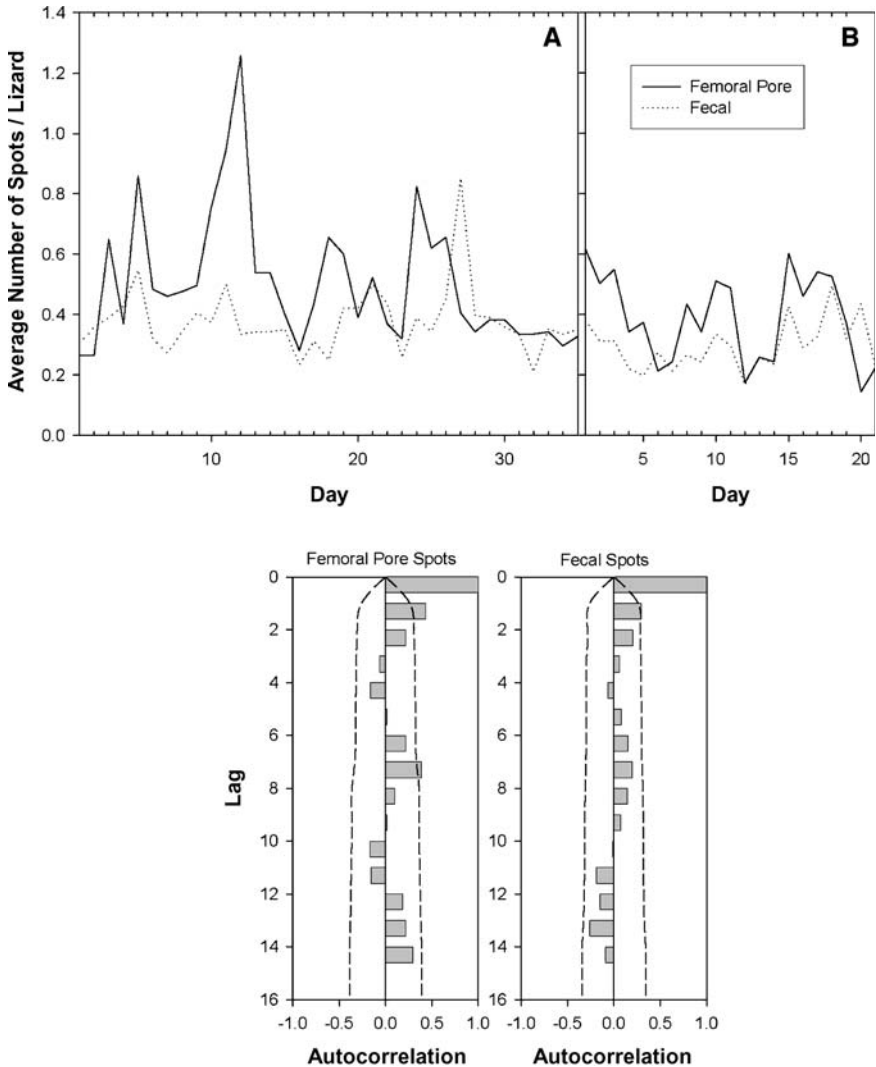


Fig. 3 Mean number of spots produced by lizards on each day of our study, averaging data for all male and female lizards. The first time sequence (A) was in October–November 2003, long after the mating season had ended. The second time sequence (B) was in January and February 2004, after animals emerged from hibernation and were beginning to show signs of reproductive activity. (C) Autocorrelation function (ACF) plots for the two time series describing relationships between the number of spots produced on different days. Bars show the magnitude of correlation coefficients calculated between the graphed data and the same time series shifted by a specified lag (up to 16 d). Dashed lines show 95% confidence intervals around a correlation of zero

territorial season (2003), but was strongly correlated with fecal spots during the subsequent mating season (2004).

Finally, assuming that lizards were synchronized with each other, time series analyses identified a cyclical pattern to the number of spots deposited, with lizards decreasing and then increasing the number of femoral pore and fecal spots produced each day (Fig. 3). This cyclical variation could be seen in the autocorrelation plots for the number of femoral pore spots, with significant positive autocorrelations at lags 1 and 7 ($r = 0.4$), with an intervening dip into negative autocorrelations at an intermediate lag 4 ($r = -0.2$). The pattern for fecal spots was similar, though weaker (autocorrelation $r = 0.2$ and 0.3 at lags 1 and 7, $r = -0.1$ at lag 4). With a series of relatively short duration (56 d), a first-order autoregressive model offered a sufficient fit to both femoral pore and fecal spot data, yielding the lowest BIC scores despite retaining much of the autocorrelation in the data.

Synchronization seems to have been stronger after hibernation than before. On days in which lizards produced more FPS, lizards also tended to produce larger spots, but this relationship was stronger during the mating season ($r = 0.7$, $P < 0.001$) than during the territorial season ($r = 0.4$, $P < 0.02$). Although there was no relationship between fecal spot number and size during the territorial season ($r < 0.1$, $P > 0.05$), the relationship was stronger but still not significant ($r = 0.35$, $P < 0.2$) during the mating season. The number of pore spots and the number of fecal spots produced by individual lizards on each day of the study were temporally linked during the mating season ($r = 0.5$, $P < 0.02$), but not before ($r = 0.2$, $P > 0.05$). There were no significant relationships between areas of femoral pore and fecal spots in either season.

Discussion

The results suggest that the amount of secretion deposited by lizards on a single perch may be an important component of the overall chemical signal, offering information on resident activity and physiological condition. The quantity of chemical deposits differed among animals of different body sizes and among animals in different part of their reproductive cycles. Deposits also varied on a weekly cycle, such that their degradation may convey information about resident lizard activity. Interestingly, a lizard could potentially gather this information from a distance, seeing as well as smelling the FPSs (Alberts, 1993). We found little difference between the sexes in the amount of chemical deposits, and no relationship between the amount of deposit produced by the same animal on different days or in different seasons of the year, suggesting that scent-marking behavior is not a good indicator of individual or sexual identity.

We found a discrepancy between gland production and chemical deposition on a single perch. Reproductively active males deposited fewer secretions than did females or territorially aggressive males, despite the known positive influence of androgens on gland productivity (Wade et al., 1993; Lindzey and Crews, 1993). This apparent contradiction can be resolved by recognizing that male sagebrush lizards are considerably more active than females, especially during the mating season, when most males defend several basking perches (Martins, 1991, 1993). Reproductively active rodents scent-mark more locations than do animals in other contexts (Wolff et al., 2002; Ferkin et al., 2004). Similarly, in a natural context, the extra

chemical secretions produced by sagebrush lizards during the mating season may be used to mark more locations rather than to mark any single location more heavily.

In mammals, seasonal increases in scent marking have been interpreted as support for the hypothesis that scent marks are used to advertise individual identity, especially at times in the seasonal cycle (i.e., mating season) when such information may be particularly important (Wolff et al., 2002; Ferkin et al., 2004). Our results extend the advertisement hypothesis by suggesting that the amount of deposition may convey information about physiological condition that supplements the information conveyed by the chemical structure of the signal. Thus, the amount of deposition may be an important element of the chemical signal, providing information about physiological condition and influencing the outcome of mate choice and aggressive interactions. We know that experimental manipulation of chemical cues on a lizard's skin alters its recognition by conspecifics (López and Martín, 2002), and the addition of femoral pore secretions to a robotic lizard model changes the behavioral response in playback experiments (Thompson, 2002). Playback studies are now also needed to determine whether receivers (lizard or mammal) can interpret effectively the information on physiological condition contained in deposit quantity.

The weekly cycle of scent-mark deposition also supports the hypothesis that femoral pore secretions function as territorial markers. Unlike in rodents, however, deposits were remarkably infrequent, with some lizards allowing several weeks to pass before depositing again, despite our daily removal of chemical secretions. Although it seems likely that the weekly cycle is required for replenishing a constant signal, further research is needed to determine whether, and how quickly, the signal is degraded and the extent this degradation may relate to the weekly cycle of deposition. Although experimental blocking of olfaction did not affect home ranges of sleepy lizards (Zuri and Bull, 2000), further studies of this sort with other lizard species may reach different conclusions. Most lizards maintain relatively exclusive territories despite considerable overlap in home ranges (Sheldahl and Martins, 2000) and behave differently when placed in an enclosure that has been previously occupied by other lizards (Labra et al., 2001). There also appears to be a relationship between the use of chemical secretions and the territorial headbob displays (Hews and Benard, 2001). Thus, experimental manipulation of olfaction with more targeted measures of changes in territory defense may yield further insights.

Finally, we found little evidence that lizards can determine individual identity, sex, or species of other animals from the quantity of chemical deposits. It seems more likely that differences in chemical composition of secretions among individuals, sexes, populations, and species (Alberts et al., 1993; Escobar et al., 2001) are used by lizards to distinguish sex, social status, and individual identity of conspecifics (Labra et al., 2001; López and Martín, 2001; Bull and Lindle, 2002; Aragón et al., 2003). We did not observe consistent individual differences in chemical deposition, as would be expected if secretion quantity were an indicator of individual identity. Moreover, an individual lizard's deposition during the prehibernation territorial season was not a good predictor of deposition during the posthibernation mating season. Further studies varying the amount and chemical composition of secretions or determining the amount of secretions produced in response to particular types of conspecifics are needed to confirm their use as territorial markers and communicative signals.

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Identification of Phytotoxic Substances from Early Growth of Barnyard Grass (*Echinochloa crusgalli*) Root Exudates

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Abstract Barnyard grass is a problematic weed worldwide. It competes with crops and causes reduction in crop yields. In this study, barnyard grass suppressed rice emergence, and the degree of rice inhibition was proportional to the density of barnyard grass. Root exudates of barnyard grass reduced germination and growth of lettuce, rice, and monochoria. Fifteen compounds potentially involved in the phytotoxic activities of barnyard grass were isolated and identified, including phenolics, long-chain fatty acids, lactones, diethyl phthalate, acenaphthene, and derivatives of phthalic acids, benzoic acid, and decane. Quantities of diethyl phthalate, decanoic acid, myristic acid, stearic acid, 7,8-dihydro-5,6-dehydrokavain, and 7,8-dihydrokavain were 2.7, 11.1, 19.6, 35.5, 10.3, and 15.5 $\mu\text{g/ml}$ of barnyard grass root exudates, respectively. The two lactones exhibited the greatest inhibition, followed by the phenolics and the derivatives of phthalic acids. Fatty acids had stronger suppression than diethyl phthalate and ethyl ester-4-ethoxy-benzoic acid. The acenaphthene and decane derivatives were the least phytotoxic. The phytotoxins released by barnyard grass roots showed strong inhibition on growth of broadleaf indicator plants and paddy weeds, but were less effective on barnyard grass itself and rice. Our study revealed that in addition to competition, barnyard grass also interferes with rice and other plants in its surroundings by chemical means.

Keywords Allelopathy · Allelochemicals · Barnyard grass · Exudates · Inhibition · Rice · Weed

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Introduction

Barnyard grass [*Echinochloa crusgalli* (L.) Beauv.], an annual grass, has been reported to cause problems in at least 61 countries and in at least 36 different crops (Holm et al., 1991). It is a major weed in paddy fields as it competes with rice (*Oryza sativa* L.) and causes reduction in rice yield. Competition from 25 barnyard grass plants/m² can cause 50% reduction in rice yield (Chin, 2001). Some weeds exude allelochemicals that suppress crop growth as well as other weed species in fields (Tang and Young, 1982; Qasem and Foy, 2001). However, differentiating between allelopathic effects and resource competition in the field is not easily achieved (Yamamoto et al., 1999). Therefore, the identification of allelochemicals exuding from roots in laboratory experiments, where the competitive factors can be eliminated, is useful to understand the phytotoxic activities of such weeds. Yamamoto et al. (1999) reported that during germination and early growth, barnyard grass inhibited the growth of cockscomb (*Celosia cristata* L. var. *kunze*), timothy (*Phleumpratense* L.), cress (*Lepidium sativum* L.), amaranth (*Amaranthus viridis* L.), rice, lettuce (*Lactuca sativa* L.), and barnyard grass. *p*-Hydroxymandelic acid, an allelochemical exuding from young barnyard grass roots, strongly inhibited the growth of rice at 59.5–178.6 μ M (Yamamoto et al., 1999). Most of the studies on rice–weed interactions focus on the weed-suppressing potential of rice, many of which concentrate on using rice allelopathy to control barnyard grass (Rimando et al., 2001; Chung et al., 2003). Major allelochemicals in rice capable of suppressing barnyard grass have been detected including phenolics, indoles, momilactones, and terpenoides (Khanh et al., 2005). Chung et al. (2003) and Jung et al. (2004) noted that barnyard grass was the least susceptible to rice allelopathy among the paddy weeds. Barnyard grass is in the same family as rice (Graminae), which may be one of the reasons for its superior ability to compete against rice. On the other hand, this noxious weed may also release growth inhibitors that inhibit emergence of rice and other paddy weeds. However, except for *p*-Hydroxymandelic acid, the composition of its root exudates, which may be responsible for such allelopathic activities, has not been investigated. Therefore, we conducted this research to isolate and identify allelochemicals in root exudates of barnyard grass and to examine their bioactivities against several important plant species.

Methods and Materials

Plants

Alfalfa (*Medicago sativa* L.), lettuce, sesame (*Sesamum indicum* L.), and rice (cv. Koshihikari) seeds from commercial sources (Wakaba company, Japan) were used. Seeds of barnyard grass, monochoria (*Monochoria vaginalis* Presl var. *plantaginea* Solms-Laub.), and Indian jointvetch (*Aeschynomene indica* L.) were collected from Miyazaki fields, Japan, in 2003. Empty and undeveloped seeds were discarded by floating in tap water. The remaining seeds were air-dried and stored hermetically at -20°C . These were sterilized with 1% sodium hypochlorite for 30 min and rinsed many times with distilled water before use. The germination percentage was randomly checked, and all were greater than 90%.

Effects of Barnyardgrass on Rice Growth

Ten healthy rice seeds were sown evenly in a Petri dish (9 cm diam) lined with filter paper and moistened with 10 ml distilled water. To the Petri dish, 10, 50, and 100 seeds of barnyard grass were added evenly between the rice seeds. Five replicates of all treatments were placed in an incubator (25°C, 4000 lx, with an 8-hr day/16-hr night cycle, humidity: 75%) using a completely random design. After 7 d, the number of germinated rice seeds was counted, and the length of shoots and roots was measured.

Barnyardgrass Exudates

Ten grams of healthy barnyard grass seeds was put in a glass pot with 100 ml of distilled water for 7 d in a growth chamber (25°C, 4000 lx, with an 8-hr day/16-hr night cycle, humidity: 75%). The distilled water was changed every 2 d. The resulting root exudates were filtered and set as the original dose, and a one-half strength solution was also prepared. Effects on emergence of rice, lettuce, and monochoria were examined by using methods similar to those described above against 20 seeds of each indicator plant. In another trial, 100 ml of the barnyard grass exudates was extracted with 70% MeOH in a shaking bath at 40°C for 24 hr. The solution was filtered, evaporated to dryness, dissolved in MeOH, and analyzed by thin-layer chromatography (TLC).

TLC Experiment

Many TLC solvent systems were tested. The combination of chloroform/ethyl acetate/acetic acid (10:3:1) gave the best separation and was selected. TLC plates were coated with 500- μ m layer of silica gel (Merck). The prepared barnyard grass root exudates were applied to the TLC plate (16 \times 20 cm). R_f values of the colored spots detected under the UV light were recorded, and the plate area was scraped and eluted with methanol. After evaporation to dryness, the residue was dissolved in distilled water, adjusted to 50 ppm, and tested for its effects on germination and growth of barnyard grass. Spots that inhibited lettuce emergence were collected, dissolved in acetone, and used for gas chromatography–mass spectrometry (GC-MS) analysis.

GC-MS Analysis

An aliquot of 2 μ l was injected (splitless) into the GC-MS (QP-2010, Shimadzu Co., Japan). The data were obtained on an ID-BPX5 column (30 m \times 0.25 mm ID, 0.25- μ m film thickness, SGE, Australia). The carrier gas was helium, and the GC oven temperature program was as follows: 50°C hold for 5 min, rate of 5°C/min to 280°C, and hold for 5 min. The injector and detector temperatures were set at 220 and 280°C, respectively. The mass range was scanned from 15 to 900 amu. The control of the GC-MS system and the data peak processing were carried out by means of the Shimadzu's GC-MS solution software, version 2.1.

Quantification of Allelochemicals in Exudates of Barnyardgrass

Phytotoxic compounds identified in the exudates were quantified. Pure reference standards were purchased from Wako (Japan). Two lactones were purified in our

laboratory. All compounds were dissolved in acetone and by using similar GC conditions to those described above to quantify the constituents of the barnyard grass root exudates by comparing the retention time and areas between the standard chemicals and the samples. However, some of the identified constituents could not be quantified as they could neither be purchased nor purified in our laboratory.

Effects of Isolated Compounds on Plant Growth

Dilution of pure reference chemicals detected in exudates of barnyard grass at 100 ppm was prepared and examined for their influence on emergence of alfalfa, monochoria, lettuce, Indian jointvetch, sesame, and rice (var. Koshihikari) using the reported bioassay method.

Statistical Analysis

All treatments were arranged in a completely randomized design with at least three replications. Bioassays were replicated five times. Data were analyzed with SAS version 6.12 (SAS Institute, 1997) using analysis of variance and least significant difference at the 0.05 probability level.

Results

Effects of Barnyardgrass on Rice Growth

Barnyard grass was sown at different densities to examine its effects on rice growth. Germination was not influenced by the presence of barnyard grass, but elongation of rice roots and shoots was suppressed when density of barnyard grass was increased (Table 1). At the rate of 10 seeds per Petri dish, no impact on root length was observed, but shoot length was significantly reduced, relative to the control. When the density of barnyard grass increased to 50 and 100 seeds per Petri dish, inhibition of rice roots was increased by 30–40%. However, shoot length was not significantly affected by the increase in barnyard grass density. This experiment found that barnyard grass had a chemical effect on rice emergence. Barnyard grass density was positively correlated with rice root and shoot length ($r = 0.94$ and 0.81 , respectively; Table 1). The inhibitory effect on rice growth was proportional to the density of barnyard grass.

Table 1 Inhibitory effects of barnyard grass on rice growth

Barnyard grass density (seeds)	Inhibition (% of control)		
	Germination	Root length	Shoot length
10	0.0a	3.0a	26.2b
50	0.0a	30.0b	31.6b
100	0.0a	39.4c	31.3b

Correlation coefficients of barnyard grass density against rice root and shoot length: $r = 0.94$ and 0.81 , respectively. Means with the same letter in a column are not significantly different at $P = 0.05$.

Table 2 Effects of barnyard grass exudates on emergence of lettuce, rice, and monochoria

Concentration	Inhibition (% of control)		
	Germination	Root length	Shoot length
Lettuce			
1/2	15.8a	44.1a	13.7a
1.0	30.6b	94.5b	88.2b
Rice			
1/2	0.0a	33.2a	26.2ab
1.0	5.0a	48.1b	27.5b
Monochoria			
1/2	7.4a	28.6a	28.7a
1.0	25.9b	65.0b	28.6a

Means with the same letter in a column are not significantly different at $P = 0.05$.

Effects of Barnyardgrass Exudates on Lettuce, Rice, and Monochoria

Barnyard grass root exudates were selected to determine potential phytotoxicity toward plants that are commonly found nearby such as rice and monochoria. The root exudates exhibited strong inhibitory effects on emergence of lettuce, rice, and monochoria (Table 2). Germination of lettuce and monochoria was reduced at the two applied doses in comparison with the controls, whereas no effect on rice germination was found. The roots of these indicator plants were more affected than their shoots. At one-half dose, the roots of monochoria and rice were suppressed by a similar level (about 30%), whereas lettuce roots were reduced by about 45% (Table 1). However, shoot length of lettuce was less affected than rice and monochoria at this concentration. At the original full dose, lettuce emergence was suppressed by about 90%. On the other hand, emergence of rice and monochoria was not significantly reduced as compared to the one-half dose, with the exception of monochoria root, which was inhibited by 65%. This experiment reveals that barnyard grass releases toxic compounds during germination that strongly reduce the growth of lettuce, rice, and monochoria.

TLC Experiment

Several spots were observed on TLC plates under UV light and examined for their activities on lettuce germination and growth at 50 ppm. Of these, only five spots suppressed lettuce emergence, with Rf values of 0.24, 0.29, 0.35, 0.42, and 0.72

Table 3 Effects of spots isolated from TLC on emergence of lettuce at 50 ppm

Spots	Rf values	Inhibition (% of control)		
		Germination	Root length	Shoot length
1st	0.24	44.4c	57.5c	33.5b
2nd	0.29	11.1ab	28.6ab	20.0b
3rd	0.35	5.6a	14.6a	2.6a
4th	0.42	22.2b	37.9b	18.0b
5th	0.72	60.6d	77.7d	54.2c

Means with the same letter in a column are not significantly different at $P = 0.05$.

(Table 3). These were selected for analysis. At 50 ppm, the compounds contained in each spot significantly reduced germination and growth of lettuce, as compared to the control, with the exception of the third spot that had no significant effect on lettuce germination. The constituents of the fifth spot exerted the greatest influence on lettuce emergence followed by the first spot (Table 3), whereas the third spot was the least inhibitory.

GC-MS Analysis

Fifteen compounds were detected in the five spots collected by TLC as shown in Table 4. Their chemical structures are described in Fig. 1. The first spot consisted of two phenolics [2-ethyl-phenol and 2,4-bis (1,1-dimethyl)-phenol] and three derivatives of phthalic acids (dimethyl ester-phthalic acid, butyl 8-methylnonyl ester-phthalic acid, and diisooctyl ester-phthalic acid). The second spot contained diethyl phthalate and a derivative of benzoic acid (ethyl ester-4-ethoxy-benzoic acid). Derivatives of decane (2,3,7-trimethyl-decane and 2-methyl-dodecane) and acenaphthene were detected in the third spot. Three long-chain fatty acids were observed in the fourth spot, including decanoic acid, myristic acid, and stearic acid. In the fifth spot, two lactones were identified [7,8-dihydro-5,6-dehydrokavain (DDK) and 7,8-dihydrokavain (DHK)]. All of these 15 constituents have been identified in barnyard grass root exudates for the first time.

Table 4 Substances identified from spots that showed inhibition on emergence of lettuce and their quantities in barnyard grass exudate

Compounds	Retention time (min)	Molecular weight	Amount in exudates of barnyard grass ($\mu\text{g/ml}$)
Spot 1			
2-Ethyl-phenol	19.4	122	–
Dimethyl ester-phthalic acid	27.8	194	–
2,4-bis (1,1-Dimethyl)-phenol	29.4	206	–
Butyl 8-methylnonyl ester-phthalic acid	39.9	223	–
Diisooctyl ester-phthalic acid	50.1	279	–
Spot 2			
Ethyl ester-4-ethoxy-benzoic acid	29.9	194	–
Diethyl phthalate	31.5	222	2.7 ± 0.01
Spot 3			
2,3,7-Trimethyl-decane	22.5	141	–
2-Methyl-dodecane	24.1	169	–
Acenaphthene	26.1	153	–
Spot 4			
Decanoic acid	30.7	200	11.1 ± 0.03
Myristic acid	35.5	228	19.6 ± 0.02
Stearic acid	43.9	284	35.5 ± 0.06
Spot 5			
7,8-Dihydro-5,6-dehydrokavain	33.4	230	10.3 ± 0.05
7,8-Dihydrokavain	43.2	232	15.5 ± 0.03

–: Measurement was not conducted.

\pm : Standard errors ($N = 5$).

Only 6 of the 15 detected compounds were quantified, as the remaining chemicals were uncommon and could neither be purchased nor successfully purified. Diethyl phthalate accounted for 2.7 $\mu\text{g/ml}$ of the root exudates (Table 4). The quantities of decanoic acid, myristic acid, and stearic acid were 11.1, 19.6, and 35.5 $\mu\text{g/ml}$, respectively. The amounts of lactones were 10.3 and 15.5 $\mu\text{g/ml}$ for DDK and DHK, respectively.

PHYTOTOXINS FROM ROOT EXUDATES OF BARNYARDGRASS

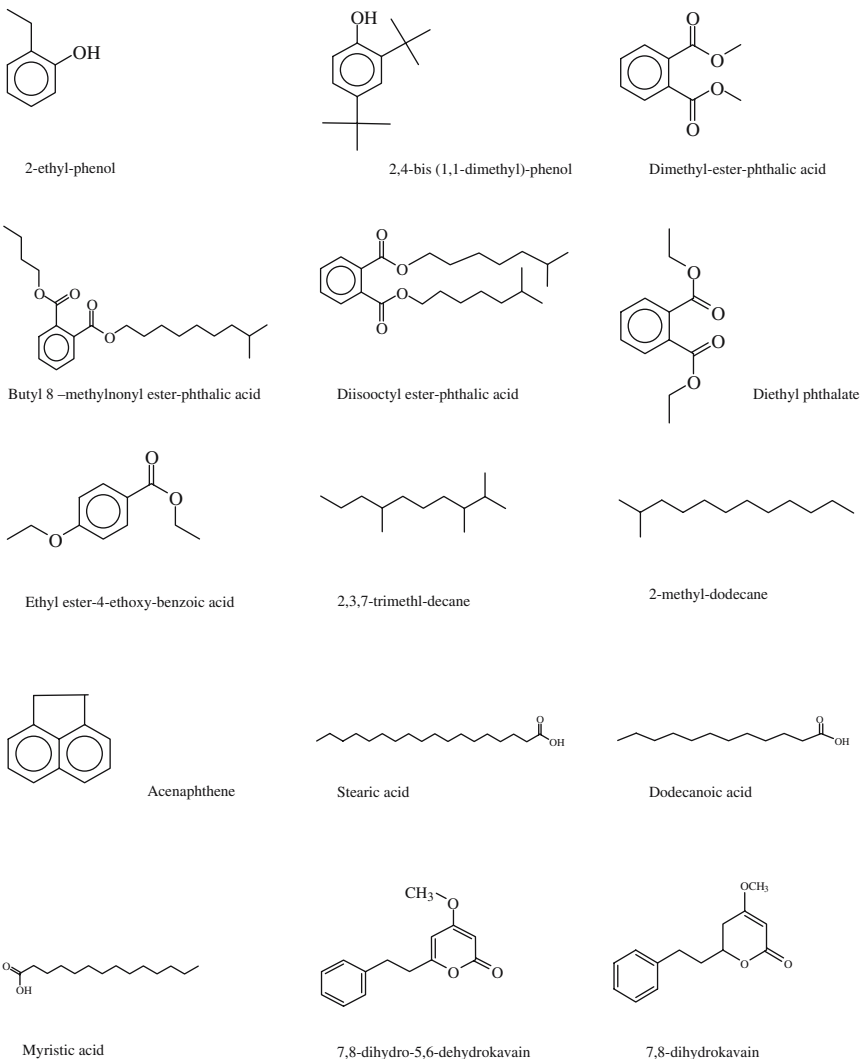
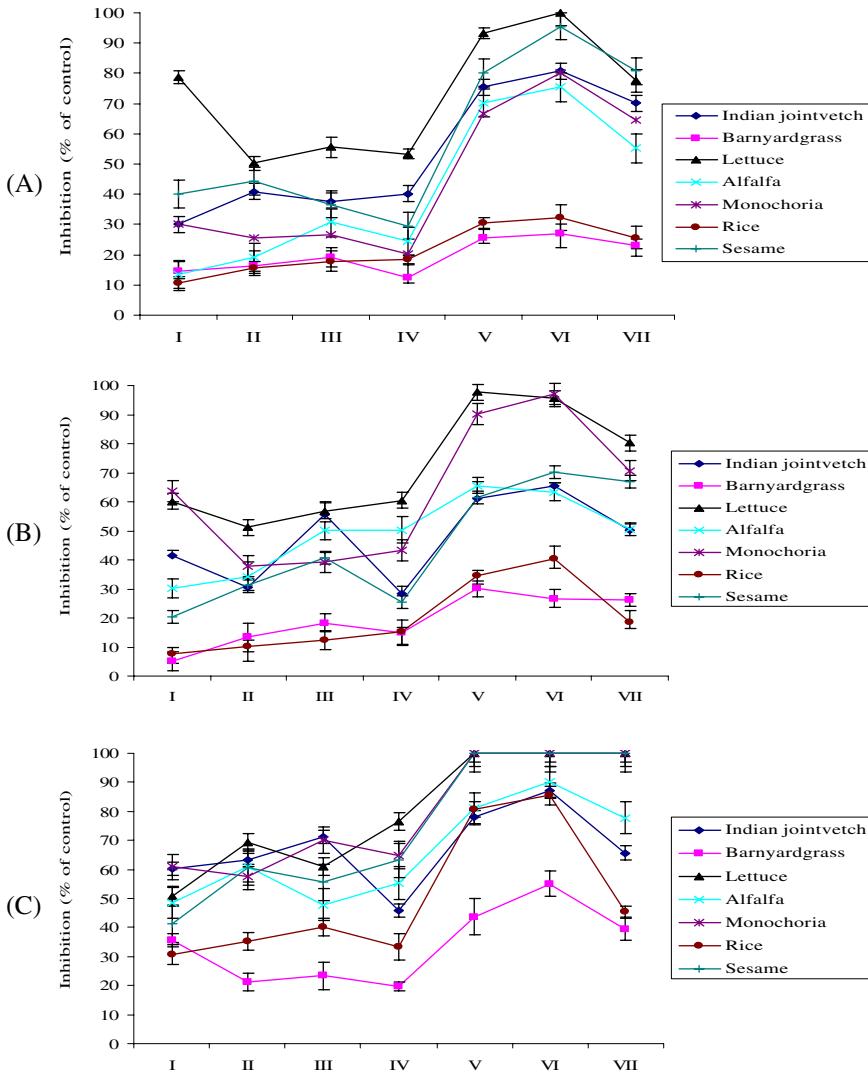


Fig. 1 Chemical structure of chemicals involved in inhibitory activities of barnyard grass root exudates

Effects of Chemicals in Root Exudates of Barnyardgrass on Plant Emergence

The effects of the two lactones on lettuce were the most detrimental, followed by the two phenolics and derivatives of phthalic acids (Tables 3 and 4). Fatty acids gave greater suppression than the derivative of benzoic acid (ethyl ester-4-ethoxy-benzoic acid; Tables 3 and 4). Acenaphthene and derivatives of decane exhibited the least effects among identified substances (Tables 3 and 4).



I: Diethyl phthalate II: Decanoic acid III: Myristic acid IV: Stearic acid
 V: 7,8-dihydro-5,6-dehydrokavain VI: 7,8-dihydrokavain VII: Mixture

Fig. 2 Effects of six individual compounds isolated from barnyard grass root exudates on emergence of seven plants [(A) germination, (B) shoot length, and (C) root length] at 100 ppm. Vertical bars represent standard errors

The six quantified compounds were tested individually for their influence on germination and growth of alfalfa, barnyard grass, Indian jointvetch, lettuce, monochoria, rice, and sesame at 100 ppm [405.5, 500.0, 438.5, 352.1, 434.8, and 431.0 μM for diethyl phthalate, decanoic acid, myristic acid, stearic acid, DDK, and DHK, respectively] (Fig. 2). In general, diethyl phthalate showed greater inhibition on growth of indicator plants than the fatty acids. The two lactones, however, inhibited indicator plant growth far more than the other compounds. The growth of all tested plants, except for barnyard grass and rice, was strongly inhibited by the quantified compounds.

The phytotoxins released by barnyard grass root exudates were least effective against rice and barnyard grass itself as compared to other broadleaf species (Fig. 2). Germination and shoot growth of barnyard grass and rice were not much affected; however, the growth of rice root was significantly suppressed by DDK and DHK and the mixture (60–70% of inhibition). The inhibitory effect of the two lactones on root elongation of barnyard grass was markedly lower than the effect on rice.

Discussion

Certain weeds are known to release allelochemicals into the environment and suppress both crops and other weeds in their vicinity (Bais et al., 2003). Therefore, the invasiveness of certain weeds may also be dependent on their allelopathic activities including the influence of exuded phytotoxins against neighboring plants. Numerous allelochemicals from weeds have been identified and reported to exhibit strong phytotoxic activity. However, most of them are from upland weeds (Qasem and Foy, 2001). Allelochemicals detected in upland weeds belong to several chemical classes, including phenolics, sesquiterpenes, flavonoids, terpenes, alkaloids, and their derivatives (Qasem and Foy, 2001). Little information on allelochemicals from weeds in rice paddies has been documented, although weeds cause serious interferences in agricultural production. Much of current research is concentrating on the biological control of rice paddy weeds in attempts to minimize the use of synthetic herbicides.

Major research efforts have focused on evaluating the ability of certain crop species to suppress barnyard grass infestation and on enhancing the allelopathic potential of these crop species, especially in rice (Chung et al., 2003). The incorporation of phytotoxic plant material into soil to suppress paddy weeds such as barnyard grass has also been investigated (Xuan et al., 2004), as well as the search for allelochemicals to control barnyard grass (Jung et al., 2004). In contrast, the allelopathic potential of this harmful weed species has not received much interest. Xuan et al. (2001, 2002, 2003, 2004) selected plants with strong weed-suppressing ability and applied them to soil 2 d after transplanting at 1–2 tons/ha. Weed biomass was controlled up to 80%, and rice yield increased by 20%. However, barnyard grass appeared to be the most difficult of the paddy weeds to control. The cause of this phenomenon is not known, although it is possible that chemical interference may have a role.

Yamamoto et al. (1999) reported that barnyard grass exhibited allelopathic potential. Root exudates of young barnyard grass suppressed root elongation of the seven plant species tested (cockscumb, timothy, cress, amaranth, rice, lettuce, and barnyard grass). A phenolic acid (*p*-Hydroxymandelic acid) was isolated and

identified in the root exudates that inhibited rice growth at concentrations of 59.5–178.6 μM . This compound has been shown to be involved in the phytotoxic activity of barnyard grass against rice (Yamamoto et al., 1999). However, to date, no further information about allelochemicals released by barnyard grass has been reported.

In this study, barnyard grass suppressed rice emergence, and the degree of rice inhibition was proportional to the density of barnyard grass. Root exudates of recently germinated barnyard grass exerted strong inhibition on germination and growth of lettuce, rice, and monochoria. Monochoria is also a noxious weed and is distributed widely in paddy fields and wet areas in the subtropics and tropics (Holm et al., 1991). Therefore, investigation of the chemical interactions among barnyard grass, rice, and monochoria, in addition to other weeds, is needed. As crop–weed interactions are better understood, the biological elimination of noxious paddy weeds may be more feasible. Lettuce is a common indicator plant used in bioassays related to allelopathy because of its high sensitivity to chemicals at low concentration (Xuan et al., 2004). Diethyl phthalate and phthalic acid derivatives are commercially important chemicals used predominantly as plasticizers in high-molecular-weight polymers, which are toxic to humans, animals, microorganisms, algae, aquatic invertebrates, and fish (Staples et al., 1997; Jonsson and Baun, 2003; Sung et al., 2003; Chen and Sung, 2005; Hu et al., 2005), as well as plants (Herring and Bering, 1988; Saarma et al., 2003). We, at first, thought the presence of diethyl phthalate and derivatives of phthalic acid might be caused by contaminants from either the sample preparation or analytical instruments. Therefore, the existence of these compounds in the root exudates of barnyard grass was examined carefully. Control experiments and samples of root exudates were repeated several times and were analyzed on two different GC-MS. Results from these experiments unequivocally confirmed that these compounds are not derived from contamination because of plasticizers used during extraction and sample preparation or due of solvent impurities. Recent reports show that diethyl phthalate is either bacterium or plant derived. Keire et al. (2001) reported that *Helicobacter pylori* secreted diethyl phthalate as a chemotactic factor. It was the first example of a phthalate ester that is produced by a bacterium. Elzaawely et al. (2006) reported that *Rumex janonicus* Houtt., a perennial herb widely distributed in the subtropics, produces diethyl phthalate. We suggest that diethyl phthalate and derivatives of phthalic acid may be derived from plants and act as a new class of plant phytotoxins. However, this needs confirming in other plant species, and the mechanism of producing these compounds by plants should be examined before concluding that these substances are natural products.

In this research, the most common phenolics found in many reported allelopathic plants were not detected in root exudates of barnyard grass. Three phenolics [2-ethyl-phenol, 2,4-bis (1,1-dimethyl)-phenol, and ethyl ester-4-ethoxy-benzoic acid] and derivatives of phthalic acids did show strong inhibition when isolated from TLC as a mixture. The amounts of these phenolics have not been quantified, but in gas chromatograms, they appeared to have lower peak areas than other identified substances (data not shown). Effects of these phenolics on plant growth need to be determined individually and compared to those of other common phenolic acids.

The two lactones were the most phytotoxic against the growth of seven plants at 431 and 434.8 μM for DDK and DHK, respectively. It is likely that lactones released by barnyard grass roots may play a major role in the allelopathic activities of the

weed. A mixture of these compounds was more inhibitory than diethyl phthalate and fatty acids, but it was lower than the effects of the two lactones individually. In this study, however, all detected phytotoxins in barnyard grass root exudates were present in low concentrations (Table 4), suggesting perhaps that in field conditions, they might not accumulate to the phytotoxic levels needed to affect rice and other weeds (Fig. 2). It is possible that phytotoxins other than those identified here may exist, and they may have allelopathic activity that accounts for the invasiveness of this harmful weed. They might be released by barnyard grass roots at stages other than the early growth stage.

This paper reveals that barnyard grass has allelopathic potential. It releases numerous phytotoxins into the environment during germination and early growth. These compounds could suppress the growth of rice and other plants, depending on the density of the grass in paddy fields. It appears that the phytotoxins that inhibit the growth of rice and other weeds have less influence on barnyard grass growth (Fig. 2). It is possible that phytotoxins released by monocots inhibit the growth of dicots more than the growth of monocots themselves and vice versa. The exudation of these phytotoxins into the environment may contribute to the success of barnyard grass as an invasive species. Both the release of these toxic compounds by barnyard grass at different growing stages and their interactions under natural conditions against crops and other weed species should be further studied.

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Tracing Pollinator Footprints on Natural Flowers

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Abstract Many insects are known to leave lipid footprints while walking on smooth surfaces. Presumably, the deposited substances improve tarsal adhesion. In bumblebees, footprint hydrocarbons also function as scent marks that allow detection and avoidance of recently depleted flowers. I used GC-MS to detect hydrocarbons deposited by bumblebee (*Bombus pascuorum*) on flowers of *Lamium maculatum*. In addition to the plants' own cuticular lipids, extracts of corollas that had been visited by bumblebees contained odd-numbered alkenes. The amount of pentacosenes ($C_{25}H_{50}$) on corollas was linearly related to the number of bumblebee visits, with workers depositing approximately 16 ng per visit (extrapolated to a total of 65 ng of bumblebee cuticular hydrocarbons). Pentacosenes were retained on visited flowers without loss for 2 hr, and probably longer. This and results from flight cage experiments suggest that flower epicuticles retain a chemical record of pollinator visitation, including information on visiting bee species. Continuous footprint accumulation necessitates new explanations concerning the reversibility of “repellent scent marks” of bumblebees.

Keywords Footprints · Cuticular hydrocarbons · Hydrocarbon signature · Scent marks · *Bombus* · *Lamium*

Introduction

Insect cuticular lipids serve a range of functions, including reducing evaporative water loss over the body surface as well as improving adhesion for movement on smooth surfaces (Lockey, 1988; Jiao et al., 2000). Additionally, the same lipids may constitute chemical signals for conspecifics, e.g., by mediating mate recognition or relating information on reproductive status (Blomquist et al., 1998). In foraging

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bees, especially bumblebees (*Bombus* sp.), cuticular lipids are thought to function as scent marks that allow foragers to detect and avoid flowers that have been previously visited and depleted (Goulson et al., 1998, 2000, 2001; Stout et al., 1998; Williams, 1998; Gilbert et al., 2001; Gawleta et al., 2005). When approaching an inflorescence, bumblebee workers briefly hover in front of a depleted flower, but then reject it without actually probing for the concealed nectar reward. Behavioral experiments (Stout et al., 1998; Goulson et al., 2000) have indicated that the remote assessment of flowers is not based on a direct perception of nectar, but rather indirectly on the perception of hydrocarbon marks deposited by previous visitors. Flowers treated with hexane extracts of bumblebee tarsi elicited a repellent effect similar to that of a natural bumblebee visit, with nectar levels remaining unaltered. Similar results were obtained when researchers applied several pure synthetic hydrocarbons (*n*-alkanes, alkenes), the main contents of cuticular lipids of bumblebees (Schmitt, 1990; Oldham et al., 1994; Goulson et al., 2000), to flowers (Goulson et al., 2000). It is unclear whether the deposition of repellent substances is an active process that merits the term “scent marking” or whether the deposits are unavoidable “footprints” (Thomson and Chittka, 2001; Gawleta et al., 2005). A further corollary is that the repellent effect is only temporary. In most cases, repellency of visited flowers is lost after 20–60 min, which has been attributed to a progressive evaporation of the deposited substances (Stout et al., 1998; Stout and Goulson, 2002).

So far, no attempts have been made to directly measure bumblebee chemical traces on natural flowers. Schmitt et al. (1991) analyzed filter paper attached to artificial feeders of *B. terrestris* laboratory colonies and found cuticular hydrocarbon profiles that were similar to those of extracts of *B. terrestris* tarsi. The same substances might be detectable on real flower petals after natural visits of bumblebees. In this case, hydrocarbon signatures of bees would have to be discernable within the hydrocarbon profile of the plant (e.g., Griffiths et al., 1999). In the present study, I compared the cuticular hydrocarbons of the common Deadnettle *Lamium maculatum* (Lamiaceae) with those of its bumblebee visitors. I asked whether bumblebee hydrocarbons are detectable on visited flowers, and explored the temporal dynamics of footprint accumulation.

Methods and Materials

The study was conducted in patches of *L. maculatum* growing in the University of Düsseldorf Botanical Gardens. At the time of the study (August 10 to September 3, 2004), the flowers were mostly visited by workers of *Bombus pascuorum*. A 1 × 1 m area of the patch was covered by a mosquito mesh tent to prevent bees from accessing these flowers.

Hydrocarbon Contrast between Bees and Flowers

The following samples were collected for GC-MS: (1) individual sets of tarsi of worker *B. pascuorum* (cut at the distal end of the tibia, combining all six legs per individual sample; $N = 6$), (2) corollas of screened unvisited inflorescences ($N = 8$), and (3) corollas of screened inflorescences that were immediately presented to for-

aging bumblebees, allowing exactly one visit per corolla ($N = 7$). For items (2) and (3), five corollas from the same inflorescence were combined in one sample. Corollas were removed by tearing them from the receptacle with clean forceps; anthers were removed at the base with scissors. Every sample was extracted for 30 sec in 1 ml *n*-hexane (p.a., Merck) containing 10 μg 2-undecanone as internal standard. In the laboratory, the solvent was reduced to 0.15 ml at room temperature.

Footprint Accumulation on Visited Flowers

Unvisited inflorescences were cut, placed individually in water-filled vials, reduced to five open flowers, and presented to bumblebees in the field. Within 15 min, 0, 5, 10, 15, 20, 25, or 30 bee visits were allowed per inflorescence, corresponding to 0 to 6 visits per flower. Larger numbers of visits were increasingly difficult to induce, because the bees strongly rejected these inflorescences. Two series were done synchronously. In one of the series, the corollas were extracted in hexane immediately after the 15-min visitation time window. In the other, the inflorescences were placed in an insectary (not accessible to foraging bees) for 2 hr and then extracted. The same procedure was replicated three times, resulting in $7 \times 2 \times 3 = 42$ samples. For a given sample, all five flowers of the inflorescence were combined and extracted for 30 sec.

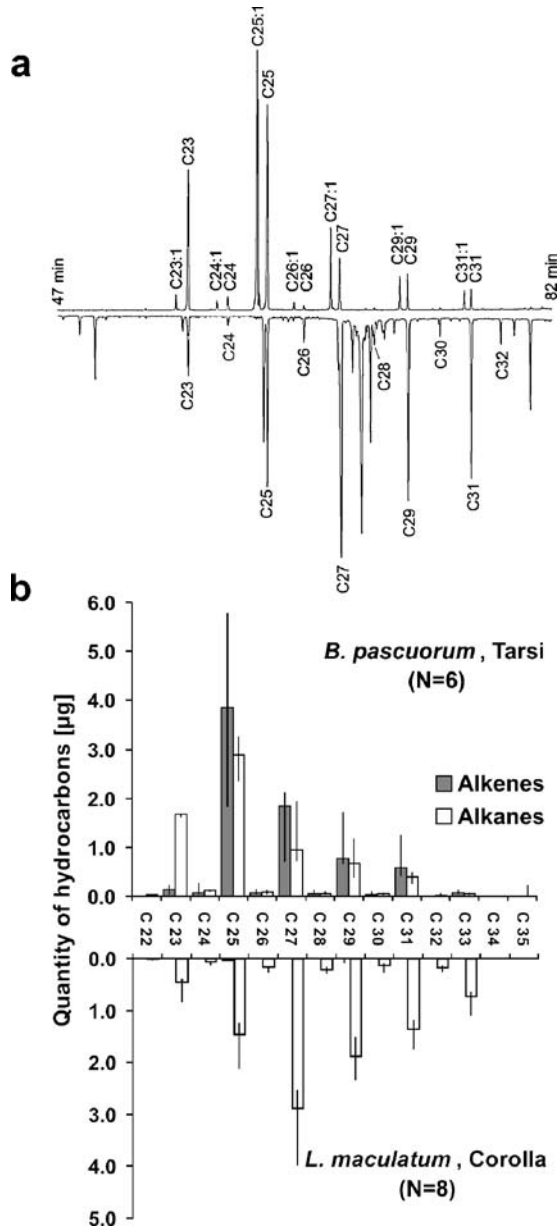
Bee-specific Hydrocarbon Signatures

Worker bumblebees were introduced into a mosquito mesh tent covering approximately 150 unvisited open *Lamium* flowers. Five bees were introduced between 08:30 and 09:30 in the morning and allowed to forage during the day. At 16:00 hr, bees were removed, and three replicate samples of five corollas each were extracted (as above). The experiment was done three times on consecutive occasions, once with *B. pascuorum*, once with *B. hortorum*, and once with *B. terrestris* workers. After an experiment with a given species was completed, all open corollas were removed from stalks with forceps and the next experiment started 2 or 3 d later when new (unvisited) flowers had opened in the tent. Tarsal extracts were taken from individuals of each of the bumblebee species ($N = 3$ per species; tarsi of all six legs pooled per sample). Effects of the (visiting) bee species on alkene composition of tarsal and corolla extracts were analyzed by using nonparametric ANOSIM permutation tests (Clarke, 1999; Clarke and Gorley, 2001). A similarity matrix based on the Bray–Curtis index (Legendre and Legendre, 1998) was calculated based on standardized data. I tested whether the factor (*visiting*) *bee species* had an effect on the rank order of between-sample similarities, separately for tarsal and corolla extracts. I also tested whether *sample type* (bee tarsi, corolla) had an effect.

Chemical Analysis

Gas chromatography-mass spectrometry (GC-MS) was performed with an HP 5890 II GC fitted with a 30-m nonpolar DB-5 column and an HP5972 mass selective detector. Injection was splitless, the oven programmed from 60 to 300°C at 3°C/min with automatic pressure programming. Characterization of hydrocarbons was done by comparison with authentic standards. Quantifications of hydrocarbon contents were based on internal (2-undecanone) and external (pentacosane) standards.

Fig. 1 (a) Total ion chromatograms of hexane extracts of *B. pascuorum* tarsi (on top) and unvisited *L. maculatum* corollas (bottom). *n*-Alkanes and alkenes are labeled and numbered with reference to the number of carbon atoms and double bonds. Unlabelled peaks in *Lamium* extracts are lipids that were not characterized. (b) Amount of hydrocarbons (median and quartile range) in tarsal and corolla extracts. Quantifications are given per individual set of tarsi ($N = 6$) and per corolla ($N = 8$), respectively. On average ($N = 6$), tarsal extract contained $8.02 \pm 2.49 \mu\text{g}$ alkanes and $8.47 \pm 3.51 \mu\text{g}$ alkenes. *n*-Pentacosane (C25) and pentacosenes (C25:1) were consistently the most abundant components, representing 19.4% and 24.0% of total individual hydrocarbons, respectively. Corolla extracts contained on average ($N = 8$) $12.27 \pm 6.44 \mu\text{g}$ alkanes (C:22 to C:33)



Results

Hydrocarbon Contrast between Bees and Flowers

Tarsal extracts of *B. pascuorum* were characterized by a series of *n*-alkanes and corresponding alkenes between C22 and C31, with odd-numbered homologs prevailing, confirming the results of an earlier investigation (Goulson et al., 2000)

(Fig. 1a, b). Corolla extracts of unvisited *L. maculatum* also contained large quantities of *n*-alkanes, but no or only traces of the respective unsaturated alkenes ($0.12 \pm 0.17 \mu\text{g}$; Fig. 1b). They also contained a range of additional lipids that were not further characterized (Fig. 1a). Four odd-numbered alkenes (C25:1 to C31:1) were well-defined markers for bumblebees. In comparison to unvisited controls, these alkenes were more abundant in samples of flowers that had received one visit per flower (Mann–Whitney *U* test: $P < 0.001$ for C25:1 and C31:1, $P < 0.01$ for C27:1, and $P < 0.05$ for C29:1; $N = 15$). As in *B. pascuorum* tarsal extracts, pentacosenes (C25:1) were the dominant alkenes on visited flowers.

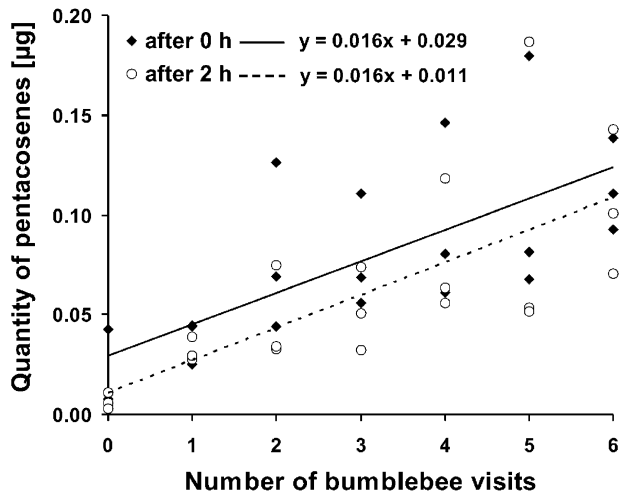
Footprint Accumulation on Visited Flowers

There were significant positive effects of the number of visits on the amount of pentacosenes extracted from corollas, both when extraction was immediate or delayed (Fig. 2). Although samples with delayed extraction contained slightly lower amounts of pentacosenes, this effect was not significant (one-way ANOVA: $N = 42$; $F = 1.40$; $P = 0.24$). Inclusion of the number of visits as a covariate did not change this result ($N = 42$; $F = 2.84$; $P = 0.10$). Judging from the slopes of the linear regressions in Fig. 2, individual bumblebees deposited on average 16 ng pentacosenes per flower visit.

Bee-specific Hydrocarbon Signatures

Extracted tarsal alkenes differed qualitatively and quantitatively between different species of bumblebees (ANOSIM: $N = 9$; $R = 1$, $P < 0.01$), as did alkenes extracted from corollas visited by the different species of bumblebees ($N = 9$; $R = 1$, $P < 0.01$). There was no overall difference of alkene composition between tarsal extracts and visited corolla extracts ($N = 18$; $R = 0.043$; $P = 0.58$), with alkene profiles extracted from corollas closely corresponding to those of the visiting species of bumblebee (Fig. 3). The chain length of the dominant alkene in deposited footprints increased

Fig. 2 Relationship between the number of bumblebee visits per flower and the amount of pentacosenes extracted from corolla samples per flower. Black diamonds represent corolla samples that were extracted immediately after bumblebee visitation (linear regression: $N = 21$; $R^2 = 0.50$; $t = 4.39$, $P < 0.001$). Circles are corolla samples that were extracted after a delay of 2 hr ($N = 21$; $R^2 = 0.53$; $t = 4.66$, $P < 0.001$)



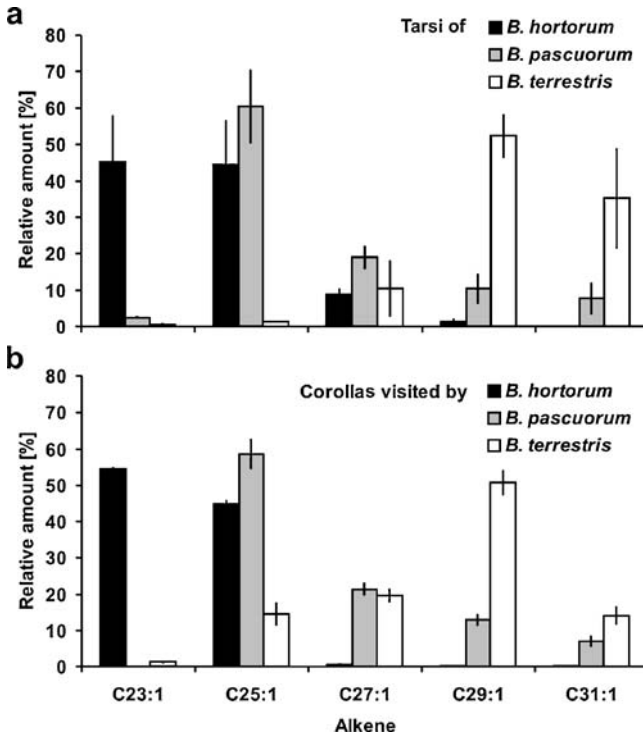


Fig. 3 (a) Alkene profiles of tarsal extracts of three species of bumblebees ($N = 3$ per species) and (b) of corolla extracts of *Lamium maculatum* visited by the three bumblebee species ($N = 3$ per visiting bee species). The mean and standard deviation are given

from tricosenes in *B. hortorum*, to pentacosenes in *B. pascuorum* and nonacosenes in *B. terrestris*.

Discussion

This is the first study to trace bumblebee “footprints” on natural flowers. Detection in corolla extracts was possible because several straight-chain alkenes served as chemical “bumblebee markers,” as they occurred abundantly on bee tarsi but not on unvisited *Lamium* flowers. Extrapolating from these markers to the entire set of tarsal hydrocarbons (see Fig. 1, assuming that deposition occurs in equal proportions), individual workers deposited approximately 65 ng of hydrocarbons per visited flower.

The origin of the deposited substances is as yet unknown. Schmitt et al. (1991) as well as the present study found that deposited substances were similar in composition to those in tarsal extracts, suggesting that tarsal glands situated in the fifth tarsomer are the secretory source (Schmitt, 1990; Schmitt et al., 1991). However, the tarsal gland reservoir has no opening to the outside in any of the several investigated bee species, which has cast doubt on their involvement in scent marking (Jarau et al., 2005). Furthermore, the hydrocarbons found on bumblebee tarsi can

also be found in similar composition on the cuticle of other body parts (Oldham et al., 1994; Goulson et al., 2000), suggesting that they are part of the hydrocarbon layer covering the entire body surface (Oldham et al., 1994). While probing *Lamium* flowers, bumblebees touch the corolla not only with their feet but also with the head, thorax, and abdomen, thus exposing the plant to a broad surface area of cuticle from which hydrocarbons may be acquired. This may be responsible for the rather large amounts of bumblebee hydrocarbons found on *Lamium* corollas in the present study.

The linear relationship between the amount of deposited pentacosenes and the cumulative number of visits per flower suggests that deposition is not reward-dependent and, therefore, probably passive (see Thomson and Chittka, 2001). Nonetheless, the depositions may be used as cues by foragers that enable them to detect recently depleted flowers (cues rather than signals; see Schmidt et al., 2005 for an example with an attractive effect). An unresolved problem exists concerning the temporal dynamics of the repellent response. In field studies with bumblebees, depleted flowers regain their original attractiveness after relatively short periods of time, in most cases between 20 and 60 min following a visit, depending on plant species and situation (Stout et al., 1998; Williams, 1998; Stout and Goulson, 2002; personal observation). It has been hypothesized that loss of repellency is observed because scent marks evaporate. In conflict with this idea, the amount of pentacosenes on visited flowers was not reduced by a 2-hr time delay before extraction, although longer delays might produce such effects. Generally, long-chain hydrocarbons such as those on bumblebee tarsi are of low volatility. This point is well illustrated by a study of Ginzel and Hanks (2002) that used combinations of synthetic alkanes (C24 to C31) to mark elytra of milkweed beetles. The profiles remained unchanged in quality and quantity over weeks, despite exposure to direct sunshine (Ginzel and Hanks, 2002). Thus, it seems unlikely that evaporation or molecular break down leads to a rapid vanishing of bumblebee footprints from flower corollas.

Why then is repellency reversed? Two alternative explanations seem plausible. First, bumblebee hydrocarbons accumulate, but are only slowly incorporated into the semiliquid layer of plant epicuticular lipids (see Jetter et al., 2000), rendering them increasingly imperceptible to foraging bees. Alternatively, bumblebee footprints may contain small quantities of low-molecular-weight volatiles that have not been detected in hexane extracts. Perception of such compounds could modify the behavioral response elicited by the hydrocarbons alone. Future studies will have to address this issue.

The present investigation shows that detection and quantification of bumblebee footprints is relatively simple and straightforward by using solvent extraction of flowers and GC. Probably the approach is also applicable to other plant–bumblebee systems. Cuticular hydrocarbons of bumblebees typically contain odd-numbered alkenes, with species-specific differences in chain length distribution (Goulson et al., 2000; this study). On the other hand, corolla extracts of bee pollinated plants lacked these alkenes (Griffiths et al., 1999, 2000; Goodwin et al., 2003; Eltz, unpublished data for *Symphytum officinale* and *Linaria vulgaris*), thus providing an unobscured background for extractive footprint detection. The finding that footprint hydrocarbons accumulate on petals without loss suggests that flowers carry a complete chemical record of bumblebee visitation, including information on visitation frequency and composition of the pollinator community. Reading this record by means of chemical analysis may constitute a powerful tool for pollination ecologists, re-

ducing time and manpower necessary for multireplicate field studies. Pilot studies will have to evaluate the feasibility and scientific potential of such an approach.

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Fragrance of Canada Thistle (*Cirsium arvense*) Attracts Both Floral Herbivores and Pollinators

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Abstract The evolution of floral scent as a plant reproductive signal is assumed to be driven by pollinator behavior, with little attention paid to other potential selective forces such as herbivores. I tested 10 out of the 13 compounds emitted by dioecious *Cirsium arvense*, Canada thistle, including 2-phenylethanol, methyl salicylate, *p*-anisaldehyde, benzaldehyde, benzyl alcohol, phenylacetaldehyde, linalool, furanoid linalool oxides (*E* and *Z*), and dimethyl salicylate. Single compounds (and one isomer) set out in scent-baited water-bowl traps trapped over 10 species of pollinators and 16 species of floral herbivores. The two dominant components of the fragrance blend of *C. arvense*, benzaldehyde and phenylacetaldehyde, trapped both pollinators and florivores. Other compounds attracted either pollinators or florivores. Florivores of *C. arvense* appear to use floral scent compounds as kairomones; by advertising to pollinators, *C. arvense* also attracts its own enemies.

Keywords *Cirsium arvense* · Trapping · Florivores · Pollinators · Scent · Herbivores · Phenylacetaldehyde · Benzaldehyde · Fragrance · Volatiles

Introduction

Insects navigate complicated odor landscapes, identifying the scent of a host plant while filtering out irrelevant chemical signals (Metcalfe, 1987; Masson and Mustaparta, 1990). Since the diversification of the angiosperms and many of their pollinators during the Cretaceous period, selection by beneficial insects has been acting on floral scent. Most research on floral volatile evolution has considered pollinators as the primary biotic selective agent (Dobson, 1994; Raguso, 2001). However, it has been proposed that the original role of plant volatiles was as deterrents against herbivores

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(Pellmyr and Thien, 1986). Mounting evidence suggests that floral herbivores, which include both florivores and predispersal seed predators, are an important factor in selection on fragrance and other floral characters. Florivores can affect fitness directly (through tissue destruction) and indirectly (by deterring pollinators) (Strauss, 1997; Krupnick and Weis, 1999; Mothershead and Marquis, 2000; Adler et al., 2001). Ultimately, these losses can affect seedling recruitment (Louda and Potvin, 1995; Kelly and Dyer, 2002). Studies have demonstrated the role of florivores in the evolution of floral morphology (Brody, 1997; Kudoh and Whigham, 1998; Campbell et al., 2002; Ehrlèn, 2002), flowering phenology (Eriksson, 1995; Pilsen, 2000; Mahoro, 2002), flower number (Ohashi and Yahara, 2000), and floral scent (Galen, 1983; Baldwin et al., 1997). If detrimental insects use floral scent to locate their hosts, then they too will contribute selective pressures that affect the evolution of floral scent. Similar scent compounds that attract both pollinators and detrimental insects may be subject to opposing selection pressures: pressure to be both apparent to pollinators and inconspicuous to florivores.

Of the studies that have investigated the attraction of herbivores to floral volatiles, many have focused on agricultural pests (Dobson, 1994). Studies have been carried out both on insects that feed on floral tissue and on those that oviposit into a flower head where their larvae feed (Cantelo and Jacobson, 1979; Wiesenborn and Baker, 1990; Haynes et al., 1991; Roseland et al., 1992; Tingle and Mitchell, 1992; Smart and Blight, 1997; Metcalf et al., 1998; Smart and Blight, 2000). Canada thistle, *Cirsium arvense* (Asteraceae), is an invasive species in the eastern United States, and native to Europe, Western Asia, and North Africa; it was probably introduced into Canada in the early 17th century (Moore, 1975). I chose to study an exotic invasive species because of its potential to demonstrate both coadapted interactions with introduced insects, as well as novel, interactions with native herbivores.

Cirsium is pollinated by a wide range of generalists, including insects from several different orders (Proctor et al., 1996). In this population, I found the most abundant pollinator on *C. arvense* to be the nonnative (but coadapted, as it is native to Europe) honeybee (*Apis mellifera*), with high visitation rates by other bees, especially *Halictus* and *Lasioglossum* ssp. (Halictidae; Theis, 2003). Other pollinators included hover flies (Diptera: Syrphidae) and common diurnal Lepidoptera, including *Vanessa atalanta* and *Pieris rapae*. A field experiment determined that nocturnal pollination is not relevant in the study population (Theis, 2003). Florivorous insects from several orders feed on *C. arvense* flower heads, including beetles (Mordellidae, Phalacridae, Meloidae, Cantharidae) and grasshoppers (Acrididae, Tettigoniidae) (Theis, 2003). Many species of *Cirsium* and other closely related groups are considered noxious weeds in the United States, and a number of biocontrol agents have been introduced to control *C. arvense*, including root, leaf, and flower feeders (McEvoy and Coombs, 1999). *Dasineura gibsoni*, an introduced cecidomyiid fly, and two introduced weevils were found on *C. arvense*; one weevil, *Larinus planus*, was quite common, whereas *Rhinocyllus conicus* was rare. With a generalist pollination system and a large number of both introduced and native florivores, it is an ideal choice for studies on potential balancing or diversifying selection on floral scent.

I quantified the chemical composition of both staminate and pistillate *C. arvense* plants and used scent-emitting traps to determine the olfactory preferences of both pollinators and florivores to *C. arvense* fragrance components. If beneficial and

detrimental insects use different fragrance components, then directional selection should minimize the compounds attractive to the detrimental insects.

Methods and Materials

Taxa and Field Sites

Cirsium arvense (L.) Scopoli, Canada thistle, (Asteraceae), is an erect perennial whose height at reproduction ranges from 0.3 to 2.0 m. Unique among thistles, *C. arvense* has dioecious flower heads of pink-purple disk flowers, which bloom from July through September (Nuzzo, 1997). I conducted this study at the U.S. Fish and Wildlife Service Wallkill River National Wildlife Refuge. Located on the border of Sussex, NJ, and Warwick, NY, USA (74°31'W, 41°17'N), the site is a former sod farm. It is dominated by mixed communities of native and exotic invasive plants in open fields, including *Asclepias syriaca*, *Ambrosia artemisiifolia*, *Solidago* sp., *Lythrum salicaria*, and *Carduus nutans*.

Emission Rates

Volatiles were collected from *C. arvense* by using dynamic headspace sampling in the field. Intact flower heads were enclosed within a nylon resin oven bag (Reynolds Consumer Products, Richmond, VA, USA). Ambient air flowed into the bag across the flower head and into Porapak® Q (80–100 mesh) packed cartridges at a flow rate of ca. 200 ml/min, via either an Air Check 52 or Air Check 2000 diaphragm pump (SKC Inc., Eighty Four, PA, USA). Cartridges were eluted with 3 ml hexane, and an internal standard of 3 µl of 0.01% anisole in hexane was added. Samples were concentrated to 75 µl with N₂. To ascertain the fragrance production of cut flower heads (*N* = 2 staminate, 3 pistillate), scent was collected for 4 hr before cutting and 4 hr immediately after cutting. Emission rates from the traps were measured for 10 min by using the same protocol described above, but in a growth chamber with controlled light and temperature (24°C) to mimic average field conditions (*N* = 3).

Fragrance Analysis

Combined capillary gas chromatography-mass spectrometry (GC-MS), with a Shimadzu GC-17A equipped with a Shimadzu QP5000 quadrupole electron impact MS as a detector (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA), was used to analyze the fragrance. A 1-µl aliquot was injected splitless onto a polar column (EC WAX) (30 m × 0.25 mm; Alltech Associates, Deerfield, IL, USA) at an initial temperature of 60°C for 3 min, which was increased by 10°C/min until 260°C, where it was held for 7 min (Theis and Raguso, 2005). Compounds were identified by using retention time (from previously injected standards) and mass spectral libraries [Wiley (1995) and NIST (1998)], with greater than 120,000 mass spectra. Quantification was achieved by relating the mass ion of each scent compound to the mass ion of the internal standard with serial dilution curves of the scent compounds run as external standards.

Scent-Baited Traps

I used scent-emitting insect traps, during July and August 2002, to attract both pollinators and florivores by using 10 of the 13 floral compounds identified from the scent blend of *C. arvensis* (Andersson et al., 2002; Theis and Raguso, 2005). Each trap consisted of a clear bowl (8-in. plastic bowls; Hummert International, Topeka, KS, USA) fixed to a tier of a tomato cage at a similar height level with that of the blooming plants. A second bowl filled with soapy water (Alconox liquid detergent 1 ml/5 l; Alconox, White Plains, NY, USA) was set inside the fixed bowl. Suspended above the soapy water by a wire was a microcentrifuge tube filled with either scent or an unbaited control tube. A hole was cut into the microcentrifuge tube, and a wick of single-stranded embroidery floss was submerged in the chemical and projected 7 mm above the top of the tube, in order to emit scent. All wicks were renewed (cut and pulled another 7 mm) once a day. Scents were refilled at least once a week. As a visual display, a cut staminate or pistillate *C. arvensis* flower head (set into a water-pik) was suspended above water bowls. Flower heads also were renewed daily.

Two field experiments were run simultaneously for 4 wk, spanning the peak of *C. arvensis*'s flowering season. One experiment had six treatments, including three pure compounds (phenylacetaldehyde, methyl salicylate, 2-phenylethanol), racemic linalool, one blend of two linalool oxide furanoid isomers, and a dry control tube. The second experiment consisted of seven treatments: two pure compounds (dimethyl salicylate, benzyl alcohol), two compounds diluted by 1/2 with mineral oil (*p*-anisaldehyde, benzaldehyde), one control tube containing mineral oil, and two cut flower heads (one staminate, the other pistillate). The cut flower heads were not used until the second week of the experiment. I did not test the pyranoid linalool oxide isomers or benzyl benzoate in the traps. The treatments were arranged in a circular array surrounding a patch of *C. arvensis*. Each circle contained two replicates of each treatment, and the arrangement of compounds was randomized once a week. Traps were placed 3 m apart and a replicate circle was set up at least 10 m away, for a total of four replicate traps. Trapped insects were identified daily and collected every 2 d.

Chemicals

The synthetic compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA; 2-phenylethanol 100%, methyl salicylate 99%, *p*-anisaldehyde 99%, benzaldehyde 99%, benzyl alcohol 99%, phenylacetaldehyde >90%, (±)-linalool 97%, furanoid linalool oxide mixture of isomers >97%) and from Quest International (Ashford Kent, UK; dimethyl salicylate).

Statistical Analyses

Trap catch was not normally distributed for any insect species. For groups with more than 24 trapped individuals, I used Systat 10.0 to test for differences in trap catch between each treatment and the control within each circle (replicates were lumped) by using the Wilcoxon's signed-ranks test for paired comparisons (Sokal and Rohlf, 1995).

Results

Trapped Species

In two field experiments, I tested the attraction of pollinators and florivores to 10 components of the *C. arvensis* blend (eight compounds and one isomeric mixture). Emission rates from the traps ranged from 343 to 769 $\mu\text{g/hr}$ (Table 1). For comparison, the average total emission rate from *C. arvensis* is 19 $\mu\text{g/hr}$ for staminate flower heads and 4 $\mu\text{g/hr}$ for pistillate. I captured a total of 15 species of florivores and nine species of pollinators, seven of which were bees, including three genera of Halictidae (Table 2). Butterflies pollinate *C. arvensis*, although infrequently in this population, but were not captured within water bowl traps.

Pollinator Attractants

A pollinator assemblage, summed for all species of pollinators with greater than 10 representative individuals, was tested for fragrance preference. Three compounds were more attractive to pollinators than the control: benzaldehyde, phenylacetaldehyde, and *p*-anisaldehyde (Wilcoxon's signed-ranks test, $P < 0.05$; Table 2, Fig. 1). Of these, *p*-anisaldehyde and phenylacetaldehyde were by far the most attractive compounds, trapping on average 48% and 37% of all pollinators within each of the respective experimental arrays. Only honeybees and *Lasioglossum* were caught in sufficient numbers to be able to detect significant differences in trap preference for individual pollinator genera; both were trapped by *p*-anisaldehyde. Phenylacetaldehyde and linalool were also attractive to honeybees.

Florivore Attractants

Florivores were attracted to floral scent compounds from *C. arvensis*. I summed the data for all florivores (that had greater than 10 individuals trapped) and determined that 26% of all florivores were trapped by benzaldehyde (Wilcoxon's signed-ranks test, $P < 0.05$; Table 2, Fig. 1). In contrast, more florivores were trapped by the control than by linalool. Specific florivores also demonstrated significant prefer-

Table 1 Scent Emitted from Traps ($N = 3$)

Compounds	Emission ($\mu\text{g/hr}$)
(<i>E</i>)-Furanoid linalool oxide ^a	582 \pm 152
(<i>Z</i>)-Furanoid linalool oxide ^a	484 \pm 100
Benzaldehyde	703 \pm 75
Linalool	430 \pm 208
Phenylacetaldehyde	453 \pm 77
Methyl salicylate	769 \pm 55
Benzyl alcohol	415 \pm 73
2-Phenylethanol	363 \pm 35
<i>p</i> -Anisaldehyde	343 \pm 57
Dimethyl salicylate	631 \pm 87

^a. Trap contained both furanoid linalool oxides.

Table 2 Insect Total Trap Catch Contrasted with Control Traps

	Exp. 1					Exp. 2					Exp. 2		Grand total	
	Phenylace taldehyde		Furanoid linalool oxides		Control Exp. 1	Benzaldehyde		Dimethyl salicylate		Control Exp. 2	Pistillate inflor	Staminate inflor		Control Exp. 2 (3 wk)
	Methyl salicylate	2-Phenyle thanol	linalool	2-Phenyle thanol		dehyde	alcohol	dehyde	aldehyde					
Pollinators ^a	31	52	42	52	37	59*	33	18	238*	26	40*	58*	13	837
<i>Apis mellifera</i>	58*	10	5	20*	6	14	7	4	54*	5	26	18	5	234
<i>Lasoglossum</i>	73	30	33	24	27	26	18	4	141*	17	7	26	5	439
<i>Augochlorella</i>	9	0	0	3	0	13	1	1	11	2	3	6	2	53
<i>Halicictus</i>	4	0	1	0	1	1	2	2	11	0	0	0	0	24
Syrphidae	1	1	1	7	3	0	2	2	14	2	2	9	1	46
<i>Ceratina</i>	0	3	0	0	1	3	2	3	6	0	0	0	0	18
<i>Hylaetus</i>	4	1	0	2	1	0	1	1	0	0	0	1	0	13
<i>Bombus</i>	2	0	2	0	1	0	0	1	1	0	2	0	0	10
Florivores ^a	43	27	22	20	30	64*	42	35	31	20	37	38*	15	426
Formicidae	13*	6	5	2	2	11	13	4	1	1	18	6	0	85
Tetrigoniidae	4	4	2	0*	4	20*	6	8	9	5	3	10*	3	75
Mordellidae	1	3	6*	3	2	14*	9*	13	2	2	6	7	1	70
Acrididae	2	0	1	1	1	10	9	2	6	8	9	10	7	60
<i>Tetraopes</i>	13	2	3	2	8	0	1	0	0	0	0	1	0	33
<i>tetophthalmus</i>														
<i>Popillia japonica</i>	2	3	1	5	3	4	1	1	4	3	0	1	3	31
<i>Lygus lineolaris</i>	4	3	2	3	5	3	0	2	1	0	0	1	0	27
<i>Lygaeus kalmii</i>	0	3	0	1	2	1	0	1	2	1	0	1	1	17
<i>Corimelaena</i> sp.	2	0	0	3	0	0	1	2	0	0	0	0	0	8
Cantharidae	1	0	0	0	0	0	0	1	4	0	0	0	0	6
<i>Larinus planus</i>	1	2	0	0	0	0	1	0	0	0	0	0	0	6
Geometridae	0	1	0	0	0	1	0	0	2	0	0	1	0	5
<i>Epicautta</i> sp.	0	0	0	0	0	0	1	1	0	0	1	0	0	3

^a "Pollinators" and "florivores" represent summed data, excluding groups with ten or fewer individuals.

^b Wilcoxon's signed-ranks test for paired comparisons between treatments and control, * $P < 0.05$, significance not tested for any group with fewer than 24 individuals.

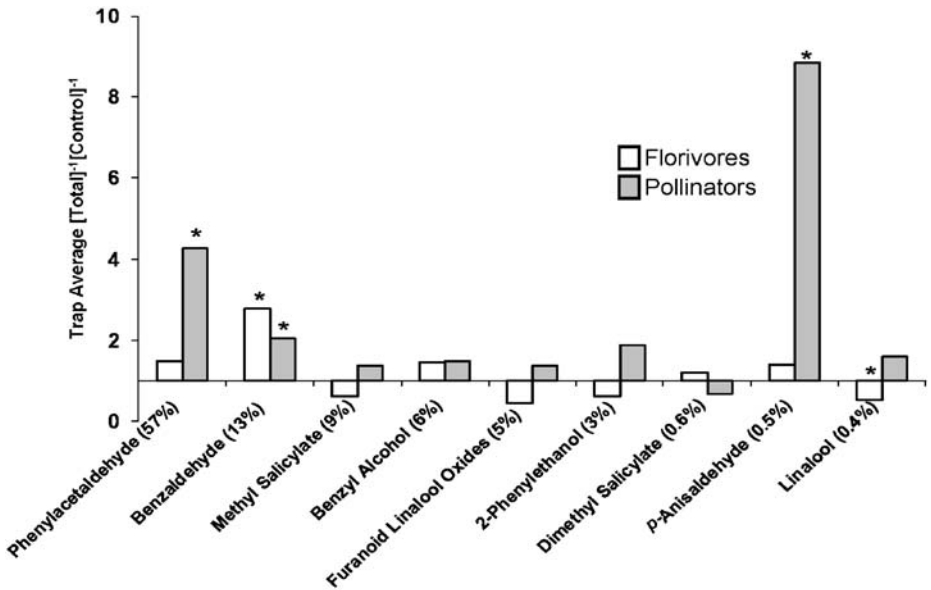


Fig. 1 The average number of trapped pollinators and florivores in each scent trap contrasted with the abundance of each of these compounds in the floral blend of staminate *Cirsium arvense*, in parentheses next to compound name. Trap catch was normalized by total trap catch and control trap catch. Significance tested by Wilcoxon’s signed-ranks test paired comparisons between treatments and control * $P < 0.05$

ences, both positive and negative. Ants (Formicidae) were attracted to phenylacetaldehyde, and mordellids were attracted to benzyl alcohol and to the furanoid linalool oxide isomers. In the first 2 wk of the experiment, mordellids were frequently trapped by dimethyl salicylate, but this preference was not consistent throughout the experiment, and it is not significant. The tettigoniid grasshoppers (predominantly nymphs) were never trapped by linalool or 2-phenylethanol.

Visual Display

Cut flower heads also trapped both pollinators and florivores. Cut flower heads emitted less than half the total emission of intact flower heads (approximately 44% for staminate flower heads and 35% for pistillate flower heads). In spite of fragrance reduction, staminate and pistillate cut flower heads were attractive to pollinators (Wilcoxon’s signed-ranks test, $P < 0.05$; Table 2). Summed data for all pollinators was strongly influenced by *Lasioglossum*, which were more frequently trapped by cut pistillate flower heads, and by honeybees, which were more frequently trapped by cut pistillate flower heads, although these preferences were not significant. Florivores and particularly the tettigoniids were attracted to cut staminate flower heads.

Discussion

Floral Scent Traps Both Pollinators and Florivores

The fragrance emitted by *C. arvensis* attracts both pollinators and florivores (Table 2, Fig. 1). Some compounds significantly attracted only pollinators (i.e., *p*-anisaldehyde), while others significantly attracted only particular species of florivores (i.e., benzyl alcohol). Some compounds were attractive to both pollinators and florivores (benzaldehyde and phenylacetaldehyde). Attractive compounds were not necessarily the most abundant components of the *C. arvensis* fragrance blend (Fig. 1). For example, *p*-anisaldehyde is 0.5% of the total blend, but a highly attractive compound for pollinators. Although trapping experiments using blends would convey a more complete picture of selection on the fragrance phenotype, pure compounds, nonetheless, should be informative. From studies on the neurophysiology of honeybees, Galizia and Menzel (2001) have demonstrated that a mix of two compounds results in a signal in the insect brain that is not novel, but rather additive of the component parts with some degree of deviation, both positive and negative (inhibition). There could be synergistic effects of compounds in the blend that result in an increase in attraction, reducing our ability to identify the intensity of selection on any one of these compounds, but not changing the direction of selection. Overall, these results demonstrate that the fragrance emitted from *C. arvensis* is perceived and utilized not just by beneficial insects, but by detrimental insects as well. As a result, components of the fragrance blend should be shaped by opposing selection pressures.

Diversity of Pollinators in Odor Traps

The dominant pollinators of *C. arvensis* were caught in the traps, including species from five families in two insect orders (Table 2). The largest numbers of individuals were caught from the family Halictidae, a sample dominated by *Lasioglossum* (*Dialictus*) bees. Also trapped were honeybees, the dominant pollinators of *C. arvensis* at this study site, as indicated in a census (Theis, 2003). Lepidopteran pollinators were excluded from analysis for two reasons: the water bowl traps did not attract butterflies, and nocturnal pollination does not occur in this population (Theis, 2003). Therefore, the moths that were trapped in these experiments, while indicative of the general attraction to aromatic floral compounds (see Cantelo and Jacobson, 1979; Plepys et al., 2002), are omitted from further discussion.

Diversity of Florivores in Odor Traps

A large diversity of dominant florivores were caught in the traps, including species from 14 families in five insect orders. All of the insects defined as florivores were seen feeding on *C. arvensis*, and many are recognized as highly detrimental, including the Japanese beetle (*Popilla japonica*), the tarnished plant bug (*Lygus lineolaris*), and blister beetles (*Epicauta* sp.) (Borror et al., 1992). Two of the florivore groups in this system have been implicated as selective forces driving the evolution of floral scent in other plant species: Negro bugs (*Corimelaena* sp.) on *Nicotiana* (Baldwin et al., 1997) and ants (Formicidae) on *Polemonium* (Galen,

1983). Nevertheless, the classification of these insects as detrimental is only putative. This applies particularly for the Cantharidae, which may be predatory on other florivores, resulting in a net positive effect. Many of the florivores that are attracted to *C. arvensis* floral odors are specialists on Asteraceae (Borrer et al., 1992). Some insects, such as *L. planus*, were frequently found on *C. arvensis*, but were infrequently captured in the traps, including control traps. It is possible that these insects are attracted by scent, but the trapping apparatus does not efficiently trap them. With such a high diversity of florivores on *C. arvensis*, none of which is completely specialized or devastating, selection pressure from this group is likely to be somewhat diffuse.

Repellent Compounds

Only florivores were significantly less attracted to some components of the floral scent blend (e.g., linalool) than to the unbaited control. A trap with the full blend minus the putative repellent could establish repellence for individual components. Repellent compounds have infrequently been reported in the literature (Dobson, 1994; Theis and Raguso, 2005), perhaps because florivores may adapt to perceive a compound as attractive if there is a reward associated with the cue.

Selection on Floral Scent Biosynthesis

Selection acting on quantitative variation in the emission of specific components of the floral scent blend may result in selection on the biochemical pathways from which those compounds are produced. The 13 compounds emitted by the flowers of *C. arvensis* are products of two biosynthetic pathways. Phenylacetaldehyde, benzaldehyde, and *p*-anisaldehyde are all products of the shikimate pathway (Dey and Harborne, 1997). In the shikimate pathway, phenylalanine ammonia lyase (PAL) is the “branch-point” enzyme between primary and secondary metabolism (Dixon and Paiva, 1995). The monoterpenoids, linalool and the linalool oxides, are produced in the DOXP/MEP pathway localized to plastids (Lichtenthaler et al., 1997; Raguso and Pichersky, 1999). Multiple enzymes may be at work in producing the diversity of volatiles, but single terpene synthase enzymes are sometimes responsible for the synthesis of several major and minor volatile products (Bohlmann et al., 1998). If selection acting on one compound affects the production of a different compound in the blend, a number of outcomes could arise. For example, honeybees are attracted by linalool, whereas florivores may be repelled. With this simple model, I would expect that linalool should increase in the blend. However, the mordellid beetles are attracted by the furanoid linalool oxide isomers (Table 2), and it is possible that positive selection acting on linalool could be constrained by negative selection on the linalool oxides, which are produced from linalool (Raguso and Pichersky, 1999). If the pollinator attractant increases, perhaps its associated oxides would also increase, resulting in the attraction of florivores. Only a few of the enzymes involved in the production of floral scent compounds have as yet been identified (Dudareva et al., 2003). However, understanding the selection pressures on the components of the floral scent blend will require knowledge of enzymatic pathways in order to obtain a full picture of selection pressure on the blend.

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Analysis of the Volatiles Emitted by Whole Flowers and Isolated Flower Organs of the Carob Tree Using HS-SPME-GC/MS

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Abstract The volatiles emitted by fresh whole flowers and isolated flower organs of male, female, and hermaphrodite carob trees (*Ceratonia siliqua* L.; Leguminosae) were analyzed by headspace solid-phase microextraction followed by capillary gas chromatography and mass spectrometry. The headspace of carob flowers is mainly constituted of high amounts of monoterpenes and sesquiterpenes, and more than 25 compounds were identified. The gender and cultivar affected both the qualitative profile and the relative abundances of the volatiles of whole flowers and isolated floral organs. Linalool and its derivatives (*cis*-linalool furan oxide, 2,2,6-trimethyl-3-keto-6-vinylnorbornene, *cis*-linalool pyran oxide, and *trans*-linalool furan oxide), α -pinene, and α -farnesene were the dominant volatiles. Female flowers had a higher diversity of volatile compounds than males and hermaphrodites, but a lower abundance of the major ones. Similarly, the floral scent of female flowers of cv. Mulata had a higher content of volatiles but a lower abundance of the major ones, when compared to cv. Galhosa. In each of the three gender types of flowers, the nectary disks seemed to be the major source of volatiles.

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Introduction

Animal-pollinated plants advertise their flowers to attract pollinators, and this can take the form of visual (color and shape) and olfactory (scents) cues (Dobson, 1994). Floral scents may function as long-distance and/or short-distance attractants (reviewed by Dobson, 1994) not only to insect pollinators (Ômura et al., 1999; Wright et al., 2002) but also to key insect pests (Nojima et al., 2003; Tasin et al., 2005).

In flowering plants, inbreeding avoidance and resource reallocation favor the evolution of gender dimorphism (i.e., females and males or hermaphrodites; reviewed in Charlesworth, 1999). However, females, which produce no functional male organs, often suffer inadequate visitation and pollen limitation (Ashman, 2000; Williams et al., 2000; Ashman and Diefenderfer, 2001). The reduction in visual floral cues (petal size and flower number) or rewards (pollen and nectar) are usually referred to as the factor responsible for the lower attractiveness to pollinators as displayed by females rather than males or hermaphrodites (Ashman et al., 2000). However, Ashman et al. (2000) evaluated the characters responsible for sex-differential visitation in *Fragaria virginiana* and found that visual cues or reward status could not fully explain the male bias in pollinator service.

Given the differences in total floral investment (reviewed in Eckhart, 1999) and in allocation to floral organs (Ashman, 1994; Jones and Burd, 2001), sex morphs of gender dimorphic plants are expected to exhibit differences in floral scent because the emission of volatiles can be biomass-dependent, and the compounds emitted can vary among the floral organs (Dobson and Bergström, 2000). In spite of the high degree of similarity usually observed in the type of compounds present in the scent profile between sexual types, differences in the abundances of the main constituents are common, namely, in some species of Salicaceae (Tollsten and Knudsen, 1992), Arecaeae (Ervik et al., 1999), and in *Geonoma macrostachys* (Knudsen et al., 1999) and *Ficus carica* (Grison-Pige et al., 2001). However, it is not yet known if this variation has functional implications since information about the source of sex differences is scarce (Dobson and Bergström, 2000), and there are no reports on whether or not they cause differential pollinator attraction.

The solid-phase microextraction (SPME) process is a fast and a powerful solventless technique, allowing the establishment of the equilibrium between the analytes from the headspace of the sample matrix and the stationary phase coating of a fused silica fiber, followed by thermal desorption of the concentrated analytes into the gas chromatograph (GC) inlet (Flamini et al., 2003; Pellati et al., 2005). Furthermore, this technique is sensitive enough to identify plant odors from specific tissues (Barták et al., 2003), such as flower organs, pollen (Flamini et al., 2003), and nectar (Raguso, 2004). The use of headspace techniques to sample volatiles in the air surrounding a plant has revealed that olfactory stimuli differ not only among species, but also among different organs within a single flower (Flamini et al., 2003; Dötterl and Jürgens, 2005). These spatial fragrance patterns within the flowers can guide insects to both nectar and pollen (Dobson, 1994; Dötterl and Jürgens, 2005). Studies of floral scents and of their patterns within a single flower are important to

better understand the chemical bases of plant–animal relationships and pollination ecology (Flamini et al., 2003) because quantitative and qualitative differences in the volatiles emitted from flower parts may be important in effecting pollinator attraction (Dobson et al., 1990). Some papers have ascribed this to distinct volatile compounds or blends emitted by different flower parts (Dobson et al., 1990, 1996; Knudsen and Tollsten, 1991; Pichersky et al., 1994; Bergström et al., 1995; MacTavish and Menary, 1997; Flamini et al., 2003; Azuma et al., 2004; Ashman et al., 2005).

Floral scent can be crucial in ensuring fertilization and in determining fruit or seed set, thus having a substantial impact on the yield of agronomically important crops (Dudareva et al., 2000), such as the carob tree (*Ceratonia siliqua* L.; Leguminosae), where the economical value relies on the fruit production and seed yield. The economic importance of this species is a result of the locust bean gum (LBG) extracted from the seeds and used in the food industry as a viscosifier, stabilizer, and gelling agent in products such as juices, dietetic beverages, desserts, baby foods, and pet foods (Batlle and Tous, 1997).

The carob tree is a polygamo-dioecious or monoecious species (Tucker, 1992). Individual trees may be male (with inflorescences carrying only staminate flowers), female (with inflorescences carrying only pistillate flowers), and hermaphrodite (with some inflorescences carrying staminate and others pistillate flowers, or with inflorescences carrying hermaphroditic flowers).

The carob is the only Mediterranean tree that has its main flowering season in autumn (September–November) with a peak in October (Martins-Loução and Brito de Carvalho, 1989; Retana et al., 1994; Batlle and Tous, 1997), when only few species are in flower (Arroyo, 1988). Moreover, carob flowers offer high amounts of nectar and pollen (Ortiz et al., 1996). Both the lack of alternative flowering species and the presence of abundant rewards mean that carob flowers are highly visited. It has been observed that the male flowers receive a higher number of floral visitors than the female ones, although the latter produce significantly higher amounts of nectar (Ortiz et al., 1996). However, the carob displays a low percentage of fruiting per raceme in both wild (Ortiz et al., 1999) and cultivated populations (Bosch et al., 1996), and it has been pointed out that the fruit production per raceme is limited by both the availability of resources and by deficient pollination (Arista et al., 1999). This evidence posed the question of whether there are differences in the scent emitted by flowers of the different genders of carob tree, which could lead to different patterns of floral visitations. To answer this, in a previous report, Custódio et al. (2004) analyzed the volatile composition of the scent emitted *in vivo* by the flowers of each of the three gender types in carob, and by flowers at different developmental stages, by using headspace SPME followed by capillary gas chromatography coupled with mass spectrometry (HS-SPME-GC/MS). More than 25 compounds were identified, and both gender and developmental stages affected the volatile composition. In male and hermaphrodite flowers, the total volatile compounds were more abundant in stage III (when the mature flowers show nectar secretion and pollen has not been shed), and in female flowers, in stage IV (when nectar and stigmatic fluids are secreted; the color of the stigma turns from greenish-yellow to yellow and brownish-yellow; brownish spots appear until the whole stigma turns brown and black). The most abundant volatile compounds reported in the headspace of carob flowers were linalool and *trans*-linalool oxide and were present in higher abundance in male flowers.

In this work, we expanded upon this earlier study by analyzing the main volatiles in the scent emitted *in vivo* by whole flowers and isolated flower organs of male, hermaphrodite, and by the two most economically important female carob cultivars (Mulata and Galhosa) to evaluate the contribution of the volatiles emitted by the different floral organs to the scent of whole flowers and to assess if the differences in the scent are linked to specific emissions of certain compounds by different organs. Additionally, the influence of the mean weight of whole flowers, nectary disks, stigmas, and stamens on the emission of volatiles was studied.

Methods and Materials

Plant Material

Inflorescences were collected from 14-year-old male, female, and hermaphrodite carob trees, growing in a nonirrigated and nonfertilized orchard, located in the south of Portugal (37°07'N; 7°39'W). Male trees were seminal, whereas female and hermaphrodite ones were grafted. Two female cultivars were studied: the Galhosa and the Mulata. The climate of the region is typically Mediterranean (Mitrakos, 1981), and the annual rainfall (400–500 mm) is mainly distributed throughout winter and early spring.

Floral Analysis

For each gender, 3 trees were selected and 25 individual flowers were randomly collected per tree from all canopy orientations. The average weight of whole flowers and of the stamens (including filaments and anthers), nectary disks (including pedicel), and stigmas was determined.

For the headspace analysis, 500 mg of each floral sample type was used. The floral material was collected at mid-day, at stages found by Custódio et al. (2004) to emit the most volatiles (Fig. 1): (1) male whole flowers at stage III, (2) hermaphrodite whole flowers at stage III, (3) female whole flowers at stage IV, and (4) separate floral organs (stamens, nectary disks, and stigmas) from male (stage III), hermaphrodite (stage III), and female flowers (stage IV).

Flowers from each sample were collected and introduced into a 30-ml amber glass vial with screw caps (Supelco). The SPME device (Supelco Inc., Bellefonte,

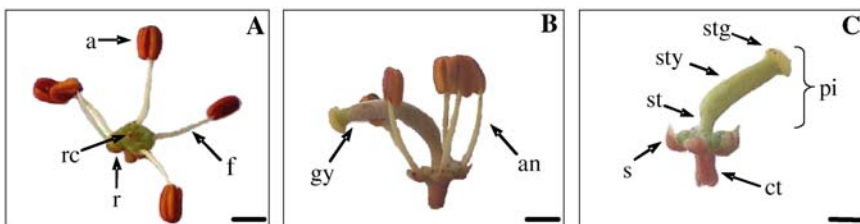


Fig. 1 (A) Male flower with anthers (a), filament (f), rudimentar carpel (rc), and receptacle (rc; bar = 8.75 mm). (B) Hermaphrodite flower with androecium (an) and gynoecium (gy; bar = 6.96 mm). (C) Female flower with a calyx tube (ct), sepals (s), and a pistil (pi) formed by a short stipe (st), an abbreviated style (sty), and a peltate stigma (stg; bar = 13.88 mm)

Table 1 Weight (mg) of whole flowers and isolated flower organs of the three genders of carob

	Hermaphrodite	Male	Female
Whole flower	100 ± 10Aa	30 ± 1Ba	30 ± 2Ba
Stamens	40 ± 1Ab	20 ± 1Bb	–
Nectary disks	30 ± 1Abc	10 ± 0Bd	10 ± 2Bb
Stigmas	20 ± 0c	–	20 ± 2c

In each column, statistical comparisons were made between different floral parts of the same sexual type and are shown in lower case letters. In each row, statistical comparisons were made between genders, for the same flower organ, and are shown in capital letters. Values represent means ± SE of three replicates with 25 flowers each. Values followed by different letters are significantly different at $P < 0.05$ (one-way ANOVA, Duncan's new multiple range test). Absence of letters indicates no significant differences at $P \geq 0.05$.

PA, USA), coated with polydimethylsiloxane fiber (100 μm), was first activated by inserting it into the GC injector port at 250°C for 1 hr. For sampling, the fiber was inserted into the headspace for 15 min at room temperature (20°C). Following sampling, the SPME device was introduced into the injector port for chromatographic analysis, where it remained in the inlet during the run, thus preparing it for the next collection of volatiles. The analyses were performed in triplicate, and blank assays using empty vials were conducted as controls.

Capillary GC/MS analyses were performed on an Agilent 6890 series gas chromatograph interfaced to an Agilent 5973 *N* mass selective detector (Agilent Technologies, Little Falls, DE, USA). A vaporization injector was used in the splitless mode (2 min) at 250°C, with a fused silica capillary column, 30 m \times 0.32 mm ID \times 0.25 μm d_f (HP-5MS; 5% diphenyl, 95% dimethyl polydimethylsiloxane, Agilent Technologies). The oven temperature program was 40°C for 2 min and then increased at 5°C min^{-1} to 180°C, followed by 15°C min^{-1} to 240°C, and held isothermally for 15 min. Helium was used as carrier gas at 30 cm sec^{-1} .

Electron ionization mass spectra in the range 40–400 Da were recorded at 70 eV. The quadrupole, source, and transfer line temperatures were maintained at 150, 230, and 280°C, respectively, and a turbo molecular pump (10^{-5} torr) was used. All data were recorded with an MS ChemStation (G1701CA; Rev C.00.00; Agilent Technologies).

The identity of each compound was determined by comparison of its retention index relative to C_6 – C_{24} n-alkanes (Adams, 2001), as well as of its spectral data with the Wiley's library spectral data bank (G1035B; Rev D.02.00; Agilent Technologies) or homemade libraries (Flavour2.1). For semiquantification purposes, the normal-

Fig. 2 Contribution of stamens (\square), stigmas (\blacksquare), and nectary disks (\blacksquare) to the mass of whole hermaphrodite, male, and female flowers of carob

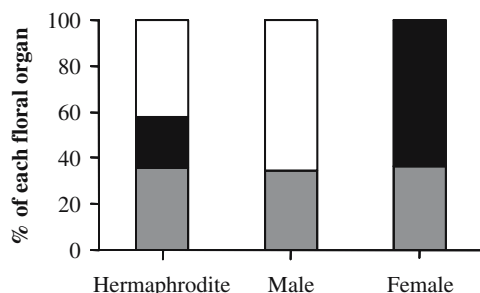


Table 2 Constituents and average compositional percentage of the volatiles of whole flowers (WF) and isolated flower organs (ND: nectary disk; Sta: stamens; Sti: stigmas) from field-grown male, hermaphrodite and female carobs (cv. Mulata and Galhosa) by HS-SPME-GC/MS.

Compounds	RI ^a	Composition (%) ^b												
		Hermaphrodite				Male			Female (Galhosa)			Female (Mulata)		
		WF	ND	Sta	Sti	WF	ND	Sta	WF	ND	Sti	WF	ND	Sti
Monoterpene hydrocarbons														
α -Pinene	902	16.3	7.5	13.4	38.8	3.2	13.4	20.7	9.8	12.3	–	59.4	4.4	20.8
Sabinene	944	0.5	0.2	–	0.2	–	0.7	–	0.3	0.7	–	0.9	–	–
β -Pinene	947	2.0	0.9	–	3.0	–	1.8	–	0.9	1.3	–	5.1	–	–
β -Myrcene	966	–	1.4	–	1.3	–	2.1	–	2.1	2.6	–	1.4	–	–
β -Terpinene	991	–	–	–	0.6	–	0.1	–	–	–	–	0.5	–	–
Limonene	1006	1.7	2.0	–	2.5	0.9	0.9	–	12.6	12.9	3.8	3.1	–	–
<i>cis</i> -Ocimene	1014	–	0.8	–	0.5	–	0.7	–	2.4	2.3	–	0.5	–	–
<i>trans</i> - β -Ocimene	1026	0.6	–	–	8.9	–	0.2	–	–	–	–	–	–	–
γ -Terpinene	1037	–	–	–	1.4	–	0.2	–	–	0.3	–	0.8	–	–
Oxygenated monoterpenes														
<i>trans</i> -Linalool furan oxide	1051	5.5	7.6	1.1	2.6	7.8	8.4	–	7.5	6.1	5.5	2.4	9.1	3.3
<i>cis</i> -Linalool furan oxide	1068	26.4	21.8	44.4	17.5	32.9	21.8	14.9	23.5	15.8	31.8	10.3	26.9	28.7
Linalool	1080	6.7	15.4	1.0	2.4	9.9	22.5	2.3	4.4	11.5	2.3	1.7	11.1	1.3
2.2.6-Trimethyl-3-keto-6-vinyltetrahydropyran	1088	9.1	11.0	5.0	7.4	13.5	6.6	3.1	3.7	–	14.1	1.0	3.7	7.8
<i>cis</i> -Linalool pyran oxide	1154	2.1	1.3	4.0	1.0	1.7	1.3	3.4	0.8	0.7	2.0	0.3	0.9	2.5
<i>trans</i> -Linalool pyran oxide	1158	6.3	6.3	15.6	2.2	7.3	5.8	9.8	3.7	6.8	9.4	1.0	5.1	9.6
4-Terpineol	1164	–	–	–	0.8	–	–	–	–	–	–	0.4	–	3.2
Sesquiterpene hydrocarbons														
α -Copaene	1363	0.1	0.3	–	0.1	–	–	–	0.6	0.7	0.9	0.8	0.7	–
β -Bourbonene	1375	–	–	–	–	0.4	–	–	–	–	–	0.5	0.7	–
β -Guaiene	1453	–	0.3	–	–	–	0.2	–	–	0.2	–	0.5	–	–
Valencene	1453	–	–	–	–	–	–	–	0.4	1.6	–	–	–	–
Germacrene-D	1461	–	–	–	–	–	–	–	–	–	–	–	1.6	–
α -Farnesene	1477	5.9	8.5	2.3	2.9	3.1	3.0	–	12.2	9.5	1.2	1.8	6.6	4.2
δ -Cadinene	1494	0.8	1.3	–	–	–	0.1	–	2.3	1.5	7.2	1.7	2.4	2.6
Others														
Theaspirane A ^c	1288	0.5	0.4	0.9	0.3	–	0.1	–	0.3	0.2	2.5	0.5	0.5	–
Theaspirane B ^c	1304	0.4	0.3	0.6	0.3	–	0.1	–	0.3	0.2	1.6	0.5	0.5	–
Methyl eugenol ^d	1384	–	0.8	–	–	2.8	1.7	–	3.2	5.7	–	1.5	4.7	–
Phytol ^e	1553	0.7	–	–	0.5	1.2	–	–	–	–	–	–	–	–
Monoterpene hydrocarbons		21.0	12.9	13.4	57.2	4.1	20.3	20.7	28.1	32.4	3.8	71.6	4.4	20.8

Table 2 (continued)

Compounds	RI ^a	Composition (%) ^b														
		Hermaphrodite				Male			Female (Galhosa)			Female (Mulata)				
		WF		ND		Sta		Sti	WF		ND	Sta	WF		ND	Sti
		WF	ND	Sta	Sti	WF	ND	Sta	WF	ND	Sti	WF	ND	Sti		
Oxygenated monoterpenes		56.2	63.4	71.1	33.9	73.1	66.3	33.5	43.8	40.8	65.2	17.1	56.8	56.4		
Sesquiterpene hydrocarbons		6.9	10.3	2.3	3.0	3.5	3.4	–	15.0	12.0	9.3	5.7	13.6	6.8		
Others		1.5	1.5	1.5	1.1	4.0	1.9	–	3.8	6.0	4.0	2.6	5.7	–		
Total identified		85.6	88.1	88.3	95.2	84.7	91.9	54.2	90.6	91.2	82.4	97.0	80.4	84.0		

^a Retention index relative to C₉–C₁₇ n-alkanes on the HP-5MS capillary column.

^b Normalized peak areas abundances without using the correction factors.

^c Carotenoid derivative.

^d Phenylpropanoid derivative.

^e Diterpene alcohol.

ized peak area of each compound was used without any correction factors to establish abundances.

Results

Weight of Whole Flowers and Floral Organs

The gender significantly affected the weight of whole flowers and isolated flower organs ($P < 0.05$), except for stigmas. Male and female flowers had similar mean weights, and the hermaphrodites were heavier (Table 1). In the hermaphrodites, the stamens were heavier than the stigmas, and in males and females, the heavier flower organs were the stamens and the stigmas, respectively (Table 1).

In the male flowers, the stamens and nectary disk comprised 64.6 and 33.7% of the total flower weight, whereas in the females, 58% of the total floral weight corresponded to the stigma and 33.1% to the nectary disk (Fig. 2). In the hermaphrodite flowers, the contribution of stamens, nectary disk, and stigma to the total flower weight was 38.4, 32.6, and 20.4%, respectively (Fig. 2).

Headspace Emission from Whole Flowers

More than 25 compounds were identified, representing 54.2–97.0% of the total volatile constituents (Table 2). The scent of the carob flowers is richer in monoterpenes than in sesquiterpenes, and linalool and its derivatives are the most abundant compounds. Differences in volatile composition were observed between the genders and the two female cultivars (Table 2).

The headspace of whole flowers of hermaphrodites, males, and female Galhosa were mainly constituted of oxygenated monoterpenes, accounting for 56.2, 73.1, and

43.8% of the total volatile emission, respectively (Table 2). The sesquiterpene compounds were present in higher abundances in the scent of whole flowers of hermaphrodites (6.9%) and females (Mulata: 5.7% and Galhosa: 15.0%; Table 2). The major components of the scent from the hermaphrodite and male flowers were *cis*-linalool furan oxide (26.4 and 32.9%, respectively), *trans*-linalool furan oxide (5.5 and 7.8%), *trans*-linalool pyran oxide (6.3 and 7.3%), linalool (6.7 and 9.9%), α -pinene (16.3 and 3.2%), α -farnesene (5.9 and 3.1%), and 2,2,6-trimethyl-3-keto-6-vinyltetrahydropyran (9.1 and 13.5%; Table 2 and Fig. 3).

In the two female cultivars, differences in the number and abundance of the compounds emitted by whole flowers were observed (Table 2 and Fig. 3). The Galhosa cultivar had a lower content of volatiles than the Mulata cultivar, dominated by *cis*-linalool furan oxide (23.5%), limonene (12.6%), α -farnesene (12.2%), and α -pinene (9.8%; Table 2 and Fig. 3). In the Mulata, 23 compounds were identified, and more than half of the fragrance of whole flowers consisted of α -pinene (59.4%), a highly volatile compound; other important constituents were *cis*-linalool furan oxide (10.3%) and β -pinene (5.1%; Table 2 and Fig. 3).

Headspace Emission from Flower Organs

There were differences in the composition of the volatiles emitted by the different floral organs (Table 2 and Fig. 3). Moreover, differences were observed between the female cultivars, in the spatial fragrance patterns within the flowers (Table 2 and Fig. 3). Cultivar Mulata emitted a higher number of volatiles compared to Galhosa, but a lower abundance of the principal compounds (Table 2 and Fig. 3). Similar to the patterns observed in hermaphrodites, the male and both female cultivars had nectary disks that showed a richer blend of compounds compared to the sexual organs (Table 2).

Nectary Disks

These structures were the major source of volatiles, and the relative abundances of the major ones were higher in the males, followed by the hermaphrodites, Galhosa and Mulata (Fig. 3). In the hermaphrodites, the main compounds in the scent of nectary disks were *cis*-linalool furan oxide (21.8%), linalool (15.4%), *trans*-linalool furan oxide (7.6%), α -pinene (7.5%), and *trans*-linalool pyran oxide (6.3%). In the cultivar Galhosa, the volatiles profile of whole flowers and nectary disks were similar, except for γ -terpinene and β -guaiene, which are only present in nectary disks in very low amounts (0.3 and 0.2%, respectively; Table 2).

Stamens

In the hermaphrodite flowers, the fragrance of the stamens consisted almost exclusively of monoterpenes, and only one sesquiterpene (α -farnesene) was detected (Table 2). Almost half of the fragrance consisted of *cis*-linalool furan oxide (44.4%; Table 2 and Fig. 2). Other important compounds were *trans*-linalool pyran oxide (15.6%) and α -pinene (13.4%; Table 2 and Fig. 3). In hermaphrodite and male flowers, only one component of the monoterpene hydrocarbon fraction (α -pinene) was emitted by the stamens (Table 2). In the males, the stamens did not produce sesquiterpenes (Table 2).

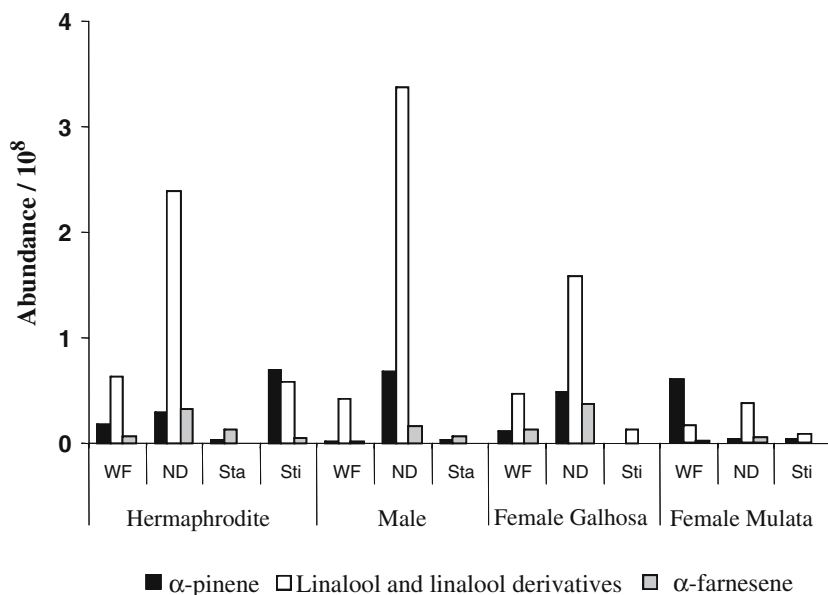


Fig. 3 Average abundance of α -pinene, linalool, and linalool derivatives, and α -farnesene, found in the volatiles of whole flowers (WF) and isolated flower organs (ND: nectarial disk; Sta: stamens; Sti: stigmas) from field grown male, hermaphrodite, and female carobs by HS-SPME-GC/MS

It is worth noting that, in the male flowers, only six monoterpene compounds were positively identified in the headspace of the stamens compared to those in the hermaphrodites. The three main volatiles, representing 45.4% of the total constituents, were α -pinene (20.7%), *cis*-linalool furan oxide (14.9%), and *trans*-linalool pyran oxide (9.8%; Table 2 and Fig. 3).

Stigmas

In the hermaphrodite, α -pinene dominated the headspace of the stigma (38.8%), together with *trans*- β -ocimene (8.9%) and *cis*-linalool furan oxide (17.5%; Table 2 and Fig. 3).

In female Mulata, the emission of its major volatile compound (α -pinene) depended largely on the emission by the stigma (20.8%). In female Galhosa, only one monoterpene hydrocarbon is present in the headspace of the stigma (Table 2).

Discussion

The scent of carob flowers is mainly attributed to a high abundance of monoterpenes and to a less content of sesquiterpenes, and linalool and its derivatives were the most abundant compounds, as previously reported in this (Custódio et al., 2004) and in other species (Azuma et al., 2001). Both the gender and the cultivar affected the number and abundance of the compounds in the headspace of whole flowers.

Contrary to previous observations (Custódio et al., 2004), female flowers had a higher diversity of volatile than males and hermaphrodites. The flower volatile composition differed from that obtained in earlier findings: linalool was in much lower abundance, while amounts of *trans*-linalool oxide were comparatively higher. Furthermore, besides linalool and *trans*-linalool pyran oxide, other derivatives of that acyclic monoterpene alcohol were identified, namely, *cis*-linalool pyran oxide, 2,2,6-trimethyl-3-keto-6-vinyltetrahydropyran, *cis*-linalool furan oxide, and *trans*-linalool furan oxide. Both studies were based on samplings taken in the field. Therefore, environmental conditions, such as temperature, humidity, light, and time of the day, may have affected the total amount and chemical composition of the floral scents (Azuma et al., 2001). Furthermore, those differences could be ascribed to the different germplasm tested, sampling period, or physiological status of the trees (Siani et al., 2002).

In spite of the fact that the female Mulata emitted a higher number of volatiles compared to female Galhosa, the abundance of the principal volatiles was lower, and there were remarkable differences between the female cultivars in spatial fragrance patterns within flowers. In the Mulata, emission of its major volatile compound (α -pinene) depended largely on emission by the stigma. In the Galhosa, the volatiles profile of whole flowers and nectary disks were similar, which may indicate that the scent of the nectary disks resembled more the fragrance of the whole flowers rather than the gynoecium. Genetic variation within a species may modify the amount and type of compounds in an odor profile (Wright et al., 2002), which may explain the differences in the volatile composition between the two female cultivars. Intraspecific variation in floral scent chemistry has been reported in other species, namely, in *Platanthera bifolia* and *P. chlorantha* (Tollsten and Bergström, 1993), *Clarkia brewerii* (Nam et al., 1999), *Antirrhinum majus* (Dudareva et al., 2000), and *Tanacetum vulgare* (Keskitalo et al., 2001). There are many reasons for intraspecific chemical variation, namely, adaptation to different pollinator species, introgression effects involved in hybridization events, random genetic drift, adaptations to disruptive learning processes in pollinators among deceptive (nonrewarding) flowers, etc. (Tollsten and Bergström, 1993; Barkman et al., 1997). In the carob tree, there are few studies about floral visitors and pollination (Ortiz et al., 1996; Arista et al., 1999), and only one (Ortiz et al., 1996) reports differences between males and females. Nothing is known about pollinator assemblages of different female cultivars, although this information is crucial for the ecological investigation of reproductive traits (Johnson and Steiner, 2000).

The female Portuguese cultivars Galhosa and Mulata are economical and ecologically interesting (Martins-Loução and Brito de Carvalho, 1989; Batlle and Tous, 1997), and the Mulata, in particular, is considered a very productive cultivar (Batlle and Tous, 1997). Pollination is a critical step for the successful production of fruits and seeds, and thus, the absence of efficient pollination can lead to low crop yields. Different scent compounds or chemical compositions may attract different pollinator species, which could cause reproductive success to vary among scent types, and the types will encounter different selective pressures from pollinators (Azuma et al., 2001). The higher amounts of volatiles identified in flowers from cultivar Mulata could be related to a more successful mechanism of reproduction leading to more efficient pollination and thus to higher production. This hypothesis needs further research through field observations of natural pollinators and

bioassays using scent compounds. It is also necessary to assess the different scent compounds and chemical profiles found in the genders and female cultivars of carob to determine if they are equally effective in attracting pollinators.

In this work, the dominant volatiles (linalool, linalool derivatives, and α -pinene) were found in all floral organs, except in the stigma of the female Galhosa, which did not include α -pinene in its scent. Usually all floral organs contain volatiles typical of that species (MacTavish and Menary, 1997). However, quantitative differences between attractive, nonfood structures (perianth) and attractive food sources, such as the androecium and gynoecium, are common (Dobson et al., 1990). Generally, the scent composition emitted by female organs is markedly different from stamens (Pichersky et al., 1994). Spatial scent patterns within a flower may function like visual patterns. Thus, differences in the strength and/or quality of emitted volatiles between floral organs may serve as guides to insects, assisting them in finding food rewards or leading them to position themselves suitably on a flower for effective pollination (Bergström et al., 1995). Furthermore, the existence of organ-specific synthesis of certain compounds was observed, which can be related to the fact that the pathway(s) for synthesis and pattern of accumulation of terpenes differs considerably between the floral organs (Dudareva and Pichersky, 2000).

Despite the fact that sexual organs accounted for higher percentages of the total flower weight compared to the nectary disks, these structures were the major source of volatile emission. This observation, together with previous findings (Custódio et al., 2004), suggests the presence of scented nectar in carob tree flowers.

Nectar is a multicomponent aqueous solution and contains aromatic compounds and other substances (Tóth et al., 2003). Among these, the most important are sucrose, glucose, and fructose (Thornburg et al., 2003). The various types of nectars can be ordered into three groups, according to sugar content: sucrose prevalent, glucose and fructose prevalent, and almost equal amounts of sucrose, glucose, and fructose (Roshchina and Roshchina, 1993). In the carob, glucose is the major nectar sugar, regardless of the tree genders, and it is present in higher concentration in male flowers, followed by the female Galhosa, the female Mulata, and the hermaphroditic ones (Custódio, 2005).

Scented nectar was found on four out of seven tested angiosperms (Raguso, 2004) and would provide an honest signal to nectar-feeding pollinators (Raguso, 2004; Dötterl and Jürgens, 2005), or serve a defensive function, such as protecting the plant crops from harmful visitors (Galen, 1999; Raguso, 2004). Additionally, scented nectar may also have an indirect impact on plant fitness, through antimicrobial activity, pleiotropic interactions with plant defense, and communication with predators and parasitoids (Pichersky and Gershenzon, 2002; Raguso, 2004).

In summary, the HS-SPME-GC/MS method allowed the collection and the analysis of volatiles emitted from whole fresh flowers and from different flower organs of three genders and two female cultivars of the carob. The reason for the chemical variation observed in floral scent between the genders and female cultivars of the carob is unknown. The results raise some pertinent questions, namely, the nature of the pollinators of the different genders and female cultivars and their reproductive success. It is also still necessary to assess if the different scent compounds and chemical profiles found in the genders and female cultivars of carob are equally effective in attracting pollinators.

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Antifeedants in the Feces of the Pine Weevil *Hylobius abietis*: Identification and Biological Activity

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Abstract Egg-laying females of the pine weevil, *Hylobius abietis* (L.), regularly deposit feces adjacent to each egg. Egg cavities are gnawed in the bark of roots of recently dead conifer trees. After egg deposition, the cavity is sealed by feces and a plug of bark fragments. Root bark containing egg cavities with feces is avoided as food by pine weevils, which indicates the presence of natural antifeedants. Here we present the first results of the isolation and chemical analyses of antifeedant compounds in the feces of *H. abietis*. In feeding bioassays, methanol extracts of the feces revealed strong antifeedant properties. Methanol extracts were fractionated by medium-pressure liquid chromatography and the antifeedant effects were mainly found in the fractions of highest polarity. Volatile compounds in the active fractions were identified by gas chromatography–mass spectrometry (GC–MS) and the nonvolatile compounds were characterized by pyrolysis–GC–MS. Based on mass spectra, a number of compounds with various chemical structures were selected to be tested for their antifeedant properties. Antifeedant effects were found among compounds apparently originating from lignin: e.g., a methylanisol, guaiacol, veratrol, dihydroxybenzenes, and dihydroconiferyl alcohol. A weak effect by fatty acid derivatives was found. The types of naturally occurring antifeedant compounds identified in this study may become useful for the protection of planted conifer seedlings against damage by *H. abietis*.

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Introduction

Adult pine weevils, *Hylobius abietis* (L.) (Coleoptera: Curculionidae), feed on tender bark of a wide range of conifer trees, with Scots pine (*Pinus sylvestris* L.) as the preferred host (Day et al., 2004). Bark of living as well as of recently dead trees is utilized, and weevils feed both below and above ground, although a sheltered feeding position is preferred (Nordlander et al., 2005). Planted conifer seedlings are frequently girdled and killed by weevil feeding, which makes *H. abietis* a severe pest of reforestation in large parts of Europe and Asia (Långström and Day, 2004).

Pine weevil larvae develop under the bark of recently dead conifer roots, in managed forests usually the roots of fresh stumps (Day et al., 2004). Large numbers of flying weevils of both sexes arrive during spring migration in areas with fresh stumps for reproduction (Örlander et al., 2000), attracted by the degradation product ethanol together with α -pinene and other host monoterpenes (Nordlander et al., 1986; Schlyter, 2004). Females lay their eggs either in the soil near roots or in specific egg cavities gnawed into the root bark (Nordlander et al., 1997).

The habit of inserting eggs into host plant tissues with the aid of the snout is an ancestral trait of the weevil family (Marvaldi et al., 2002). To construct the egg cavity, female *H. abietis* chew through the outer bark and continue into the phloem tissue along the xylem, about as far as reached by her snout (Fuchs, 1912). She then turns around and deposits an egg together with feces in the cavity, after which she turns once again and pushes the egg and feces deeper into the cavity with her snout (Nordlander et al., 2000). The opening of the cavity is finally sealed with a plug made of fragments nibbled from the outer bark (*H. Nordenhem*, personal observation). Similar egg-laying behavior has been observed in other *Hylobius* species (Wen et al., 2004), as well as in species of the weevil genus *Pissodes*, ovipositing in bark of aboveground parts of conifer trees (Wallace and Sullivan, 1985; Langor and Williams, 1998; Zhang et al., 2004), and in the boll weevil, which oviposits in flower buds (Stansly and Cate, 1984).

It is not known why female *H. abietis* place her feces close to or on top of her eggs. A likely function of the feces is that it contains oviposition deterrents (Anderson, 2002), as suggested for *Pissodes terminalis* (MacLaughlan and Borden, 1995). Feces might also contain substances that give protection to the egg against predation (Blum and Hilker, 2002) or the destruction by feeding pine weevils (Nordlander et al., 1997). Indeed, it has been reported that most feeding is done on the opposite side of pine bolts from where the majority of eggs were laid (Bylund et al., 2004). If feces in the oviposition cavity have a feeding-deterrent effect on *H. abietis*, this may be either a mechanism to avoid feeding that destroys eggs in the bark or a mechanism to avoid making oviposition cavities adjacent to existing ones, which may later cause competition for the newly hatched offspring.

A primary aim of our research was to find the chemical cues that deter pine weevils from feeding at oviposition sites. In this study, we investigated whether constituents in the feces from *H. abietis* could inhibit or stop the initiation of feeding by other individuals of this species. The antifeedant activity in feces extracts and

fractions thereof was measured in a laboratory feeding bioassay by using twigs of Scots pine as food.

Methods and Materials

Collection and Maintenance of Weevils

Pine weevils of both sexes were collected during spring migration at a sawmill in southern Sweden, where they landed in large numbers as a response to a massive emission of attractive conifer volatiles. After collection, weevils were stored in darkness at 10°C and provided with fresh Scots pine branches or stems with tender bark as food. These storage conditions interrupted their reproductive development, so that no oviposition started until about a week after the weevils had been transferred into experimental conditions, i.e., 22°C and 18:6 (L/D) regime. This transfer of weevils was made about 10 d before their use in a bioassay or for the production of feces.

Feeding Bioassay

Various extracts, fractions, and single compounds were tested for antifeedant effects on the *H. abietis* by using a two-choice laboratory bioassay described by Bratt et al. (2001). Fresh pieces of Scots pine twigs (50 mm long, 15 mm diam) were split, and each half (= test twig) was wrapped in aluminum foil. On each test twig, two sharp-edged metal rings (5 mm diam) were placed at 25 mm distance and punched through the foil into the bark. The rings and the pieces of aluminum foil inside them were then removed. The thin outer layer of cork bark inside two circular areas was carefully removed with a scalpel. Thereafter, new rings were fitted into the bark around the two bark areas exposed and 100 µl of the extract/compound to be tested were applied to the bark in one of the two rings. In the other ring, the same amount of the solvent was applied as control. When the solvent had evaporated, the metal rings were removed. Each test twig was placed onto moistened filter paper in a 142-mm-diam Petri dish and one weevil was placed into each dish. Forty replicates were used, 20 with females and 20 with males. The weevils were all in the reproductive phase of their life cycle and were starved for 24 hr in the Petri dishes prior to the test period. Each weevil was used only once. The bioassays were conducted at room temperature (ca. 22°C).

The amount of feeding on treatment and control areas of each test twig was recorded after 6 and 24 hr. The proportion of available bark area that had been penetrated by the test weevil was estimated under a stereomicroscope and with the aid of a transparent millimeter grid. In most of the tests, there was no significant difference in response between the sexes, and most of the data presented were, therefore, pooled. Effects of the various treatments were described by two variants of the antifeedant index (AFI; Blaney et al., 1984): $(C - T) \times 100 / (C + T)$. (1) In AFI area (AFIa), C represents the mean area of control surfaces consumed and T the mean consumed area of treated surfaces. (2) In AFI number (AFIn), C is the number of control surfaces with feeding scars and T the number of treated surfaces with feeding scars.

Hence, AFIn indicates to what extent feeding is completely inhibited on the treated area during 24 hr, whereas AFIA also includes the reduction in feeding where it has been initiated. The two indices are fairly well correlated, but AFIA tends to be higher than AFIn because the antifeedant substances generally affect both the initiation of feeding and the amount consumed, if feeding has started. For both indices, an antifeedant effect gives positive values up to a maximum of 100. Statistical differences in feeding/no feeding between treatment and control were tested with Fisher's exact test of a 2×2 table: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Collection and Extraction of Feces

Feces were collected by placing weevils of either sex into clean glass Petri dishes for 24 hr. No food was provided in the Petri dishes but the weevils had been feeding on fresh Scots pine bark until just before the feces collection started. After the 24 hr period, the weevils were removed and the feces were scraped off the glass surfaces with a razor blade. The collecting procedure was repeated during several days and the feces were stored in a sealed vial at 5°C for up to a week before the entire material from each sex was subjected to extraction. Feces were divided into two equal parts: one was extracted with methanol (pa Merck) and the other with n-hexane (pa Merck). The methanol extract was first washed with hexane and then subjected to chromatography. Extracts were tested in the feeding bioassay at a concentration corresponding to the amount of feces produced by three individuals during 24 hr. This was a high concentration, more than what the female deposits on an egg, but used to compensate for the anticipated loss of activity due to absorption effects during the solvent extraction.

Isolation and Fractionation

The hexane-washed methanol extracts from 1.5 to 1.8 g of feces were evaporated under reduced pressure at ambient temperature. The gummy residue obtained was dissolved in a minute amount of methanol, mixed with silica gel (Merck 60, 230–400 mesh ASTM), and the solvent was evaporated to dryness. Medium-pressure liquid chromatography (MPLC) of the extracts was carried out on silica gel, using a laboratory pump and preparatory chromatography columns (Baeckstrom Separo AB, Stockholm, Sweden). A representative fractionation by MPLC started with pure hexane, followed by a gradient of hexane/ethyl acetate, to obtain fractions containing compounds with increasing polarity. Subsequent changing of the gradient to ethyl acetate/methanol and finally elution of the column with pure methanol gave fractions with increasing polarity. In all, more than 80 chromatography fractions were collected. These were pooled into 10 major fractions, guided by thin-layer chromatography (TLC), and selected by obvious chemical resemblance. An attempt to fractionate the most polar fraction resulted in partial solvolysis during the chromatography procedure, and a new medium polar fraction was thereby obtained. The most polar compounds had an R_f value of 0–0.05 on TLC (using 40% ethyl acetate in hexane as eluent). The hydrolyzed medium polar fraction had a broad R_f value of 0.4–0.7. TLC was visualized by spraying with a solution of 3% vanilla (by weight) in 1% conc. sulfuric acid (by volume) dissolved in ethanol. Hexane and methanol extracts, and the MPLC fractions dissolved in methanol were tested for their antifeedant properties.

Chemical Identification of Volatile Constituents

Separation and identification of components in the various extracts and fractions were made by gas chromatography–mass spectrometry (GC–MS) using a Varian 3400 GC connected to a Finnigan SSQ 7000 quadrupole mass spectrometer. The GC was equipped with a split/splitless injector (splitless mode, 30 sec; injector temperature, 220°C; carrier gas, He). A DB-5 or a DB-wax capillary column (30 m, 0.25 mm i.d., and 25 μ m film thickness) was used with a temperature program: 40°C (1 min)–5°C/min–230°C (in 20 min). The MS ion source temperature was 150°C; mass spectra were obtained at 70 eV with a mass range of 30–400 *m/z*. Mass spectra of unknown compounds were compared to the Finnigan NIST MS library, and available authentic standards by comparing GC retention times and by co-injections.

Chemical Identification of Nonvolatile Constituents

The gummy mixture obtained from the most polar MPLC fraction was dissolved in methanol and analyzed by a pyrolysis–GC–MS both with and without the alkylating reagent tetramethylammonium hydroxide (Hardell and Nilvebrandt, 1996). The injector temperature was kept either at 400°C or at 600°C. A DB-5 fused silica capillary GC column (30 m, 0.25 mm i.d., and 0.25 μ m film thickness) was used with the temperature program 50°C (5 min)–10°C/min–310°C.

Reference Compounds

Synthesis of dihydroconiferyl alcohol, 3-(4-hydroxy-3-methoxyphenyl)propan-1-ol: Methyl 3-(4-hydroxy-3-methoxyphenyl)propanoate (1.57 g, 7.48 mmol, obtained from previous work by Drs. H. Erdtman and T. Norin at the Department of Chemistry, Organic Chemistry, KTH, Stockholm, Sweden) was reduced by lithium aluminum hydride (570 mg, 15 mmol) in tetrahydrofuran (THF, 20 ml). The reaction mixture was neutralized with diluted sulfuric acid and partitioned between *t*-butyl methyl ether and water. The organic phase was separated and dried with MgSO₄, filtered and then mixed with silica gel, after which the solvent was evaporated. Dihydroconiferyl alcohol (1.089 g, 5.98 mmol) was obtained after chromatography. The yield was 80%. ¹H NMR δ : 6.83 (1H, d, *J* = 7.6 Hz), 6.70 (1H, s), 6.68 (1H, d, *J* = 7.5 Hz), 3.86 (3H, s), 3.67 (2H, t, *J* = 6.4 Hz), 2.64 (2H, t, *J* = 7.6 Hz), 1.86 (2H, m).

Synthesis of 1-(4-hydroxy-3-methoxyphenyl)-3-methoxypropane and 1-(3,4-dimethoxyphenyl)-3-methoxypropane: Sodium hydride in oil (91 mg, 2.28 mmol) was rinsed with hexane and then a mixture of 3-(4-hydroxy-3-methoxyphenyl)propan-1-ol (215 mg, 1.18 mmol) in 1.5 ml THF, was added. After the evolution of hydrogen had ceased, methyl iodide (170 mg, 1.20 mmol) diluted with 1.5 ml THF was added. The reaction mixture was heated at 38°C for 4 hr. Toluene (1 ml), ammonium chloride (125 mg, 2.38 mmol), and silica gel (1.2 g, Merck 60 type) were added; the reaction mixture was impregnated to dryness and the impregnated silica gel was put on top of a silica gel column. Two methylated products (109 mg) and some unreacted starting material were obtained after chromatography. The mono-methylated product was separated from the dimethylated product in a subsequent chromatography: 1-(4-hydroxy-3-methoxyphenyl)-3-methoxypropane (major product): ¹H NMR δ : 6.83 (1H, d, *J* = 7.9 Hz), 6.70 (1H, s), 6.68 (1H, d, *J* = 7.8 Hz), 5.50

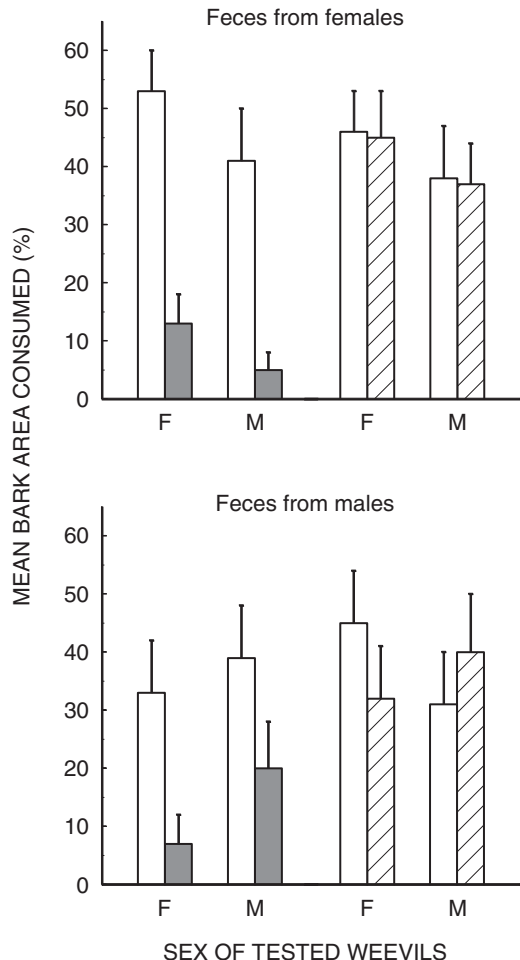
(1H, s), 3.86 (3H, s), 3.38 (2H, t), 3.35 (3H, s), 2.61 (2H, t, $J = 7.5$ Hz), 1.86 (2H, m). 1-(3,4-Dimethoxyphenyl)-3-methoxypropane (minor product): $^1\text{H NMR } \delta$: 6.81–6.71 (3H, m), 3.87 (3H, s), 3.86 (3H, s), 3.39 (2H, t, $J = 6.2$ Hz), 3.35 (3H, s), 2.63 (2H, t), 1.87 (2H, m).

All reference compounds were purchased from commercial suppliers (i.e., Lancaster or Sigma-Aldrich) except the two substances for which we have described the synthesis.

Results

Pine weevils of both sexes avoided feeding on pine twigs treated with a methanolic extract of pine weevil feces (Fig. 1). Feces from both sexes had this antifeedant effect, although males tended to be less deterred from feeding by feces from males than by feces from females. In the crude methanol extract of female feces (Fig. 2), a complex mixture of aromatic compounds (Table 1) and saturated and unsaturated

Fig. 1 Mean proportion of bark area consumed by *H. abietis* in experiments with 20 single weevils of either sex choosing between an area treated with feces extract and a control area treated with the solvent only. White columns, control; black columns, methanol extracts; hatched columns, hexane extracts. Bars denote SE



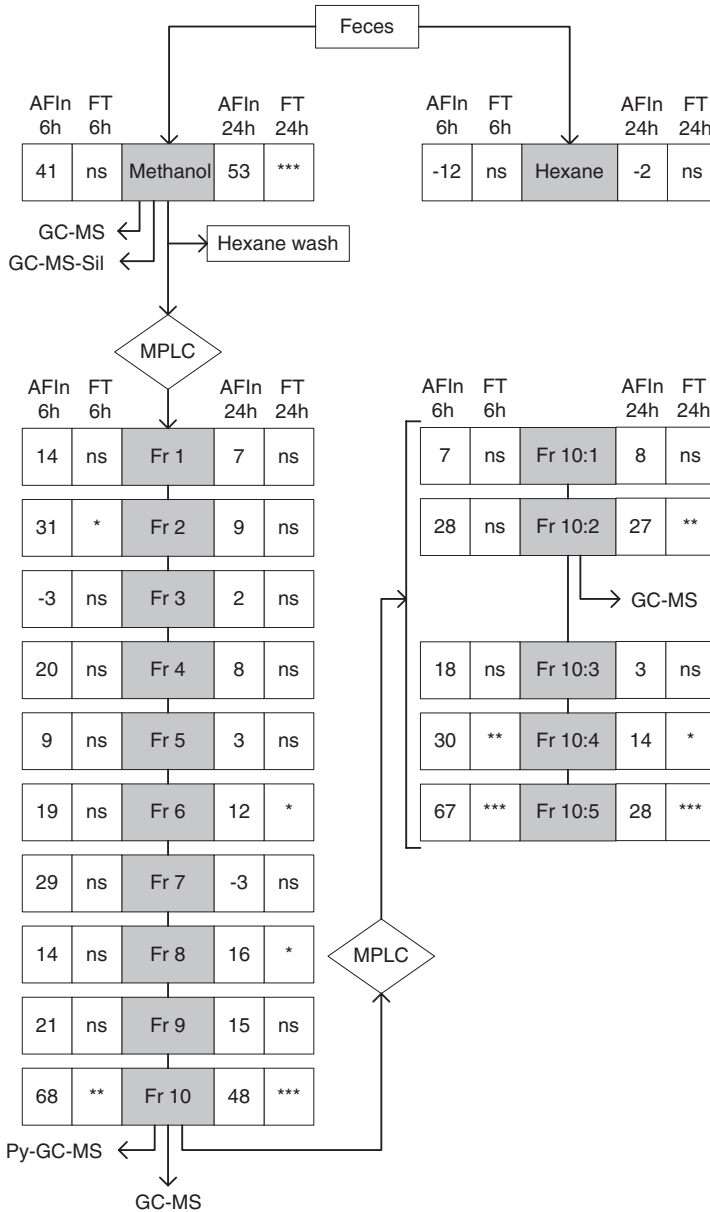


Fig. 2 Scheme showing the procedures of extraction and fractionation of feces collected from *H. abietis* females. The response by *H. abietis* to each fraction is shown as AFIn values after 6 and 24 hr (0 = no effect, 100=complete feeding inhibition). Significant differences from control are indicated as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Fisher's exact test)

Table 1 Main aromatic constituents identified in the most active extracts and fractions by GC–MS and pyrolysis–GC–MS (and some additional related substances tested for antifeedant properties)

MW	Identified substances	Structures	Methanol extract GC-MS	Most polar fraction 10 PY-GC-MS	Hydrolyzed MPLC-fraction 10:2 GC-MS	AFIa 6 hr	AFIa 24 hr	AFIn 6 hr	AFIn 24 hr
94	Phenol			x	x	90	36	84 ***	15 ns
108	1-Hydroxy-2-methylbenzene; <i>o</i> -Cresol			x	x	100	58	100 ***	44 ***
108	1-Hydroxy-4-methylbenzene; <i>p</i> -Cresol			x		92	12	81 ***	22 *
110	1,4-Dihydroxybenzene			x		87	73	85 ***	42 ***
120	Dihydrobenzofurane			x					
122	1-Methoxy-2-methylbenzene; 2-Methylanisol			x		73	12	79 ***	9 ns
122	1-Methoxy-3-methylbenzene; 3-Methylanisol					42	35	44 ***	23 ***
122	1-Methoxy-4-methylbenzene; 4-Methylanisol					58	14	54 ***	5 ns
124	1-Hydroxy-2-methoxybenzene; Guaiacol; 2-Methoxyphenol			x		97	42	93 ***	14 ns
124	1-Hydroxy-3-methoxybenzene; 3-Methoxyphenol			x					
136	1,2-Dihydroxy-4-etenylbenzene; 3,4-Dihydroxystyrene			x					
138	<u>An isomer of methylguaiacol</u>			x					
138	1,2-Dimethoxybenzene; Veratrol			x		29	2	39 ***	2 ns
138	1,4-Dimethoxybenzene					93	45	87 ***	31 ***
148	(<i>E</i>)-1-Methoxy-4-prop-1-enylbenzene; Anethole			x		95	61	94 ***	35 ***
164	3-(4-Hydroxy-3-methoxyphenyl)-1-propene; Eugenol			x		88	47	88 ***	31 ***
182	Dihydroconiferyl alcohol		xxx	xxx	xxx	32	16	27 *	9 ns
196	A 151,178,196; Identified by MS		x		x				
196	B 137,138,196; Identified by MS		x		x	57	21	60 ***	9 ns
196	1-(4-Hydroxy-3-methoxyphenyl)-3-methoxypropane			x	x	98	94	93 ***	76 ***
196	3-(3,4-Dimethoxyphenyl)-propan-1-ol					72	38	52 *	18 ns

x, minor component > 5% of total amounts according to total ion chromatogram (TIC)–GC–MS; xx, between 5 and 50%; xxx, main compound. Antifeedant effect shown by the antifeedant indices AFIa and AFIn (0 = no effect, 100 = complete feeding inhibition; see [Methods and Materials](#)). Asterisks refer to significance levels of the Fisher test.

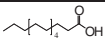
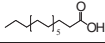
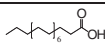
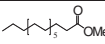
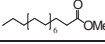
fatty acids with a length of 14–18 carbon atoms were identified by means of GC–MS (Table 2). In addition, oxygenated terpenes and resin acids were present in trace amounts. Among the structurally related aromatic compounds, the main constituent was identified as dihydroconiferyl alcohol (Fig. 3). Hexane extracts of both male and female feces contained large numbers of monoterpenes and sesquiterpenes. These extracts had no effect on feeding by any of the sexes (Fig. 1). Therefore, no further fractionations of the hexane extracts were made.

MPLC Fractions

The crude methanol extract was fractionated by MPLC and the 10 fractions were all tested for antifeedant activity (Fig. 2). The most polar fraction (fraction 10) showed significant and relatively strong antifeedant effect after testing periods of 6 and 24 hr. Fraction 2 had a significant, but weak, antifeedant effect after the first 6 hr, whereas fractions 6 and 8 showed weak antifeedant effects after 24 hr. No significant feeding differences were observed between the sexes.

The most polar fraction (10, Fig. 2) was characterized by the use of pyrolysis–GC–MS. A large number of possibly lignin-related aromatic compounds were present, e.g., phenol, dihydroxybenzene, methylanisols, guaiacol, veratrol, hydroxyl- and methoxy-substituted benzoic acids, a phenylacetate ester, and some phenyl-

Table 2 Main aliphatic constituents identified in the most active extracts and fractions by GC–MS and pyrolysis–GC–MS, cont. of Table 1

MW	Identified substances	Structures of identified substances	Methanol extract GC-MS	Most polar fraction 10 PY-GC-MS	Hydrolyzed MPLC-fraction 10:2 GC-	AFIa 6 hr	AFIa 24 hr	AFIn 6 hr	AFIn 24 hr
238	Tetradecanoic acid; Myristic acid		x			21	8	22 ns	5 ns
256	Hexadecanoic acid; Palmitic acid		x	x	trace				
254	(9Z)-Hexadecenoic acid; Oleic acid					(-) 11	2	(-) 14 ns	(-) 2 ns
270	Octadecanoic acid; Stearic acid		xx		trace				
268	An octadecenoic acid		xx		trace				
266	An octadecadienoic acid		x		trace				
264	An octadecatrienoic acid		x		trace				
272	Methyl hexadecanoate			x					
298	Methyl octadecanoate			x					
296	A methyl octadecenoate			x					
294	A methyl octadecadienoate; A methyl linoleate			x					
294	(9Z,12Z)-Methyl octadecadienoate; Methyl linoleate					57	34	58 **	31 ***

x, minor component >5% according to TIC–GC–MS; xx, between 5 and 50% of total amounts. Antifeedant effect shown by the antifeedant indices AFIa and AFIn (0 = no effect, 100 = complete feeding inhibition; see [Methods and Materials](#)). Asterisks refer to significance levels of the Fisher test.

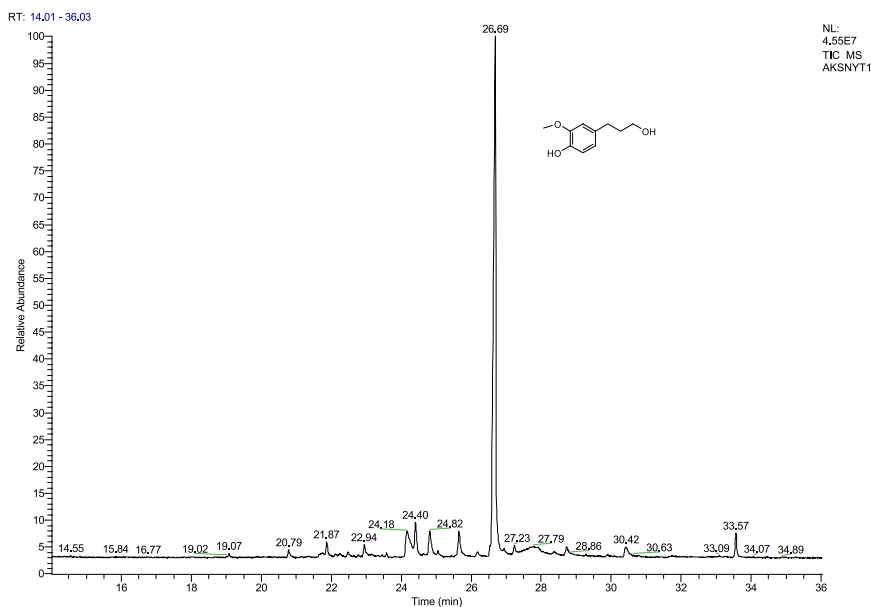


Fig. 3 Total ion chromatogram of fraction 10:2 obtained by GC–MS. The main compound is dihydroconiferyl alcohol

propanoids (Table 1). One of the main constituents was a compound with a mass spectrum similar to that of dihydroconiferyl alcohol.

This active MPLC fraction (10) was rechromatographed to give five fractions (10:1–10:5, Fig. 2). The strongest activity was still shown by the most polar fraction (10:5), apparently containing the major part of the lignin-related compounds already identified in fraction 10. In addition, weak antifeedant activities were found in fractions 10:2 and 10:4. Fraction 10:2 consisted of compounds that increased in concentration or formed during the collection and chromatography of fraction 10. These compounds appeared as a streaking blue spot with vanilla reagent in TLC (R_f value 0.4–0.7). The fraction contained more than 80% of one aromatic compound that was also present in the crude methanol extract (Fig. 3, peak at R_T 26.69). The following ^1H NMR data were found for this main constituent: ^1H NMR δ ; 6.83 (1H, d, $J = 7.9$ Hz), 6.70 (1H, s), 6.69 (1H, d, $J = 8.2$ Hz), 3.88 (3H, s), 3.68 (2H, t, $J = 6.4$ Hz), 2.64 (2H, t, $J = 7.5$ Hz), 1.87 (2H, m). These data and the mass spectrum suggest that the compound is dihydroconiferyl alcohol. However, the mass spectrum is also similar to that of homovanillic acid, commonly present in lignin fractions. Dihydroconiferyl alcohol was synthesized (see Reference Compounds), and by comparing MS data it was confirmed that the main constituent in the blue spot in fact was dihydroconiferyl alcohol (Table 1).

Test Results of Separate Constituents in the Active Fractions

Based on the GC–MS analyses of the crude extract and the active fractions, some identified compounds and some additional structurally related ones were selected for further testing of antifeedant properties (Table 1). The main component in the female extract was dihydroconiferyl alcohol [3-(4-hydroxy-3-methoxyphenyl)propan-

1-ol] (Fig. 3), but this compound showed only a weak antifeedant effect during the first 6 hr (AFIn = 27). The methyl ether of dihydroconiferyl alcohol [1-(4-hydroxy-3-methoxyphenyl)-3-methoxypropane], a slightly more hydrophobic compound, showed a strong antifeedant effect (AFIn = 93 after 6 hr and AFIn = 76 after 24 hr). Strong but short-lasting antifeedant effects were obtained with anethol (AFIn = 94, 6 hr), found in the methanol extract, and eugenol (AFIn = 88, 6 hr), present in the pyrolysis extract of the most polar MPLC fraction.

Monoaromatic molecules with one or two oxygen atoms showed a strong antifeedant effect during the first 6 hr of the test period. However, 1,4-dihydroxybenzene still had a significant antifeedant effect after 24 hr (AFIn = 42), as did the 1,4-dimethoxybenzene (AFIn = 31). The more volatile 1,2- and 1,4-anisoles (methoxymethylbenzenes) had a high effect, lasting for 6 hr, whereas 1,3-anisole remained active as antifeedant after 24 hr (AFIn = 23).

The fatty acids showed no significant antifeedant effect; hence, they seemed to be less active than the corresponding methyl ester, methyl linoleate (AFIn = 31, 6 hr).

Discussion

In this study, we demonstrate the antifeedant properties of fractions from pine weevil feces and chemical cues (semiochemicals) that deter adult pine weevils from feeding. Guided by a bioassay developed for testing antifeedant effects on the pine weevil (Bratt et al., 2001; Legrand et al., 2004), we first investigated the activity of total extracts of male and female feces, respectively. We found that both sexes were strongly deterred from feeding by the methanol extracts but not by the hexane extracts. Females tended to be more deterred than males by the methanol extract of female feces. By further bioassay-guided fractionation of the methanol extracts of female feces, several compounds with antifeedant effects were identified. As the most polar fraction was the most active one, we used both GC–MS and pyrolysis–GC–MS to identify antifeedant components in the extracts. The results of the pyrolysis–GC–MS enabled us to verify that molecular fragments related to lignin were present in the extract. The brown gummy mixture obtained from the methanol extracts and from the most polar MPLC fraction largely consisted of fragments that looked like catabolized lignin, e.g., *o*-cresol, resinol, guaiacol, veratrol, dihydroxybenzenes, anethole, and eugenol. However, no trace of coniferyl alcohol was found, which has been reported previously to be a common lignin constituent (Hardell and Nilvebrandt, 1996, and references therein). Instead, we identified dihydroconiferyl alcohol, which was also present in the active fractions identified by GC–MS with standard injector temperature. Dihydroconiferyl alcohol is possibly formed during degradation of lignin in the pine weevil gut, as it has not been found in methanol extracts of fresh Scots pine phloem (A.-K. Borg-Karlson, unpublished data). Thus, enzymes capable of cleaving carbon–carbon bonds or of hydrogenation of double bonds might be present in the gut of *H. abietis*.

The results obtained by the GC–MS and the pyrolysis–GC–MS techniques provided us with a number of candidates to be used for antifeedant tests. Also, the highly polar compounds ferulic acid and *p*-hydroxycinnamic acid were identified by silylation of the methanol extract and subsequent GC–MS analyses (Borg-Karlson et al., unpublished data), which revealed additional aromatic structures with possible antifeedant activities. The major components and chemically related compounds

were selected for tests of their antifeedant activity. The strongest antifeedant effects were obtained with compounds having one aromatic ring, preferably with hydroxyl, methyl, and/or methoxy groups. Lignans and related compounds with a methylenedioxyphenyl structure have a feeding-deterrent effect on storage pests such as *Tribolium* species (Harmatha and Nawrot, 2002). In the present study, simple metabolites of lignin (KEGG: Kyoto Encyclopedia of Genes and Genomes, 2005) as several aromatic compounds, e.g., *o*-cresol and 1,4-dihydroxybenzene, showed long-lasting antifeedant effects. The structurally related 1,4-dimethoxybenzene had a similar antifeedant effect. The main compound in the volatile part of the female feces, dihydroconiferyl alcohol, showed a weak antifeedant effect, which initiated further investigations on the effects of other substituents on the aromatic ring (Unelius et al., submitted).

Early studies with direct stimulation, using a single-cell recording technique (Mustaparta, 1975), revealed that *H. abietis* can sense over 130 compounds belonging to various compound classes, such as oxygenated aliphatic compounds, terpenoids, and aromatic compounds. Recordings from single neurones in the antenna combined with gas chromatography (GC–SC) have shown that the receptors are selectively tuned to each of the main constituents in conifer volatiles, especially to (+)- and (–)- α -pinene and (+)- and (–)-limonene (Wibe et al., 1998). These monoterpenes are also known to be important for the orientation toward the host (Nordlander, 1990, 1991). Ground traps baited with α -pinene attract large numbers of pine weevils, but when limonene is added, this attraction is almost completely inhibited (Nordlander, 1990). Monoterpenes, mainly α -pinene and β -pinene, were present in the hexane extract in this study. This extract did not negatively affect pine weevil feeding on the pine bark, although it in itself contained high amounts of feeding stimulant compounds.

The chemical perception of an insect is restricted by the molecular receptive ranges of its odor and taste receptors. The antennal receptors serve as detectors for the volatile constituents of the hosts. We expect that taste receptors on the palpi and the mouth parts are involved in the reception of the low volatility and highly polar compounds, but specific sensillae on the antennae are also known to contain taste receptors (Mustaparta, 1975; A. Wibe, personal communication). We have limited knowledge of the molecular receptive range of these receptors. Only a few compounds, such as glucose, certain amino acids, and methyl salicylate, are known to activate the taste receptors in *H. abietis* (A. Wibe, personal communication). Aromatic compounds as those we have identified in the feces, i.e., anethole, eugenol, guaiacol, *p*-cresol, and anisaldehyde, all elicited high responses using antennal single-cell recordings by direct stimulation (Mustaparta, 1975). Recent investigations using the GC–electroantennogram technique have confirmed the strong antennal activity of anethole and also of 2-, 3-, and 4-methylanisol (Wibe et al., 2001). These receptor responses can now be tentatively linked to a behavioral response, as our feeding tests with 2-methylanisol show a clear antifeedant effect during the first 6 hr.

The fatty acid derivatives we tested showed no antifeedant effect. In the earlier study by Mustaparta (1975), a 9,12,15-octadecatrienoic acid and its corresponding methyl ester elicited strong responses in single-cell recordings by direct stimulation of receptor cells in *sencilla basiconica*. In our antifeedant tests, methyl linolenate showed only short-term effects. The acids present in the methanol extract, with carbon atom chain lengths of 14 and 16, showed the same patterns with minute antifeedant effects. Fatty acids and their corresponding methyl esters were present

in large amounts in the active methanol extract (ca. 40%), and in a silanized methanol extract of pine weevil feces (A.-K. Borg-Karlson, unpublished data). These acids did not seem to elicit any persistent antifeedant effect, although they may be active in combination with other constituents identified in the extracts. Månsson et al. (2005) have shown that aliphatic acids with shorter chain lengths, i.e., octanoic, nonanoic, and decanoic acid, have an antifeedant effect on *H. abietis*, whereas acids with longer carbon chains, in accordance with our results, have low or no effect. Nonanoic acid and acids with similar chain lengths are frequently present in solvent extracts of plant tissues and can be formed during degradation of unsaturated fatty acids (Borg-Karlson, 1990).

The compounds identified in this study are not unique to our pine weevil–conifer system. Most of the compounds have been identified previously in other plants and show a variety of functions as pollinator attractants or bacteriostatics (e.g., Anderson et al. 1993; Hilker and Meiners, 2002). Fungal fruiting bodies emit aromatic compounds related to the ones we have identified, e.g., anisole, benzaldehyde, methyl-anisate, and methyl 4-methoxyphenylacetate (Rösecke et al., 2000; Rösecke and König, 2000). These constituents may indicate that the host is infested with fungi and, thus, unsuitable for egg laying, similar in function to that of pine weevil feces. The odor might be a negative signal for the female. Similarly, it has been suggested that the oxygenated monoterpene verbenone, which is frequently present in volatiles of old sawdust (Wibe et al., 1997), serves as a chemical cue to the pine weevil indicating an old and unsuitable host (Lindgren et al., 1996).

We searched for chemicals in the feces of *H. abietis*, which might act as cues deterring pine weevils from feeding at oviposition sites. A large number of aromatic compounds were present in the most active extracts and fractions. The most active compounds were structurally related to the building blocks of lignin and most probably the results of lignin degradations in the gut, either by bacteria (Dillon and Dillon, 2004) or by fungal symbionts. Feces constituents formed by bacterial symbionts have recently been shown to affect the behavior of *Blatta germanica* (Schal et al., 2002). The compounds we have identified are biologically relevant for the pine weevil because they are present in the feces of this species and because feces are deposited at the oviposition sites. Promising antifeedant candidates for future studies can be selected among the aromatic compounds, e.g., ethers of dihydroconiferyl alcohol, methyl-, hydroxy-, or methoxybenzene, or benzoic acid derivatives. The compounds we have tested, showing high antifeedant effect, will be key compounds in the search for even more active substances and may prove to be useful in finding a sustainable method for protecting planted conifer seedlings against damage by the pine weevils.

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Effect of Racemic and (+)- and (–)-Gossypol on the Survival and Development of *Helicoverpa zea* Larvae

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Abstract Gossypol is a sesquiterpene that occurs naturally in seed and other parts of the cotton plant. Because of restricted rotation around the binaphthyl bond, it occurs naturally as enantiomeric mixtures with (+)-gossypol to (–)-gossypol ratios that vary between 97:3 and 31:69. Commercial cotton varieties (*Gossypium hirsutum*) normally exhibit an approximate 3:2 ratio. (+)-Gossypol is significantly less toxic than (–)-gossypol to nonruminant animals; thus, cottonseed containing high levels of (+)-gossypol might be safely fed to nonruminants. Gossypol, however, is an important component in the cotton plant's defense against insect herbivores, but it is not known how cotton plants that exhibit high levels of (+)-gossypol in the foliage might be affected by insect herbivory. To address this question, 1-d-old *Helicoverpa zea* larvae were fed diets with 0.16, 0.20, and 0.24% racemic, (+)-, and (–)-gossypol. Larval pupal weights, days-to-pupation, and survival were adversely affected by all gossypol diets compared with the control diet. Statistical differences were determined by comparing the compounds among themselves at the three levels and between the three compounds at the same level. When the compounds were compared among themselves, no large differences were observed in pupal weights or in days-to-pupation among any of the diets. Among the three compounds, at the 0.16% level, the diet containing racemic gossypol was the most effective at reducing survival. At the 0.20 and 0.24% levels of racemic (+)- and (–)-gossypol, survival was not statistically different. The overall results indicate that (+)-gossypol is as inhibitory to *H. zea* larvae as racemic or (–)-gossypol, and thus, cotton plants containing predominantly the (+)-enantiomer in foliage may maintain significant defense against insect herbivory.

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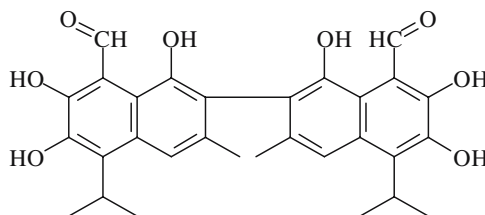
Introduction

World cottonseed production for 2004/2005 is estimated to be 42.9 million metric tons. Cottonseed is a highly nutritious source of protein; however, it is considered a by-product of fiber production because it contains gossypol (Fig. 1), a naturally occurring toxin. Complete elimination of gossypol from the plant is not practical because plants lacking gossypol are highly susceptible to a host of insect herbivores (Jenkins et al., 1966; Lukefahr et al., 1966; Oliver et al., 1971; Meisner et al., 1977; Zur et al., 1979). Moreover, high levels of gossypol in the foliage are associated with resistance to insects.

Gossypol exhibits optical activity because of restricted rotation around the binaphthyl bond (Jaroszewski et al., 1992a). Of the two possible enantiomers, (-)-gossypol is significantly more toxic to nonruminant animals. In commercial *Gossypium hirsutum* cottonseed, the ratio of (+)- to (-)-gossypol is usually about 3:2. However, in some cultivars such as Texas Marker 1, the ratio is approximately 1:1 (actual 53:47). In contrast, some moco cotton varieties that are native to Brazil have (+)- to (-)-gossypol ratios as high as 96:4. Feeding experiments have demonstrated that (+)-gossypol had little effect on mean cumulative weight gain and feed conversion ratios of chickens (Bailey et al., 2000; Stipanovic et al., 2001; Lordelo et al., 2005). This implies that increasing the (+)- to (-)-gossypol ratio in commercial cottonseeds could overcome the impediment to utilizing this high-quality protein as a nonruminant feed source.

Through a traditional backcross breeding program that used these native varieties, the percent (+)-gossypol in the commercial seed was increased to over 90% (Bell et al., 2000). However, little is known concerning how high levels of (+)-gossypol in the foliage might affect resistance to herbivorous insects. It is known that the total concentration of gossypol varies significantly in different plant tissues depending on environmental conditions (Stipanovic et al., 1988). For example, the mean concentration of gossypol in various plant tissues for 14 *G. hirsutum* varieties grown at five locations from south Texas to north Texas (~1000 km) varied as follows: leaves, 0.04–0.10%; flower buds, 0.8–1.0%; seed 0.9–1.1%. Yang et al. (1999) reported that *Helicoverpa armigera* larvae raised on artificial diets containing (+)-gossypol matured more slowly, and the percentage of larvae surviving to the adult stage was lower as compared to those raised on (-)-gossypol. Herein, we report the results on racemic gossypol [a 1:1 mixture of (+)- and (-)-gossypol, designated as (±)-] and (+)- and (-)-gossypol in artificial diet feeding studies on the

Fig. 1 Structure of gossypol



generalist herbivore *Helicoverpa zea* (Boddie). Because the (+)- to (–)-gossypol ratio varies between approximately 3:2 and 2:3 in commercial cotton cultivars (Cass et al., 1991; Jaroszewski et al., 1992b; Percy et al., 1996), we included (±)-gossypol as a check to approximate commercial cottons.

Methods and Materials

Preparation of (+)-, (–)-, and (±)-Gossypol

A crystallization process to separate the gossypol enantiomers as gossypol–acetone (1:3) followed by storage of the products under vacuum for 3 d to remove essentially all of the acetone was used to obtain chemically pure chiral gossypol (Dowd, 2003). Purified racemic gossypol acetic acid (1:1) was obtained by repeated recrystallization of gossypol from acetone and acetic acid. The gossypol samples were analyzed by high-performance liquid chromatography as previously described (Puckhaber et al., 2002) to confirm enantiomeric content. The racemic gossypol was 49.9% (–)-gossypol and 50.1% (+)-gossypol, whereas the enantiomer samples were >99.5% optically pure.

Preparation of Diets

Five diet sets were prepared. Four contained Alphacel (ICN Nutritional Biochemicals), which is a nonnutritive polysaccharide that was used as a carrier, and one control diet set contained no Alphacel. Each gossypol sample was dissolved in 35 ml of ethyl acetate and was quantitatively added to 15 g of Alphacel. The suspensions were evaporated to dryness. Hexane (~50 ml) was added to the dry samples and evaporated to remove traces of ethyl acetate. The samples were left under vacuum for 36 hr. Six gossypol/Alphacel preparations were made with 800, 1000, or 1200 mg of either (+)- or (–)-gossypol; 893, 1116, and 1339 mg of (±)-gossypol–acetic acid (1:1), which is 89.62% gossypol by weight, was used to make the three (±)-gossypol/Alphacel preparations. To account for the acetic acid in the (±)-gossypol/Alphacel preparations, an equivalent amount of acetic acid was added to the Alphacel/chiral gossypol and Alphacel control (i.e., Alphacel with no gossypol) preparations.

Diets were prepared from a dry soy bean–wheat germ premix (Instant Soybean–Wheat Germ Insect Diet; Stonefly Industries, Inc., Bryan, TX, USA) with a slight modification of the manufacturer's instructions. Specifically, a dilute vinegar solution was prepared from water (75%) and 5% vinegar (Albertson's, white vinegar; 25%). This dilute vinegar solution (390 ml) was added to 15 g of Alphacel with or without gossypol and 100 g of the diet premix. This provided 505 g of finished diet. One additional control diet was prepared according to the manufacturer's instructions (i.e., 100-g dry diet, 284 ml water, and 16 ml 5% vinegar) with no Alphacel. The final levels of gossypol were 0.16, 0.20, or 0.24%.

Larvae

Larvae were reared from *H. zea* (Boddie) moths collected from the wild and raised in rearing chambers at College Station, TX. Newly hatched larvae were allowed to feed on the artificial diet with neither Alphacel nor gossypol for 1 d before transfer

to the test diets. This delay assured their fitness and more closely mimics their behavior in nature. That is, other researchers have noted that newly hatched *Heliothis virescens* larvae carefully avoid consumption of cotton glands (Hedin et al., 1992).

Feeding Trials

Larvae were raised on the 11 diets [i.e., soybean–wheat germ diet, soybean–wheat germ diet plus Alphacel, and the latter diet with 0.16, 0.20, or 0.24% of (+)-gossypol, (-)-gossypol, or racemic gossypol]. A single 1-d-old *H. zea* (Boddie) larva was placed in a 22-ml plastic cup that contained 4–5 g of a specific diet. Each treatment consisted of 40 larvae, which were raised in incubators at 27°C on a 12-hr light/12-hr dark regime. The following parameters were measured: number of survivors, days-to-pupation, and pupal weight. After 10 d, cups were inspected daily to check for larval survival and pupation. Pupae were allowed to harden for 1 d and then weighed.

Statistical Analyses

Logistic regression and frequency analysis (Proc Logistic and Proc Freq, respectively) were performed to determine the comparative probability of survival on different diets. Results of logistic regression are the ratio of survival odds for larvae consuming two different diets. For example, the odds of survival [$p/(1 - p)$] on the 0.16% (-)-gossypol diet is $[0.70/(1 - 0.70)]$ (see Table 1), whereas the odds of survival in the 0.20% (-)-gossypol diet is $[0.40/(1 - 0.40)]$. To compare the survival on these diets, the ratio of these two odds was calculated (Hosmer and Lemeshow, 2000; Agresti, 2002). This provides a measure of the likelihood of survival between these two groups. Thus, the odds ratio of larval survival on the 0.16% (-)-gossypol diet compared to the 0.20% (-)-gossypol diet shown in Table 1 is calculated as follows:

$$\left(\frac{0.7}{1 - 0.7}\right) / \left(\frac{0.4}{1 - 0.4}\right) = \left(\frac{0.7}{0.3}\right) / \left(\frac{0.4}{0.6}\right) = 3.5$$

Table 1 Odds ratios of survival and probability of significance for survival of *Helicoverpa zea* larvae fed with racemic, (+)-, or (-)-gossypol at different concentrations

Gossypol form	Odds ratio comparison between gossypol concentrations		
	0.16/0.20	0.16/0.24	0.20/0.24
(±) ^a	nsd ^b (0.470) ^c	nsd (0.352)	4.8 (0.036)
(-)	3.5 (0.008)	16.3 (<0.001)	nsd (0.742)
(+)	10.5 (<0.001)	22.7 (<0.001)	2.2 (0.075)

^a (±)- denotes gossypol racemate.

^b nsd denotes not statistically different (logistic regression analysis with a 95% Wald confidence level).

^c Value in parentheses is the probability that the difference within the survival comparison is statistically significant.

Statistical differences between days-to-pupation on different diets and least-square mean weights of pupae on different diets were determined using Proc Mixed in SAS version 9.1 (SAS Institute, Cary, NC, USA, 2003) software computations. Survival odds and differences in days-to-pupation and least-square mean pupae weights were made between the control diet with no Alphacel and the control diet with Alphacel, but no gossypol. Similarly, all gossypol diets were compared to the two control diets. In addition, in designing the experiment, an *a priori* decision was made to evaluate the gossypol diet data by dividing the study into 18 groups. Nine groups considered how a concentration of each enantiomer or the racemate affected the larvae compared to other concentrations of the same enantiomer or racemate. The other nine groups considered how each enantiomer or racemate at a specific concentration affected the larvae compared to the other enantiomer or racemate at the same concentration. These were the only pairwise comparisons evaluated among the gossypol diets.

Results

We found a clear dose response to gossypol for survival and, to a lesser extent, pupal weight. Furthermore, overall survival, days-to-pupation, and pupal weights were not different when the larvae were fed with racemic, (+)-, and (-)-gossypol at the same concentration. Specific analyses of the data are given below.

Survival

The survival results of the feeding experiment are shown in Fig. 2. Contingency table analysis indicated no significant difference in survival between the diet and the diet with Alphacel. The same analysis showed that larval survival on all of the 0.20% and 0.24% gossypol diets was lower than the diet with Alphacel without gossypol. For the 0.16% diet, this was only true for the (\pm)-gossypol diet.

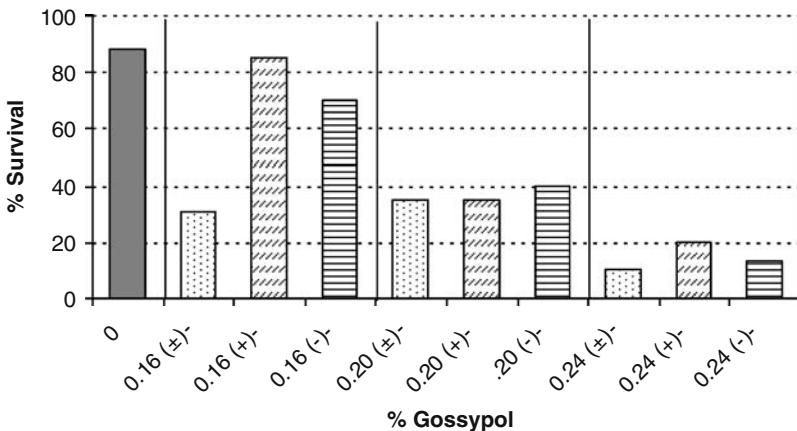


Fig. 2 Percentage survival of *Helicoverpa zea* larvae raised on artificial diet containing Alphacel with various amounts of racemic, (+)-, and (-)-gossypol

The odds ratio of survival at different levels of racemic, (+)-, or (–)-gossypol is given in Table 1. When the effect of feeding racemic gossypol at different levels was compared, the only statistically significant difference in survival was between the larvae fed with the 0.20 and 0.24% diets (Table 1). That is, larvae fed with the diet containing 0.20% racemic gossypol were 4.8 times more likely to survive as compared with larvae fed with the diet containing 0.24% racemic gossypol. Among the (–)-gossypol diets, larvae fed with the 0.16% diet were 3.5 times more likely to survive than larvae fed with the 0.20% diet and 16.3 times more likely to survive than larvae fed with the 0.24% diet. The (+)-gossypol diets showed a similar trend with larvae fed with the 0.16% diet being 10.5 times more likely to survive than larvae fed with the 0.20% diet and 22.7 times more likely to survive than larvae fed with the 0.24% diet, whereas larvae fed with the 0.20% diet were 2.2 times more likely to survive than those on the 0.24% diet. All other comparisons of survival with concentration differences were not statistically different. When survival was compared between the two enantiomers and racemic gossypol treatments at equivalent concentrations, the only significant differences were at the 0.16% level (Table 2). Specifically, larvae fed with the (–)-gossypol diet at the 0.16% level were 6.2 times more likely to survive compared with larvae fed with the racemic diet at this level, and larvae fed with the (+)-gossypol diet at the 0.16% level were 14.9 times more likely to survive than larvae fed with the racemic diet at this level. This same set of comparisons at the 0.20 and 0.24% diets showed no statistical differences in survival. Odds ratios of survival of the (+)-gossypol diets compared to the (–)-gossypol diets at the same levels were not statistically different.

Days-to-Pupation

There was no statistical difference in the time needed to reach pupation between the diets with and without Alphacel (data not shown). However, there were differences between the Alphacel diet without gossypol and the Alphacel diets that contained gossypol (Fig. 3). In all of the latter, the days-to-pupation increased. Among the various gossypol diets, moderately significant different delays in pupation were noted between (–)-gossypol at the 0.16 and 0.24% levels ($P = 0.03$) and between the 0.20 and 0.24% levels ($P = 0.02$). The only other pupation delay was noted for the (+)-gossypol diets between the 0.16 and 0.24% levels ($P = 0.06$), and this difference

Table 2 Odds ratios of survival and probability of significance for survival of *H. zea* larvae fed with racemic, (+)-, or (–)-gossypol at equivalent concentrations

Percent gossypol in diet	Odds ratio comparison between gossypol forms		
	(–)- ^a /(±)-	(+)-/(±)-	(–)-/(+)-
0.16	6.2 (<0.001) ^b	14.9 (<0.001)	nsd (0.298)
0.20	nsd ^c (0.664)	nsd (0.792)	nsd (0.592)
0.24	nsd (0.724)	nsd (0.385)	nsd (0.791)

^a (±)- denotes gossypol racemate.

^b Value in parentheses is the probability that the difference within the survival comparison is statically significant.

^c nsd denotes not statistically different (logistic regression analysis with a 95% Wald confidence level).

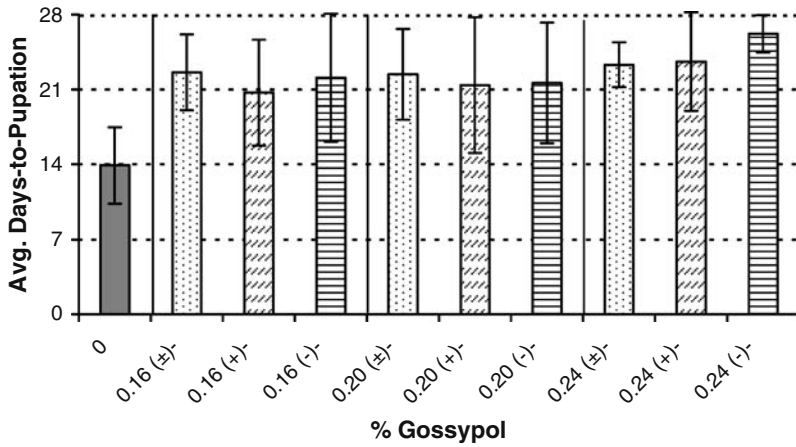


Fig. 3 Average days-to-pupation of *H. zea* larvae raised on artificial diet containing Alphacel with various amounts of racemic, (+)-, and (-)-gossypol

was also moderate. No significant delays to pupation were noted among the three racemate levels. Furthermore, there were no significant differences in days-to-pupation between the racemate and the (+)-gossypol or (-)-gossypol diets or between the (+)-gossypol and (-)-gossypol diets at the same levels of gossypol.

Pupal Weights

There was no large statistical difference in pupal weights between the diet alone and the diet plus Alphacel (data not shown). Pupae from larvae raised on all diets

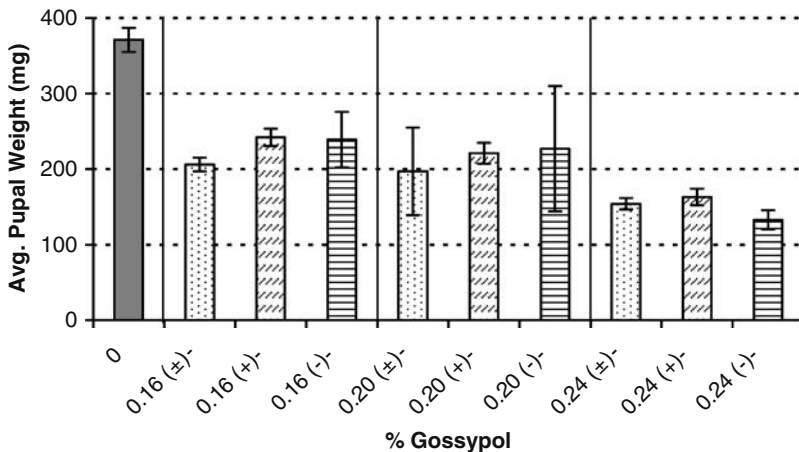


Fig. 4 Average pupal weights (mg) of *H. zea* larvae raised on artificial diet containing Alphacel with various amounts of racemic, (+)-, and (-)-gossypol

containing gossypol were smaller than those raised on the diet plus Alphacel (Fig. 4). Statistical differences in pupal weights were observed when comparing (\pm)- or (+)- or ($-$)-gossypol under our *a priori* criteria. For the (\pm)-gossypol diets, pupae from larvae raised on the 0.24% diet weighed less than those raised on the 0.16% diet ($P = 0.1$); no difference was found when the other racemate diets were compared. Pupae weighed less for larvae raised on the 0.24% ($-$)-gossypol diet vs. the 0.20 and 0.16% ($-$)-gossypol diets ($P < 0.001$). Pupae from larvae raised on the (+)-gossypol diets were different at all three concentrations ($P < 0.1$). There were no significant differences in pupal weights between the racemate and the (+)-gossypol or ($-$)-gossypol diets at the same levels of gossypol except at the 0.16% level where pupae weights were less for the racemate vs. (+)- or ($-$)-gossypol ($P = 0.06$ and $P = 0.09$, respectively). Additionally, there were no significant differences between the (+)-gossypol and ($-$)-gossypol diets at the same levels.

Discussion

These results show that racemic, (+)-, and ($-$)-gossypol were comparable at reducing survival, days-to-pupation, and pupal weight. One exception was in the survival study where the 0.16% racemic gossypol diet reduced larval survival more effectively than did either of the individual enantiomers at that level. In addition, ($-$)-gossypol was more effective in extending the days needed to reach pupation at the 0.24% level than either (+)-gossypol or the racemate. No large significant differences in pupal weights were observed when racemic, (+)-, or ($-$)-gossypol were compared at the same levels.

These results are somewhat different from those reported by Yang et al. (1999) for *H. armigera*, who, in their artificial diet study, found that larvae matured more slowly, and the percent survival was lower when (+)-gossypol was incorporated into the diet as compared with ($-$)-gossypol. Yang et al. did not include (\pm)-gossypol in their study.

Others have reported enhanced biological activity of ($-$)-gossypol in a variety of enzyme, cell, and whole animal studies (Matlin et al., 1985; Lindberg et al., 1987; Benz et al., 1990; Sang et al., 1991; Gonzalez-Garza et al., 1993; Li and He, 1993; Lin et al., 1993; Blackstaffe et al., 1997; Shelley et al., 1999). Therefore, one might anticipate that the ($-$)-enantiomer would be a more potent inhibitor of insect herbivory. However, we did not find meaningful differences between the enantiomers or between the enantiomers and the racemate in this study. Thus, these results are consistent with the theory that cotton plants with high levels of either (+)- or ($-$)-gossypol in the foliage will not be substantially more susceptible to generalist insect herbivores than cotton plants with ratios of (+)- to ($-$)-gossypol that vary between 3:2 and 2:3. However, these results are based on the generalist herbivore *H. zea*, and an additional study on at least one other species that utilizes cotton as a host will be conducted. Ultimately, validation of this hypothesis awaits field studies.

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Role of the Lipoxygenase/lyase Pathway of Host-food Plants in the Host Searching Behavior of Two Parasitoid Species, *Cotesia glomerata* and *Cotesia plutellae*

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Abstract To elucidate the role of the plant lipoxygenase (LOX)/lyase pathway for host search behavior of two parasitic wasps attacking herbivorous larvae, an *Arabidopsis* mutant (*all84*) was isolated with a mutation somewhere in the LOX/lyase pathway. Detached leaves of the mutant were shown to release less (*Z*)-3-hexenal, a first green leaf volatile (GLV) product of the LOX/lyase pathway. The braconid larval parasitoids studied, *Cotesia glomerata* and *Cotesia plutella*, differ in their ability to discriminate among plant volatiles induced by feeding of lepidopteran hosts and nonhosts: *C. plutella* only responds to plant volatiles induced by hosts (*Plutella* larvae), whereas the response by the more generalist *C. glomerata* is not host specific. The *Arabidopsis* mutant *all84* infested by *Pieris* larvae was less attractive to *C. glomerata* than *Arabidopsis* wild type (wt) infested by the host larvae. *C. glomerata* was attracted by two of the GLV biosynthesized through the LOX/lyase pathway, (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate. However, attraction of *C. plutellae* to volatiles from *Plutella*-infested *all84* plants did not differ from attraction to host-infested wt *Arabidopsis*. Both wasp species were arrested to the respective host-infested edge of the wt leaf by showing characteristic antennal searching behavior on the edge. In *C. glomerata*, the duration of this searching behavior at the infested leaf edge was significantly shorter on *all84* plants than on wt plants. By contrast, the duration of the searching behavior of *C. plutellae* on the host-infested leaf edge of *all84* was not significantly different from that on the wt

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leaf. These data suggest that the LOX/lyase pathway is directly involved in the production of attractants and arrestants important for host search behavior of the more generalist *C. glomerata*, but not for the specialist *C. plutellae*.

Keywords *Cotesia glomerata* · *Cotesia plutellae* · *Pieris rapae* · *Plutella xylostella* · *Arabidopsis* · Green leaf volatiles · Lipoxygenase/lyase pathway

Introduction

Plants show induced responses to herbivory. Induced responses that benefit plants are called induced defenses (Karban and Baldwin, 1997). Induced defenses can be divided into two categories: induced direct defenses (e.g., induced production of toxic compounds, digestibility reducers, repellents, trichomes, etc.) and induced indirect defenses (i.e., enhancing the effectiveness of carnivorous natural enemies of herbivores). An example of an induced indirect defense of a plant is the attraction of carnivorous natural enemies to herbivores by the production of herbivore-induced volatiles. Induced indirect defense responses by plants have attracted much attention from ecologists (for reviews, see Takabayashi and Dicke, 1996; Dicke and Vet, 1999; Dicke and van Loon, 2000; Kessler and Baldwin, 2002).

Studies on the mechanisms involved in the production of herbivore-induced volatiles in plants (e.g., Hopke et al., 1994; Dicke et al., 1999; Ozawa et al., 2000; Horiuchi et al., 2001; Van Poecke and Dicke, 2002; Schmelz et al., 2003) are important in understanding ecological aspects of induced indirect defense responses by plants (Kessler and Baldwin, 2002). *Arabidopsis thaliana* is a model plant for molecular biology and genetic engineering, and mutants related to induced-defense responses are available. Therefore, *Arabidopsis* is also a model plant species for the study of the molecular mechanisms involved in the production of herbivore-induced volatiles by plants. By using *Arabidopsis* mutants, Van Poecke and Dicke (2002) reported that the octadecanoid and the salicylic acid pathways of host-infested plants were involved in attracting the parasitic wasp *Cotesia rubecula*, one of the most important parasitoids of cabbage white butterfly larvae, *Pieris rapae* (Lepidoptera: Pieridae), in Eurasia.

The so-called “green leaf volatile compounds” (GLV) are produced by green plants in response to mechanical wounding by herbivores, and some GLVs are known to be involved in interactions between plants and parasitic wasps (e.g., Takabayashi et al., 1991; Whitman and Eller, 1992; Birkett et al., 2003; Gouinguéné et al., 2005). GLV are produced through the lipoxygenase (LOX)/lyase pathway from linolenic and linoleic acid in green plants (Matsui et al., 2000). Here, our first objective was to investigate the role of the LOX/lyase pathway in the production of herbivore-induced plant volatiles that attract parasitic wasps. We isolated an *Arabidopsis* mutant (*all84*) with a mutation somewhere in the LOX/lyase pathway that results in changes of the amount of (*Z*)-3-hexenal, the first biosynthesized GLV of the LOX/lyase pathway. (*Z*)-3-Hexenal is further converted into other GLV such as (*E*)-2-hexenal (leaf aldehyde), (*Z*)-3-hexenol (leaf alcohol), and (*Z*)-3-hexenyl acetate, all of which are in the LOX/lyase pathway (Hatanaka, 1993; Matsui et al., 2000). After identifying the mutant, we studied whether the mutation would affect the interaction between host-infested plants and their parasitoids mediated by

herbivore-induced plant volatiles by focusing on the following two tritrophic systems: (1) *Arabidopsis*, cabbage white butterfly larvae (*P. rapae*) and its parasitoid *Cotesia glomerata* and (2) *Arabidopsis*, diamondback moth larvae (*Plutella xylostella*) and its parasitoid *Cotesia plutellae*. We have previously reported that both of these parasitoid species are attracted to volatiles emitted from cruciferous plants infested by their respective host larvae (Takabayashi et al., 1998; Shiojiri et al., 2000a). Interestingly, the response of *C. glomerata* is not host specific: The wasps preferred plants infested with either the cabbage white (hosts) or diamondback moth larvae (nonhosts) to intact plants (Shiojiri et al., 2000a). In contrast, the response of *C. plutellae* was highly host specific: the wasps were attracted only to host-infested plant volatiles (Shiojiri et al., 2000a).

After finding a host-infested plant through attraction to herbivore-induced plant volatiles, *C. glomerata* and *C. plutellae* search for their hosts on the infested plants. Both wasps were chemically arrested to the host-infested edge of the leaf by showing characteristic antennal searching behavior (Sato, 1979; Horikoshi et al., 1997; Shiojiri et al., 2000b). This behavior is host specific: Neither wasp species showed the behavior on artificially damaged edges or on edges damaged by nonhost larvae (Shiojiri et al., 2000b). This behavior enhances the wasps' chance of finding their hosts (Sato, 1979; Shiojiri et al., 2000b). Neither leaf juice nor host regurgitant alone applied on a piece of filter paper elicited the same behavior in the wasps. However, immediately after leaf juice and host regurgitant were applied together on the same piece of filter paper, the wasps showed the same antennal searching behavior (Sato, 1979; Horikoshi et al., 1997; Shiojiri et al., 2000b), indicating that chemicals (arrestants) that are formed immediately after mixing leaf juice and regurgitant are an important host-specific cue in host searching by *C. glomerata* and *C. plutellae*. However, mechanisms involved in the formation of these host-specific arrestants along the host-infested edge are unknown. Thus, the second objective of this paper was to use the *Arabidopsis* mutant to elucidate whether the LOX/lyase pathway is involved in the production of *C. glomerata* and *C. plutellae* arrestants.

Methods and Materials

Insects and Plants

Adult females of *P. rapae* were collected from a field in Kyoto City, Japan, in order to obtain eggs during the experimental period. Hatched larvae, which were reared on cabbage leaves in a climate-controlled room (25 + 2°C; 50–70% relative humidity (r.h.); 16L:8D), were used for the experiments. *P. xylostella* larvae were collected from crucifer crops in the same field in Kyoto City and reared in a climate-controlled room (25 ± 2°C, 50–70% r.h., and 16 hr photophase), with cabbage leaves as food to obtain adults. We then kept the adults in the climate-controlled room to obtain eggs. The hatched larvae, which were reared in the climate-controlled room, were used for all experiments.

C. glomerata and *C. plutellae* were obtained from parasitized host larvae collected in a field in Kyoto, Japan. Adults of each parasitoid species were separately maintained in plastic cages (130 × 200 × 300 mm) with honey as food in a climate-controlled room (18 ± 2°C, 50–70% r.h., and 16 hr photophase) for 3 d to ensure

mating. Females were then individually transferred to a glass tube (20 mm diam., 130 mm long) containing honey as food, and kept in a climate-controlled room ($18 \pm 2^\circ\text{C}$, 50–70% r.h., 24 hr darkness) until used. Females were never kept for more than 10 d. At least 1 hr before the start of each experiment, oviposition-inexperienced female wasps were transferred to another climate-controlled room ($25 \pm 2^\circ\text{C}$, 50–70% r.h.) where they were kept under constant light conditions.

Arabidopsis (ecotype Landsberg) plants were grown in plastic pots (five plants per pot) in a climate-controlled room ($22 \pm 2^\circ\text{C}$, 16 hr photophase, 5000 lx) for 4–5 wk. Fully developed but nonflowering plants were used for all experiments.

Isolation of an *Arabidopsis* Mutant

In this study we isolated *Arabidopsis* plants (ecotype Landsberg), with a mutation somewhere in the LOX/lyase pathway, by characterizing the amount of (*Z*)-3-hexenal, the first biosynthesized GLV in plants. Rosette leaves of M2 plants of 3- to 4-wk-old EMS-treated *Arabidopsis* were collected. They were put individually into glass vials with 50 nmol of *n*-heptanal as an internal standard and frozen at -80°C for at least 1 hr. For each vial, a small glass column packed with 2,4-dinitrophenylhydrazine-coated silica gel (140 mg) was connected, and then the vial was heated at 100°C for 20 min. As (*Z*)-3-hexenal in the leaves vaporized and passed through the column, it was converted to the corresponding hydrazone derivatives on the surface of the silica gel. After elution of the hydrazones with hexane, (*Z*)-3-hexenal was quantitatively analyzed with HPLC as described by Matsui et al. (2000). Approximately 1000 lines were screened, and a mutant deficient in GLV formation was isolated [designated as *all* (aldehyde-less)]. In this study, the mutant used was backcrossed three times with wt plants. Details of the biochemical nature of the mutant and its volatile profiles will be reported in our forthcoming paper (Matsui et al., unpublished).

Flight Response of *C. glomerata* and *C. plutellae* to *Arabidopsis* Infested by Host Larvae

The flight response of female *C. glomerata* and *C. plutellae* were observed with respect to two groups of 4 *Arabidopsis* plants, each group in a different pot and receiving a different treatment. Two pots were positioned ca. 25 cm apart in a cage ($25 \times 35 \times 30$ cm) with three windows covered by nylon gauze and one door for introducing plants and wasps. The cage was placed in a climate-controlled room ($25 \pm 2^\circ\text{C}$, 50–70% r.h.; Shiojiri et al., 2000a). Five to ten wasps at a time were released halfway between the two pots. The first landing by each wasp on a plant in either of the 2 pots was recorded as its choice. Once the wasp landed on a plant, it was immediately removed from the cage with an insect aspirator. If the wasp did not land on any of the two pots within 30 min, it was evaluated as a no-choice result. The trial was repeated four to five times, and the pots were replaced for each trial. Four second instar *P. rapae* or ten third instar *P. xylostella* were placed on *Arabidopsis* plants in a pot. After 24 hr, they were used as infested plants. The artificially damaged plants were prepared by punching holes in leaves three times at 12-hr intervals to mimic damage found in infested plants.

Significant preferences in dual-choice tests were analyzed by using a binomial probability function under the null hypothesis that wasps had a 1:1 distribution over

the two groups of plants. The wasps that did not make a choice were ignored for further statistical analysis. In earlier experiments, we found that releasing five to ten wasps at the same time did not significantly affect the choice of the individual wasps (Shiojiri, unpublished data).

Response of *C. glomerata* to Three Synthetic GLV

To determine if the volatiles biosynthesized through the LOX/lyase pathway attract *C. glomerata* to host-infested *Arabidopsis*, we chose three GLV: (*E*)-2-hexenal (leaf aldehyde), (*Z*)-3-hexanol (leaf alcohol), and (*Z*)-3-hexenyl acetate. These components were found in the headspace of wt *Arabidopsis* infested by *P. rapae* larvae (Ozawa, unpublished data). Tested compounds (97–98% purity) purchased from Wako Chemical Industries Co. Ltd. were individually dissolved in hexane (0.01 mg/ml solution). Ten microliters of the solution (0.1 µg compound) were applied on a piece of sponge cellulose (5 × 25 × 25 mm). To facilitate a low volatilization rate, the sponge was placed in a sealed polyethylene film bag (50 × 70 mm), and the bag was then placed in another sealed bag after evaporation of the solvent for 3 min. The amount (ion intensity) of each compound vaporized from the sealed bag per unit time (1 hr) was ca 1.9×10^7 [(*E*)-2-hexenal], 4.4×10^6 [(*Z*)-3-hexanol], and 6.4×10^6 [(*Z*)-3-hexenyl acetate], which was similar to that emitted from infested wt: 1.2×10^7 [(*E*)-2-hexenal], 9.5×10^6 [(*Z*)-3-hexanol], and 3.0×10^7 [(*Z*)-3-hexenyl acetate] (Tenax trapping, flow rate; 100 ml/min, 2 l grass bottle). As a control, 10 µl of pure hexane were impregnated in a piece of sponge cellulose. The bag containing a sample or a control piece of sponge was placed on the soil in a pot with three intact wt plants. Flight preference of the wasps for plants with the sample compared with control plants was observed. Samples were renewed every 30 min. Every hour, plants were replaced by new plants to avoid the possibility that the tested volatiles induce any response in *Arabidopsis* plants themselves (Bate and Rothstein, 1998; Kishimoto et al., 2005). When *C. glomerata* showed no preference for the test compound or the control, sample solutions of different concentrations (0.1 and 0.001 mg/ml) were applied to the sponge cellulose following the same procedure as described above.

Antennal Response of *C. glomerata* and *C. plutellae* to Host-Infested Edges of *Arabidopsis* Leaves

The antennal searching behavior of *C. glomerata* and *C. plutellae* to host-infested leaf edges was observed in a test tube (20 mm diam., 130 mm long). Four second instar *P. rapae* larvae or ten third instar *P. xylostella* were placed on *Arabidopsis* plants in a pot. After 24 hr, larvae and their associated products such as silks and feces were removed from the leaves with a small fine brush. After washing the leaves with water, we cut out a piece (50 mm²) with a herbivore-infested edge ca. 8 mm long. We also prepared pieces of artificially damaged leaves. The artificially damaged plants were prepared by punching holes in leaves. The damaged area was the same as in herbivore infested plants. We then cut out a piece (50 mm²) with an artificially damaged edge ca. 8 mm long. The piece of leaf was placed in a tube into which one wasp had been introduced. When the wasp showed strong antennal contact with the piece of leaf, it was judged as showing searching behavior, and the duration of the searching behavior (searching time) was recorded. When the wasp

left the piece of leaf for more than 10 sec or started antennal cleaning on it, searching behavior was judged to be finished. When the wasp did not show searching behavior within 5 min, it was judged to have shown no response. Twenty females were used for each bioassay.

Results

Isolation of *Arabidopsis* Mutants

One mutant (*all84*) that produced significantly less (*Z*)-3-hexenal (57.7 ± 8.5 nmol/g fresh weight, $n = 11$) than wt plants (300.2 ± 108.7 nmol/g fresh weight; $n = 6$) (*t* test, $P = 1.7 \times 10^{-6}$) was isolated. Under the growing conditions used, no apparent difference in phenotype could be observed between *all84* and wt.

Flight Response of *C. glomerata* to *Arabidopsis* Infested by *P. rapae* Larvae

When comparing intact wt plants and intact *all84* plants, most *C. glomerata* showed no choice. However, wasps showed a significant preference for host-infested *all84* plants over intact *all84* plants. In addition, wasps preferred infested wt plants to infested *all84* plants, and artificially damaged wt plants to artificially damaged *all84* plants (Fig. 1).

Flight Response of *C. plutellae* to *Arabidopsis* Infested by *P. xylostella* Larvae

C. plutellae showed equal preference when comparing intact wt plants with intact *all84* plants, and infested wt plants with infested *all84* plants. However, wasps showed significantly greater preference for infested *all84* plants than for intact *all84* plants (Fig. 2).

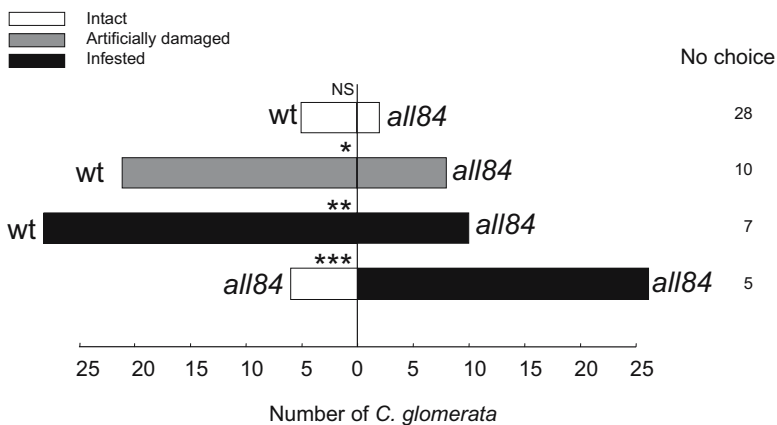


Fig. 1 Flight response of *C. glomerata* to an *Arabidopsis* wt and a GLV-deficient mutant plant (*all84*) in different treatments. NS, not significantly different; * $0.05 > P > 0.01$, ** $0.01 > P > 0.001$, *** $0.001 > P$ (binomial test)

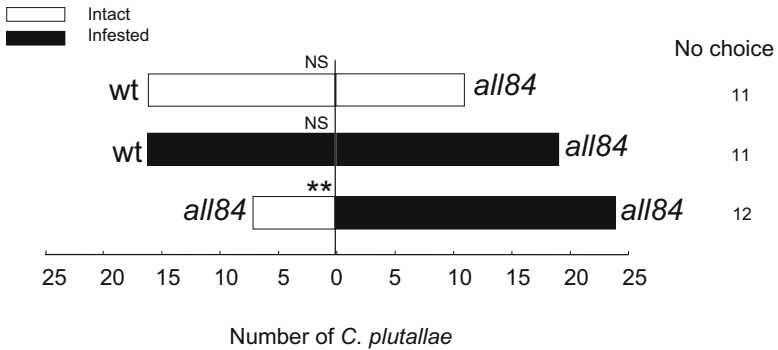


Fig. 2 Flight response of *C. plutellae* to an *Arabidopsis* wt and a GLV-deficient mutant plant (*all84*) in different treatments. NS: not significantly different; **0.01 > P > 0.001 (binomial test)

Response of *C. glomerata* to Three Synthetic GLV

Significantly more *C. glomerata* females were attracted to intact wt plants with (*E*)-2-hexenal (0.01 mg/ml concentration) than to the wt control plants. Wt plants treated with (*Z*)-3-hexenyl acetate of the same concentration were also preferred to wt control plants. However, at the same concentration, the wasp showed equal preference for wt plants treated with (*Z*)-3-hexenol and for wt control plants (Fig. 3). At higher (0.1 mg/ml) and lower (0.001 mg/ml) concentration of (*Z*)-3-hexenol, the wasps were not attracted to the compound (data not shown).

Antennal Response of *C. glomerata* and *C. plutellae* to the Edge Damaged by the Hosts of *Arabidopsis* Leaves

The searching time of *C. glomerata* on artificially damaged wt leaves and on artificially damaged *all84* leaves did not differ significantly (*t* test, P = 0.08). However, the searching time of the wasp on *P. rapae* (host)-damaged *all84* leaves was reduced compared to host-damaged wt leaves (*t* test, P = 0.005) (Fig. 4, left). No differences

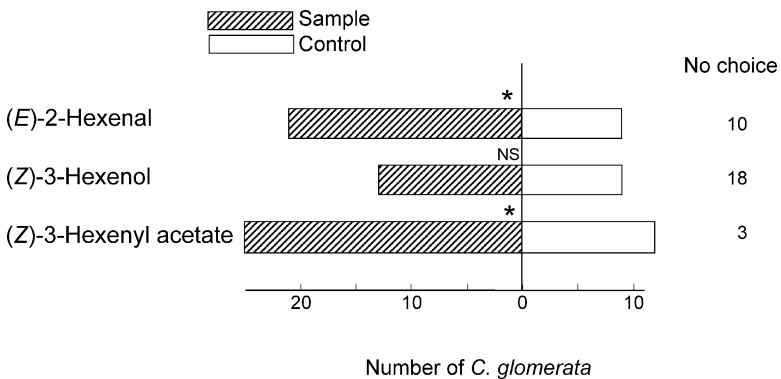


Fig. 3 Flight response of *C. glomerata* to three synthetic GLV (0.1 µg compound in a piece of sponge cellulose in a sealed polyethylene film bag) placed on intact *Arabidopsis*. NS, not significantly different; *0.05 > P > 0.01 (binomial test)

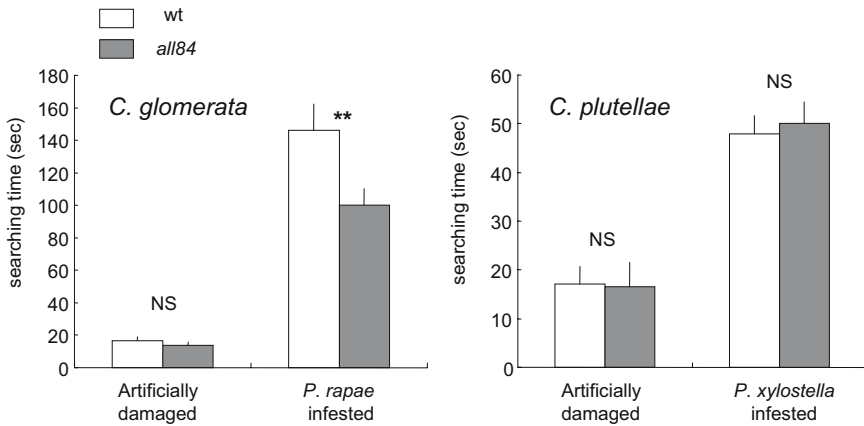


Fig. 4 Duration (seconds) of antennal searching behavior of *C. glomerata* (left) and *C. plutellae* (right) to an *Arabidopsis* leaf with different treatments. NS, not significantly different; $^{**}0.01 > P > 0.001$ (*t* test)

in searching times were found for *C. plutellae* having contact with artificially damaged wt leaves vs. artificially damaged *all84* leaves (*t* test, $P = 0.38$) or with *P. xylostella* (host)-damaged leaves vs. host-damaged *all84* leaves (*t* test, $P = 0.41$; Fig. 4, right).

Discussion

C. glomerata is attracted to host-infested cruciferous plants over uninfested cruciferous plants (Takabayashi et al., 1998; Dicke and van Loon, 2000; Shiojiri et al., 2000a). *C. glomerata* are also attracted to artificially damaged cruciferous plants, and cruciferous plants infested by nonhost larvae (*P. xylostella*) over uninfested plants (Takabayashi et al., 1998; Shiojiri et al., 2000a). Thus, not only are *C. glomerata* attracted to compounds that are produced in response to injury by their hosts, but also to injury caused by other lepidopteran larvae. As green plants emit GLVs through the LOX/lyase pathway when damaged mechanically (Matsui et al., 2000), we hypothesized that some GLV from the LOX/lyase pathway are involved in the attraction of *C. glomerata* by infested cruciferous plants. Here, we showed that the attractiveness of host-infested *all84* plants to *C. glomerata* was weaker than that of host-infested wt plants. This was also observed when comparing artificially damaged *all84* and wt plants. In addition, we also showed that the addition of the GLV (*E*)-2-hexenal or (*Z*)-3-hexenyl acetate to intact wt plants increased their attractiveness to *C. glomerata*. These results indicate the importance of GLV of the LOX/lyase pathway in the attraction of *C. glomerata* to herbivore-infested cruciferous plants.

Artificially damaged and host-infested *all84* plants were more attractive than intact *all84* plants to *C. glomerata*. This could be because the production of (*Z*)-3-hexenal was not completely suppressed in *all84* plants. (*Z*)-3-Hexenal is converted into other GLV such as (*E*)-2-hexenal (leaf aldehyde), (*Z*)-3-hexenol (leaf alcohol), and (*Z*)-3-hexenyl acetate in the LOX/lyase pathway (Hatanaka, 1993; Matsui et al.,

2000). Thus, artificially damaged and host-infested *all84* would still have produced relatively larger amounts of not only (*Z*)-3-hexenal, but also of other GLV when compared with intact *all84*. The slight increase of the wasp-attractive GLV in infested *all84* plants would in part explain the preference of *C. glomerata* to infested *all84* plants over intact *all84* plants. Furthermore, wasps might also use volatile chemicals biosynthesized in pathways other than the LOX/lyase pathway emitted from infested *all84* plants.

C. plutellae preferred host-infested cruciferous plants over uninfested cruciferous plants, artificially damaged plants, and plants infested by nonhost (*P. rapae*) larvae (Shiojiri et al., 2000a). These results indicate that *C. plutellae* responds to compounds that are produced only in response to injury by their hosts. Thus, it is unlikely that they respond only to GLV. In this paper, we showed that infested *all84 Arabidopsis* plants were more attractive to *C. plutellae* than intact *all84* plants, and there were no differences in attractiveness between infested *all84* and infested wt plants. These data indicate that the mutation in the LOX/lyase pathway in *all84* does not negatively affect *C. plutellae* attraction.

C. glomerata and *C. plutellae* show antennal host searching behavior to filter paper impregnated with a mixture of host-food plant juice and host regurgitant, but do not respond to either substance on its own (Sato, 1979; Shiojiri et al., 2000b). Thus, the plant factors as well as the host factors are involved in the production of arrestants that elicit the antennal searching behavior. Interestingly, the duration of antennal searching of *C. glomerata* on the host-infested edge of *all84* leaves was significantly shorter than that on wt leaves, suggesting the LOX/lyase pathway could also be involved in the production of arrestants for *C. glomerata*. By contrast, the duration of antennal searching by *C. plutellae* on host-infested edges of *all84* leaves did not differ significantly from that on infested wt leaves. The data indicate that the mutation in the LOX/lyase pathway in *all84* does not negatively affect the production of the *C. plutellae* arrestants.

Although the site of the mutation in *all84* is not yet clarified, our data show that the LOX/lyase pathway plays an important role in the interaction between host-infested *Arabidopsis* and *C. glomerata* mediated by attractants and arrestants. In contrast, the mutation in the LOX/lyase pathway in *all84* did not affect the chemical interaction between *Arabidopsis* and *C. plutellae*. This, however, does not necessarily mean that the LOX/lyase pathway is not involved in the production of *C. plutellae* attractants and arrestants. Identification of the mutation in *all84* is extremely important and is now under way. Van Poecke and Dicke (2002) reported that the jasmonic acid signaling pathway and the salicylic acid signaling pathway were both involved in the production of attractants for *C. rubecula*, a parasitoid of *P. rapae* larvae, by host-infested *Arabidopsis* plants. Whether these signaling pathways are also involved in the interaction between *Arabidopsis* and the two parasitic wasps in this study needs to be determined. Further detailed studies on the molecular mechanisms involved in the production of attractants and arrestants for both parasitic wasps would contribute to the understanding of the ecology and evolution of these tritrophic systems, and to the attempt to increase the effectiveness of natural enemies by using attractants and/or arrestants of plant origin in integrated pest management systems.

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Ablation of Caterpillar Labial Salivary Glands: Technique for Determining the Role of Saliva in Insect–Plant Interactions

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Abstract There has been an ardent interest in herbivore saliva due to its roles in inducing plant defenses and its impact on herbivore fitness. Two techniques are described that inhibit the secretion of labial saliva from the caterpillar, *Helicoverpa zea*, during feeding. The methods rely on cauterizing the caterpillar's spinneret, the principal secretory structure of the labial glands, or surgically removing the labial salivary gland. Both methods successfully inhibit secretion of saliva and the principal salivary enzyme glucose oxidase. Caterpillars with inhibited saliva production feed at similar rates as the untreated caterpillars, pupate, and emerge as adults. Glucose oxidase has been suggested to increase the caterpillar's survival through the suppression of inducible anti-herbivore defenses in plants. Tobacco (*Nicotiana tabacum*) leaves fed on by caterpillars with ablated salivary glands had significantly higher levels of nicotine, an inducible anti-herbivore defense compound of tobacco, than leaves fed upon by caterpillars with intact labial salivary glands. Tomato (*Lycopersicon esculentum*) leaves fed upon by caterpillars with suppressed salivary secretions showed greatly reduced evidence of hydrogen peroxide formation compared to leaves fed upon by intact caterpillars. These two methods are useful techniques for determining the role that saliva plays in manipulating plant anti-herbivore defenses.

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Introduction

Insect saliva contributes to a wide range of functions, such as digestion, water balance, circumventing animal–host defenses, maintenance of mouth parts, pheromone production, pathogen transmission, and antipredator defenses, and is suspected to have antimicrobial properties (Ribeiro, 1995; Felton and Eichenseer, 1999; Liu et al., 2004; Musser et al., 2005). Emerging data indicate that insect feeding induces plant responses that are qualitatively and quantitatively different than mechanical wounding (Felton et al., 1994; Stout et al., 1994, 1998; Alborn et al., 1997; McCloud and Baldwin, 1997; De Moraes et al., 1998; Felton and Korth, 2000; Kahl et al., 2000; Reymond et al., 2000; Musser et al., 2002a,b). A principal factor in determining the unique and specific plant responses to herbivory may be oral secretions released by the herbivore during feeding. Studies demonstrate that the application of insect regurgitant (or head extracts) to an artificial wound on plants can elicit defenses that are not associated with artificial wounding alone (Mattiacci et al., 1995; Alborn et al., 1997; McCloud and Baldwin, 1997; Felton and Korth, 2000; Kahl et al., 2000; Reymond et al., 2000; Musser et al., 2002b; but see Mithofer et al., 2005). β -Glucosidase and fatty acid–amino acid conjugates (e.g., volicitin) are elicitors of plant defenses and components of caterpillar regurgitant (Mattiacci et al., 1995; Alborn et al., 1997, 2000; Halitschke et al., 2001).

There are two principal limitations to the use of oral secretions as utilized in the studies cited above. First, the origin of the oral secretions is undefined. Regurgitant and head extracts include chemicals that may not be typically associated with caterpillar feeding (Ribeiro, 1995; Felton and Eichenseer, 1999). Regurgitant includes not only saliva from the labial and mandibular glands, but also gut enzymes, partially digested plant material, and gut microbes (Ribeiro, 1995; Felton and Eichenseer, 1999). Indeed, it has been suggested that elicitors could be of microbial origin (Spiteller et al., 2000). Caterpillar saliva originates from the labial and mandibular salivary glands (Felton and Eichenseer, 1999). For caterpillars, the spinneret functions as the secretory structure for labial saliva (Felton and Eichenseer, 1999). Second, there is a critical lack of dose response in these studies because it is not known if the amount of oral secretions applied to plant tissues is consistent with what is produced by caterpillars during feeding (see Peiffer and Felton, 2005).

Glucose oxidase (GOX), a salivary enzyme present in many lepidopteran and some herbivorous hymenopteran larvae, catalyzes D-glucose in the presence of oxygen to D-gluconic acid and hydrogen peroxide (H₂O₂) (Eichenseer et al., 1999; Felton and Eichenseer, 1999; unpublished data). GOX occurs in especially high levels in larval *Helicoverpa zea* and has been suggested to increase the caterpillar's survival through the suppression of inducible herbivore defenses in plants (Eichenseer et al., 1999; Felton and Eichenseer, 1999; Musser et al., 2002a). A wounded tobacco leaf treated with GOX had significantly less induction of nicotine, an inducible anti-herbivore defense, compared to tobacco plants that were wounded and treated with inactive GOX (Musser et al., 2002a).

In this paper we demonstrate two techniques that can be used to suppress herbivore saliva production *in situ*. These methods were then used to determine how saliva modulates induced defenses.

Methods and Materials

Plant and insect rearing

In a rearing facility with a 15-hr photoperiod, neonates of *H. zea* were fed a wheat germ and soy-protein-based artificial diet at 28°C until the sixth instar (Chippendale, 1970; Broadway and Duffey, 1986). Tobacco seeds were sown in 4-l plastic pots filled with a Redi Earth Peat-Lite soil mixture (Scott-Sierra Horticulture Products Company, Marysville, OH, USA) in a greenhouse at the University of Arkansas in Fayetteville, Arkansas. The greenhouse was maintained on a 14-hr photoperiod, with high-pressure sodium lights (1000 W) and day and night temperatures of approximately 33°C and 20°C, respectively. Plants were watered every 2 d and fertilized (N:P:K = 15:30:15, American Plant Food Co. Creve Coeur, MO, USA) once a week. When the tobacco plants exceeded a height of 0.33 m, they were used in the bioassays described below. Tomato plants (cv. Better Boy) were grown at 25°C in a greenhouse. Plants with four nodes were used for all experiments. *Pieris rapae* were reared on cabbage in a greenhouse at the University of Lausanne, Switzerland, in a manner described by Reymond et al. (2000).

Caterpillar spinneret cauterization

Spinnerets of actively feeding sixth instar *H. zea* caterpillars were cauterized in order to inhibit secretions from the labial salivary. Caterpillars were anesthetized with ice, held still with soft forceps, and viewed under a dissecting microscope while a red-hot metal probe touched the spinneret until the spinneret was cauterized sufficiently to remove the cone appendage. The probe was heated with a Bunsen burner. The spinnerets for mock-treated caterpillars had their cuticles cauterized near the spinneret and away from notable sensory structures so that the spinneret remained functional.

A 3,3'-diaminobenzidine tetrahydrochloride (DAB) assay was used to determine if the cauterization of the spinneret was successful. The caterpillars were starved for 1 hr, and then fed on glass fiber disks soaked with a mixture of 50 mg/ml sucrose and 50 mg/ml glucose solution. After feeding, damage of approximately 20% was apparent on the glass microfiber filters (Whatman International Ltd., Maidstone, UK), the fiber disk was treated with 75 μ l of DAB at a concentration of 0.5 mg/ml (dissolved in distilled water and HCL for a pH 5.0) (Sigma Chemical Co., St. Louis, MO, USA) and a solution of 75 μ l of 0.5 mg/ml of horseradish peroxidase (Sigma Chemical CO., St Louis, MO, USA). These two solutions turned the damaged disks brown if H₂O₂ was present due to catalysis of glucose. Similar cauterization was performed on an alternative species, fifth instar *P. rapae*; however, no screening method was used because we found that *P. rapae* contained no detectable GOX activity as determined by the methods described by Eichenseer et al. (1999), and their feeding activity was disrupted due to the cauterization.

Scanning electron microscopy

Samples for scanning electron microscopy were prepared as described by Hayat (1989). Briefly, the larvae's heads were removed and fixed overnight in 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) in 0.1 M phosphate buffer, pH 7.4. Larvae were rinsed in 0.1 M phosphate buffer and treated with 2% aqueous osmium tetroxide (EMS) for 2 hr. Following water rinses, the larvae were dehydrated in graded ethanols (70 to 100%), then dried in a Baltec SCD030 (Techno Trade, Manchester, NH, USA) critical point dryer. Dried samples were mounted and coated with gold/palladium to increase conductivity (Baltec SCD050, Techno Trade). Samples were imaged at 20 KV in a JSM 5400 scanning electron microscope (JEOL, Peabody, MA, USA), and images were captured by using IMIX-PC v.10 software (Princeton Gamma Tech, Princeton, NJ, USA).

Caterpillar labial gland ablation

The labial salivary glands were removed from living sixth instar *H. zea* caterpillars. Caterpillars were chilled at 4°C, submerged in antibacterial soap at 10 mg/l for 1 min, and then submerged in 0.5% solution of H₂O₂ for 1 min. The washed caterpillar was immediately placed onto a parafilm wax-coated dissection dish filled with water and then held dorsal end down by two pinned strips of parafilm. One strip was placed over the hindgut portion of the caterpillar, and the other strip was placed over the head and foregut. While the larva was submerged, the cuticle of the second abdominal segment between the true legs and prolegs was lifted slightly with forceps. A small incision was made in the cuticle that provided access to the pair of labial salivary glands. Using forceps, the labial salivary glands were completely extracted from the body cavity. The cuticle was pinched back together with the caudal portion overlapped by the anterior section of cuticle. The caterpillar was rinsed with distilled water to remove residual soap and peroxide, removed from the straps, and blotted dry with a tissue paper. The caterpillar remained lying dorsally on the tissue until the cuticle appeared sealed and the caterpillar became active (approximately 30 min). Mock-treated caterpillars had a wounded abdomen as described above, but the labial salivary glands were not removed from the cavity of the caterpillar. The caterpillars fed on glass fiber disks, and the disks were assayed with DAB as described above. *P. rapae* (fifth instar) caterpillars were ablated in a manner described above, except that the caterpillars were not washed in soap or H₂O₂.

To quantify the levels of GOX secreted by caterpillars with intact labial salivary glands vs. caterpillars without intact labial salivary glands, approximately 140 sixth instar *H. zea* caterpillars were selected; half had a surgery that removed their labial salivary glands, and the other half had a mock surgery where the glands remained intact as described directly above. After the caterpillars healed, they were individually placed into a medicine cup containing a 25-mm-diam glass fiber disk (Whatman Inc., Florham Park, NJ, USA) containing 50 mg/ml solution of sucrose, and placed into a 33°C incubator. We monitored the caterpillars for 10 hr, so that whenever approximately 30% of the glass fiber disk was eaten, the disk was stored in one of six 50-ml Corning™ polypropylene centrifuge tubes (Corning, New York, NY, USA) on ice. We evenly sorted the fed-on disks so that there were three

samples/centrifuge tubes containing disks fed on by caterpillars with intact salivary glands and three samples/centrifuge tubes containing disks fed on by caterpillars without labial salivary glands. In addition, after the above disks were eaten, the caterpillars received additional glass fiber disks that could have been added to the tubes above. Also, the caterpillar frass was removed from the disk as soon as possible to reduce the addition of gut enzymes or salivary GOX swallowed by the caterpillar before the surgery. We have determined that the frass still can have GOX activity and that may have slightly altered our quantified results. Caterpillars that appeared dead or did not feed due to the surgery were removed from the experiment.

After 10 hr, the partially eaten disks were washed by putting 5 ml of deionized water into the centrifuge tubes and vigorously shaken. The centrifuge tubes were spun in a centrifuge for 10 min at 5,000 rpm. The liquid supernatant was concentrated by cellulose 30,000 molecular weight cutoff Micron® centrifugal filter devices (Millipore Corp., Bedford, MA, USA) to approximately 1 ml per centrifuge tube. The concentrated retentate was assayed for the presence of GOX. Glucose oxidase activity was measured by the change of absorbance of the reaction mixture at 460 nm, read with a TU-1901 Double Beam Spectrophotometer (GenTech Scientific, Inc., Arcade, NY, USA) for a 1-min kinetic assay as described by Eichenseer et al. (1999).

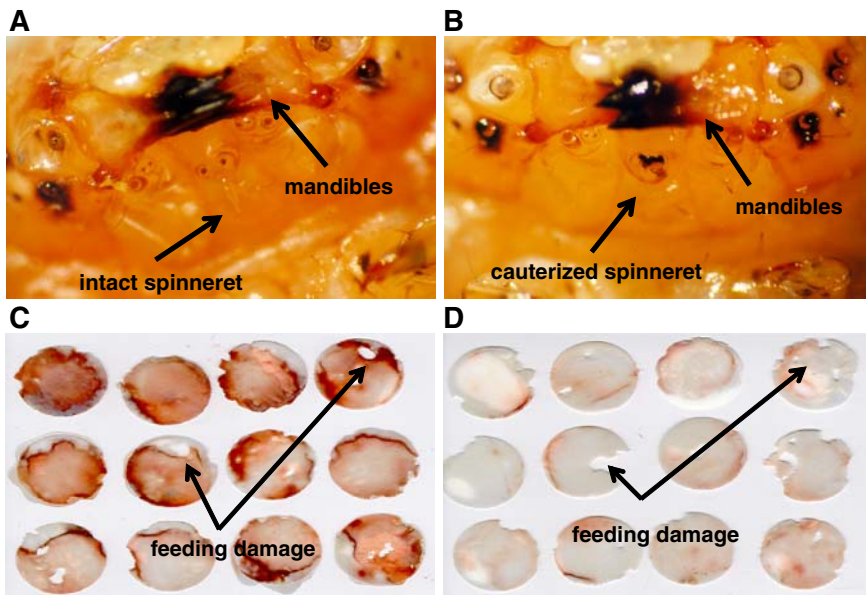


Fig. 1 The cauterization of the caterpillar spinneret. (A) Intact spinneret of *H. zea* caterpillar. (B) Cauterized spinneret. (C) Dark stain on glass fiber disks fed upon by *H. zea* caterpillars with intact spinnerets indicates that salivary GOX catalyzed the formation of H_2O_2 . (D) Glass fiber disks fed upon by caterpillars with cauterized spinnerets stained light compared to glass fiber disks fed upon by caterpillars with intact spinnerets indicating lower levels of secretion of the salivary enzyme, GOX

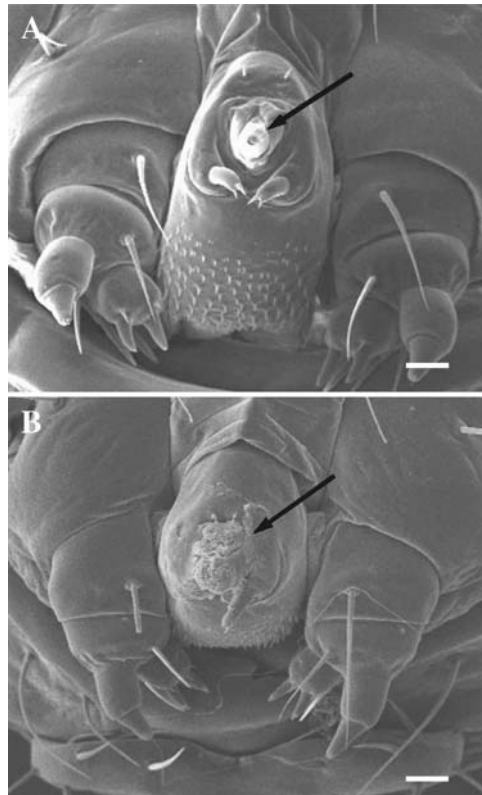
Effect of saliva on nicotine levels in situ

One *H. zea* caterpillar with either intact or ablated labial salivary glands was placed into a 9.5-mm-diam plastic Petri dish clip cage. This caged larva was attached on the second uppermost fully expanded tobacco leaf for 24 hr. Three days after the 24-hr feeding treatment, approximately 150 mg of the remaining leaf that was fed upon by the caterpillar were prepared for high-performance liquid chromatography (HPLC) analysis with C:18 column and MeOH/H₂O gradient to quantify nicotine (Saunders and Blume, 1981). A mean comparison of nicotine levels was analyzed with a Tukey–Kramer HSD test in a one-way analysis of variance with JMP (Sall and Lehman, 1996). The experiment was replicated six times.

Effect of saliva on H₂O₂ formation in tomato leaves

A DAB assay was also used for H₂O₂ production in tomato foliage fed upon by *H. zea* larvae with cauterized or intact spinnerets. The assay was slightly modified from Orozco-Cardenas and Ryan (1999). Tomato plant leaves were removed with a scalpel, and the cut end was placed in 1 mg/ml DAB (free base) for 3 hr. Sham-operated or cauterized larvae were allowed to feed on the leaves until they had eaten approximately 1 cm². Leaves remained in the DAB for an additional 2.5 hr, and were then cleared by boiling in 96% ethanol. Leaves were stored in 100% ethanol.

Fig. 2 Scanning electron micrograph of spinneret ablation in *H. zea*. Scale bars equal 100 μ m. Arrows signify the spinneret. (A) Intact spinneret of *H. zea* caterpillar. (B) Cauterized spinneret

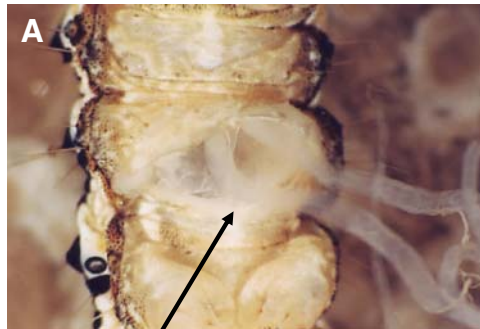


Results

Caterpillar spinneret cauterization and labial salivary gland ablation

H. zea caterpillars with cauterized spinnerets (Figures 1 and 2) or without labial salivary glands (Fig. 3) had drastically less labial salivary secretions than the mock-treated caterpillars as determined by glass fiber disk staining for H_2O_2 (Figures 1 and 4). In addition, when the glass fiber disks fed on by the caterpillars with intact labial glands were washed, we performed an enzyme assay on the retentate and determined that the collected salivary secretions showed drastically higher levels of the major labial salivary protein, glucose oxidase [2395.76 ± 1064.8 (mean \pm SE) $\mu\text{m mg}^{-1} \text{min}^{-1}$] compared to glass fiber disks fed on by caterpillars without labial glands (126.90 ± 52.2 $\mu\text{m mg}^{-1} \text{min}^{-1}$). So, although the caterpillars without labial salivary glands secreted negligible amounts of these enzymes in comparison to caterpillars with intact labial salivary glands, the feeding level for caterpillars with the suppressed salivary secretion was similar to the feeding level of their mock counterparts according to the percentage of glass fiber disk eaten per hour or the amount of artificial diet eaten ($P > 0.05$; data not shown). Both groups of caterpillars could still pupate and eclose to adults. We noted that if fifth instars had a cauterized spinneret, the sixth instar would lack a labium and the ability to secrete saliva.

Fig. 3 Labial salivary gland removal from living *H. zea* caterpillars. (A) Incision of cuticle of sixth instar *H. zea* on second abdominal segment with pair of salivary glands pulled from cavity. (B) Healed cuticle from sixth instar *H. zea* with ablated salivary glands

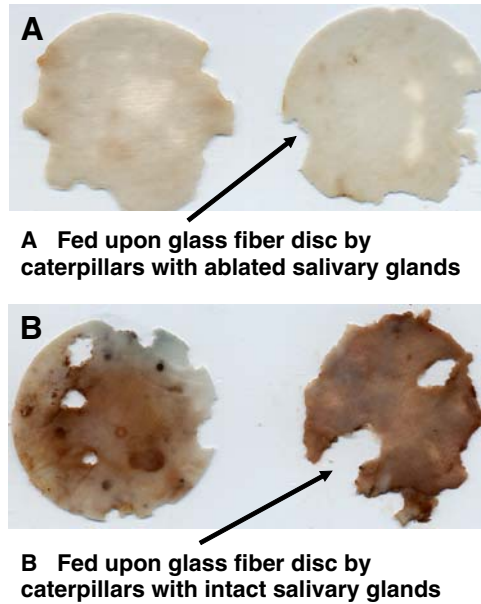


A. *H. zea* Labial Salivary Gland Extraction



B. *H. zea* Cuticle After Salivary Gland Extraction

Fig. 4 Glass fiber disk fed upon by *H. zea* caterpillars with ablated salivary glands or intact salivary glands. (A) Glass fiber disks fed upon by caterpillars with ablated salivary glands were light brown and white, which indicated low levels of H_2O_2 production compared to glass fiber disks fed upon by caterpillars with intact salivary glands (B) that were dark brown, which indicated high levels of H_2O_2 production



Other methods of salivary ablation were attempted with mixed results. Super glue[®] (Pacer Technology, Rancho Cucamonga, CA, USA) dabbed on the spinneret did not provide adequate inhibition of labial salivary secretions. Another approach involved the ablation of the labium and spinneret with dissecting scissors in the fifth instar. This resulted in adequate inhibition of labial saliva in the sixth instar because the labium and spinneret was absent after the molt. However, this dissection was more time-consuming than the procedures described above and resulted in higher levels of mortality (H. Eichenseer, personal communication).

P. rapae with cauterized spinnerets showed minimal signs of feeding and shortly died. However, labial salivary gland ablation was successful, and these caterpillars would feed at a similar rate as the mock-wounded caterpillars as determined by the number of *Arabidopsis* leaves fed on per hour per caterpillar. These caterpillars could also pupate and eclose to adults. In addition, mandibular salivary glands could

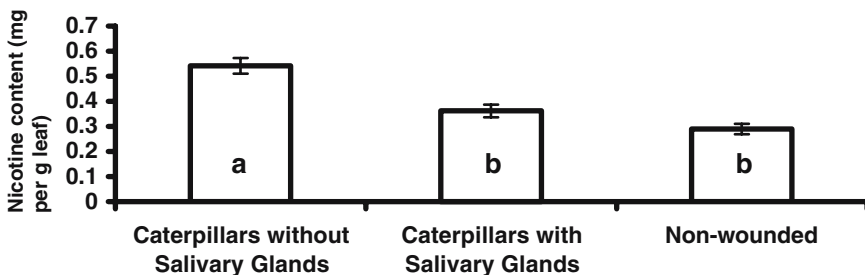


Fig. 5 Nicotine levels in tobacco leaves fed on by *H. zea* caterpillars. Nicotine content was analyzed by HPLC and expressed in milligrams per gram leaf wet weight. Different letters represent statistically significant differences between treatments at $P < 0.05$. Bars indicate mean \pm SE

be ablated in *P. rapae*, and these caterpillars could feed at a sufficient rate for experimentation. These results with *P. rapae* will allow further testing of the role of salivary secretions in eliciting plant responses.

Effect of Saliva on Nicotine Levels in Tobacco in situ

Tobacco leaves fed upon by *H. zea* caterpillars with intact salivary glands had significantly lower levels of nicotine compared to leaves fed upon by caterpillars with ablated salivary glands (Figure 5; $0.01 < P < 0.05$). These results are consistent with what was previously reported for the cauterization technique (Musser et al., 2002a).



Fig. 6 Effect of saliva on H_2O_2 formation in tomato leaves. Leaves were fed on by *H. zea* larvae with intact spinnerets or cauterized spinnerets. Dark deposits of DAB indicate the formation of H_2O_2

Effect of saliva on H₂O₂ formation in tomato leaves

The cauterization of spinnerets substantially reduced the oxidative burst in leaves fed upon by insects (Figure 6). H₂O₂ formation was visible by the brown deposits of oxidized DAB. Caterpillars with intact salivation elicited systemic peroxide formation, whereas caterpillars with compromised salivation elicited limited peroxide formation, primarily confined to the feeding sites and the vascular tissues. Tomato plants were chosen for this experiment because they showed a consistently stronger oxidative burst in response to larval feeding.

Discussion

Herbivores may elicit specific plant responses that are qualitatively and/or quantitatively unique compared to mechanical wounding (Felton et al., 1994; Stout et al., 1994, 1998). The oral secretions of herbivores are considered key components contributing to these unique and specific plant responses (e.g., Mattiacci et al., 1995; Alborn et al., 1997; Felton and Korth, 2000; Kahl et al., 2000; Reymond et al., 2000; Musser et al., 2002a,b). Techniques have not been available to determine the role of oral secretions or saliva *in situ*; to date, researchers have relied on collecting regurgitant and applying it to artificial wounds to mimic oral secretions during feeding. More recently, Mithofer et al. (2005) have developed a “mechanical” worm to simulate caterpillar feeding, and have demonstrated that volatile profiles emitted from lima bean leaves by using this device were qualitatively similar to caterpillar feeding. Here, we show that two methods could be successfully employed to suppress the production of saliva of caterpillars during feeding. By using these techniques, we show that plant defensive responses to caterpillar feeding are qualitatively different when caterpillars are able to secrete saliva compared to their treated counterparts in which salivary secretions are eliminated.

The ability to nearly eliminate the secretion of saliva *in situ* is a powerful tool that can be applied to investigate the physiology of caterpillar saliva. We have used this technique to study the role of saliva in modulating the induced responses of host plants (e.g., Musser et al., 2002a). *H. zea* caterpillars with cauterized spinnerets or ablated salivary glands had a dramatic reduction of H₂O₂ staining on the glass fiber disk in comparison to mock-treated caterpillars. This provided solid evidence that labial saliva in general and the labial salivary enzyme GOX specifically was suppressed.

Cauterization of the spinnerets prevented much of the oxidative burst in tomato foliage that follows rapidly after feeding. Mechanical damage in tomato triggers a rapid and systemic production of many signaling compounds such as jasmonic acid (Schilmiller and Howe, 2005). H₂O₂ has been shown to act as a secondary messenger for the induction of plant defense genes encoding proteinase inhibitors and polyphenol oxidases (Orozco-Cardenas and Ryan, 1999). The more extensive release of H₂O₂ in foliage fed upon by caterpillars with intact salivary secretion is likely due to the release of GOX during feeding. H₂O₂ is highly permeable across membranes and could easily diffuse from the initial feeding site as the insect enzyme oxidizes glucose in the plant tissues. Alternatively, GOX or other component(s) of saliva could mediate reiterative, systemic oxidative bursts by plant cells (Alvarez et al., 1998).

In most examples where the spinneret cauterization procedure was used, we concluded that saliva plays a role in suppressing plant defenses. *H. zea* with intact salivary glands that fed upon tobacco plants counteracted the stimulation of nicotine and proteinase inhibitors in comparison to feeding by caterpillars with ablated labial salivary glands (Musser et al., 2002a; unpublished data). Glucose oxidase was determined to be the principal salivary enzyme responsible for suppressing the induction of nicotine in wounded tobacco plants (Musser et al., 2002a). In addition to suppressing nicotine in tobacco, spinneret cauterization in *Spodoptera exigua* indicated that saliva and the enzyme GOX could decrease transcript levels of *Medicago truncatula* genes encoding terpenoid biosynthesis (Bede et al., 2006). The response of plants to herbivore feeding not only involves the induction of scores of defense-related genes, but also involves the repression of comparable numbers of genes (Arimura et al., 2000; Reymond et al., 2000). Saliva no doubt plays an important role in both the induction and repression of plant responses to herbivory.

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UV-Induced Mortality in Encapsulated Intertidal Embryos: Are Mycosporine-Like Amino Acids an Effective Sunscreen?

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Abstract Mycosporine-like amino acids (MAAs) are believed to protect a variety of marine organisms against the negative effects of ultraviolet radiation (UVR). However, their role in protecting developing intertidal encapsulated embryos remains untested. In the present study, we focused on the UV protective role of natural concentrations of MAAs for two intertidal gastropod species, *Bembicium nanum* and *Siphonaria denticulata*, which lay egg masses in habitats exposed to direct sunlight. We predicted that in both species, a higher concentration of MAAs within the egg mass would increase the likelihood of embryonic survivorship in the presence of UVR. Egg masses from both species were collected along the rocky shores of southeastern New South Wales, and a portion from each was subjected to one of three separate spectral treatments: full spectrum, UV-B block, and UV block. Proportions of surviving embryos were recorded following 72 hr exposure to spectral treatment. In addition, MAAs in each egg mass were quantified. Levels of variation in MAA concentration were striking, with *S. denticulata* egg masses showing more intraspecific variation than those of *B. nanum*. Surprisingly, survivorship under all spectral treatments was extremely high for both species, irrespective of MAA concentration. Under full spectrum treatments, *B. nanum* survivorship and total MAA concentration were significantly and positively correlated; however, MAA accounted for just 23.6% ($R = 0.486$) of the variation in survivorship. In contrast, survivorship in *S. denticulata* was not correlated with MAA concentration under full spectrum light. We conclude that the dependence on MAAs as photoprotection may be species-specific; however, it is likely that both species possess alternative mechanisms that minimize the negative effects of UVR.

Keywords Mycosporine-like amino acids · UV-B · Ultraviolet radiation · Egg mass · Gastropod · Embryonic survivorship · Intertidal · MAAs

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Introduction

Increasing levels of potentially harmful UVR are reaching the earth's surface due to the thinning of the ozone layer (Bjorn, 1999). Tropical areas have some of the highest levels of UVR because of the relative thinness of the ozone layer near the equator and the low zenith angle of the sun (Baker et al., 1980). However, organisms living at high latitudes have no less risk to UVR exposure, as seasonal ozone depletion has been detected above the midlatitudes (Platt, 2000). The harmful effects of solar ultraviolet radiation (UVR) are well documented in both terrestrial and marine environments, and they include DNA and protein damage, deleterious effects on behavior, reproduction, and overall ecosystem function (reviewed by Haeder et al., 1998; Paul and Gwynn-Jones, 2003). Indeed, increased exposure may already be a factor in global declines and range reductions in some aquatic populations (Belden and Blaustein, 2002).

In assessing the effects of UVR on marine organisms, particular attention has been placed on organisms inhabiting shallow waters. Potentially harmful UV-B wavelengths (280–320 nm) can penetrate to depths of up to 20 m (Booth and Morrow, 1997). Intertidal organisms are at the greatest risk of exposure and potentially experience some of the highest UVR intensities (Karentz, 2001). Even more vulnerable are the developing embryos within egg masses deposited in habitats exposed to sunlight. UVR can stunt development, produce deformities, and cause the death of encapsulated embryos of some intertidal gastropods (Biermann et al., 1992; Rawlings, 1996; Przeslawski et al., 2004); however, egg masses of species that routinely spawn in full-sun habitats are generally more resistant to the negative effects of UVR than egg masses from those species that spawn only in shaded habitats (Przeslawski et al., 2004). The reasons for these observations remain unknown, but it may be that embryos of these species are biochemically protected against UV-induced damage.

Mycosporine-like amino acids (MAAs) are known to serve many marine organisms as biochemical sunscreens (reviewed by Shick and Dunlap, 2002). They are clear, water-soluble compounds that absorb UVR maximally within the potentially harmful wavelengths 310–360 nm. MAAs encompass 19 known compounds; but some of these, such as mycosporine–taurine, are restricted to only a few taxa (Karentz, 2001). Overall, mycosporine–glycine, porphyra-334, shinorine, and palythine are the most common MAAs in marine organisms (Karentz, 2001). Plants, algae, and cyanobacteria are able to synthesize MAAs *de novo* through the shikimate pathway (Bentley, 1990). In contrast, animals lack this pathway and, therefore, likely acquire MAAs through symbioses or diet (reviewed by Karentz, 2001; Shick and Dunlap, 2002).

The biological importance of MAAs as natural sunscreens has been inferred, in part, by their widespread occurrence in algal and invertebrate species that inhabit shallow water throughout the world's oceans and lakes (Karentz et al., 1991; Shick et al., 1992; Jeffrey et al., 1999; Sommaruga and Garcia-Pichel, 1999). Three pieces of evidence underscore the important photoprotective role of MAAs. First, MAA concentrations are positively correlated with levels of UV-B radiation exposure in organisms that are able to synthesize MAAs *de novo* (Franklin et al., 1999; Shick et al., 1999). Second, MAAs accumulate in potentially vulnerable tissues such as the skin, ovaries, and eggs of marine invertebrates, likely providing UV screening protection (Shick et al., 1992; Gleason and Wellington, 1995; Adams et al., 2001).

Finally, experiments confirm that developmental abnormalities in planktonic larvae after exposure to UVR is inversely correlated to MAA concentration (Gleason and Wellington, 1995; Adams and Shick, 1996, 2001).

MAAs are prevalent in intertidal egg masses (Przeslawski et al., 2005a), although their photoprotective role in this stage of development remains untested. The present study is the first to examine the relationship between MAAs and mortality in encapsulated embryos; moreover, it represents the first study to examine this relationship in more than one intertidal organism, thus allowing interspecific comparison of photoprotective mechanisms. *Siphonaria denticulata* and *Bembicium nanum* were chosen because their egg masses are found abundantly year-round along the rocky shores of southeastern Australia (authors' unpublished data), and both species incorporate MAAs in their egg masses (Przeslawski et al., 2005a). Furthermore, *S. denticulata* and *B. nanum* spawn exclusively in habitats exposed to full sunlight (Benkendorff and Davis, 2004). In the present study, we sought to determine how natural variation in MAA concentration affects subsequent survivorship following exposure to UVR. We predicted that embryonic survivorship in both species would be positively correlated with the concentration of MAAs following exposure to full spectrum light.

Methods and Materials

Egg Mass Collection

Undeveloped (retrochophore) egg masses of *S. denticulata* and *B. nanum* ($N = 26$) were collected from three intertidal locations along the Illawarra coast, NSW, Australia during March–April 2004. *S. denticulata* deposits cylindrical gelatinous ribbon egg masses, and *B. nanum* deposits discrete connected gelatinous capsule egg masses (see Przeslawski et al., 2005b). We collected both species from rocky intertidal locations at Bass Point (34°35'S, 150°53'E), Bellambi (34°37'S, 150°92'E), and North Wollongong (34°25'S, 150°55'E). Previous research revealed no spatial or temporal differences in MAA complements within egg masses from these sites (Przeslawski et al., 2005a). All egg masses were examined under a dissecting microscope (40× magnification) to confirm the retrochophore stage of development.

Spectral Treatments

Egg masses were cut in half, and one half was further divided into three pieces. Previous research has confirmed that cutting gelatinous egg masses does not have any negative effects on embryonic mortality (Rose, 1986; Przeslawski et al., 2005b). Each piece was placed in small plastic containers (height 2.5 cm, diam 6 cm) in an outdoor 300-l recirculating aquarium. Natural sunlight and cutoff filters were used to obtain three spectral treatments: (1) full spectrum (no filter), (2) UV-B block (mylar film), and (3) UV block (polyethylene film with UV absorption) (refer to Przeslawski et al., 2004 for spectral transmission properties). Containers were randomly repositioned daily within the aquarium. Salinity was maintained at 35 ppt with the addition of distilled water. A submerged refrigeration unit maintained temperature at $19.7 \pm 0.2^\circ\text{C}$ (mean \pm SEM). This was confirmed for the duration of

the experiment with records taken at 15-min intervals with a thermal microchip (Thermochron I-Button).

After 72 hr in experimental treatments, all egg mass pieces were removed and examined with a dissecting microscope (40× magnification). Peripheral embryos were examined as they were most directly exposed to spectral treatments. Also, only peripheral embryos could be examined without destroying the integrity of the egg mass (see Przeslawski et al., 2004). Survivorship of embryos was estimated for each replicate by scoring a random sample of 100 embryos on both sides of the egg mass as alive or dead. Embryos were considered dead if they were degenerating (Przeslawski, 2004).

MAA Analysis

The other half of each egg mass was cleaned by agitation in filtered seawater for 30 sec followed by gentle blotting to remove excess water. The egg masses were lyophilized and stored at -80°C for up to 4 wk until extractions were performed. Previous research has shown that such a storage time does not affect MAA concentration (Dunlap and Chalker, 1986; Karsten et al., 1998).

Three serial extractions were performed on 20 mg dry weight of each egg mass in 0.5 ml of 80% HPLC-grade methanol for 1 hr at room temperature. Extraction efficiency was tested and was over 95% after three extractions for both species. The supernatant was pooled, and MAAs were separated by reverse-phase high performance liquid chromatography (RP-HPLC) on a Phenosphere C8 5 μm (4.6 i.d. \times 250 mm) column with guard (Phenomenex) at a flow rate of 0.8 ml min^{-1} . The aqueous mobile phase was 39.9:0.1:60 (water/acetic acid/methanol.) MAAs were identified using maximum wavelength absorption and cochromatography with prepared standards from the Australian Institute of Marine Science.

Statistical Analysis

To determine potential inter- and intraspecific differences in MAA complements, nonmetric multidimensional scaling and one-way ANOSIMs were performed by using PRIMER (v.5). Potential effects of species and spectral treatment on embryonic mortality were analyzed with a nested ANOVA with the REML technique in JMP (v.4). To satisfy assumptions of ANOVA, survivorship was transformed by using $\arcsin\sqrt{x}$ due to its binomial distribution (Zar, 1998). Potential relationships between embryonic survivorship and total MAA concentration were resolved by using correlation analyses for each species under each spectral treatment using JMP (v.4). $\alpha = 0.05$ for all statistical tests.

Results

MAA Concentrations

Mycosporine-like amino acids were found in all egg masses. Seven distinct MAAs were detected (Table 1), and the chromatograms revealed no unidentified MAAs. We recorded the presence of seven MAAs in egg masses of *S. denticulata* and four in *B. nanum* egg masses (Table 1). Egg masses from *B. nanum* had the lowest total

Table 1 Mean concentrations of mycosporine-like amino acids (nmol mg⁻¹ dry weight) in *Siphonaria denticulata* and *Bembicium nanum* egg masses (*N* = 26)

	<i>Siphonaria denticulata</i>	<i>Bembicium nanum</i>
Mycosporine-glycine	3.41 ± 0.38	6.30 ± 0.41
Shinorine	0.88 ± 0.40	0.69 ± 0.16
Porphyra-334	3.76 ± 0.68	1.30 ± 0.18
Mycosporine-2-glycine	0.01 ± 0.01	0.04 ± 0.04
Palythene	1.56 ± 0.32	NP
Palythine	0.52 ± 0.09	NP
Asterina-330	3.04 ± 0.60	NP
Total	13.17 ± 1.31	8.33 ± 0.45

NP indicates a compound was not present. Error is standard error of mean.

concentrations of MAAs (8.33 nmol mg⁻¹ ± 0.45 SE), while total concentrations of MAAs in egg masses of *S. denticulata* averaged 13.17 nmol mg⁻¹ (± 1.31 SE). The highest individual total MAA concentration recorded was 23.20 nmol mg⁻¹ in an egg mass of *S. denticulata*.

An n-MDS plot revealed that MAA complements differed among species (Fig. 1), and an ANOSIM confirmed that these differences were significant (*R* = 0.598, *P* = 0.001). Intraspecific variation of MAAs was relatively high in egg masses of *S. denticulata* as revealed by the relatively large spread of data points in the n-MDS plot (Fig. 1). In contrast, egg masses of *B. nanum* showed much lower levels of intraspecific variation in MAA complements (Fig. 1).

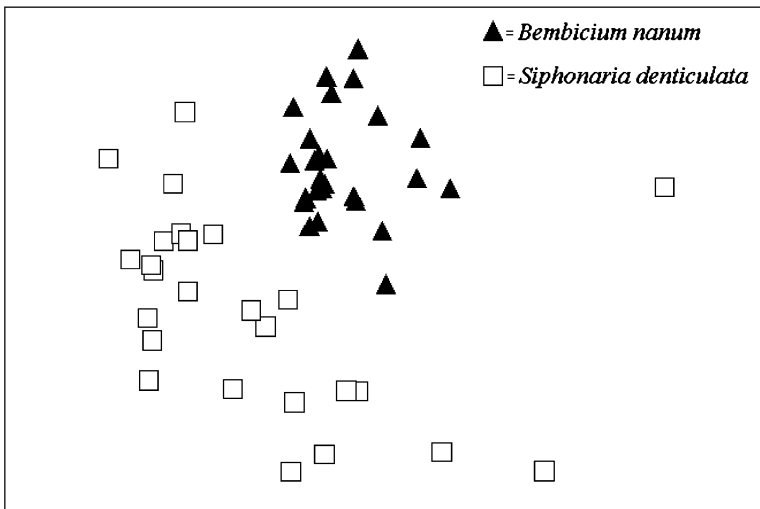


Fig. 1 A nonmetric multidimensional scaling of MAA complements in egg masses from two species of intertidal mollusk (*N* = 26). Points that are relatively close denote similarities in their MAA complements. Stress = 0.12

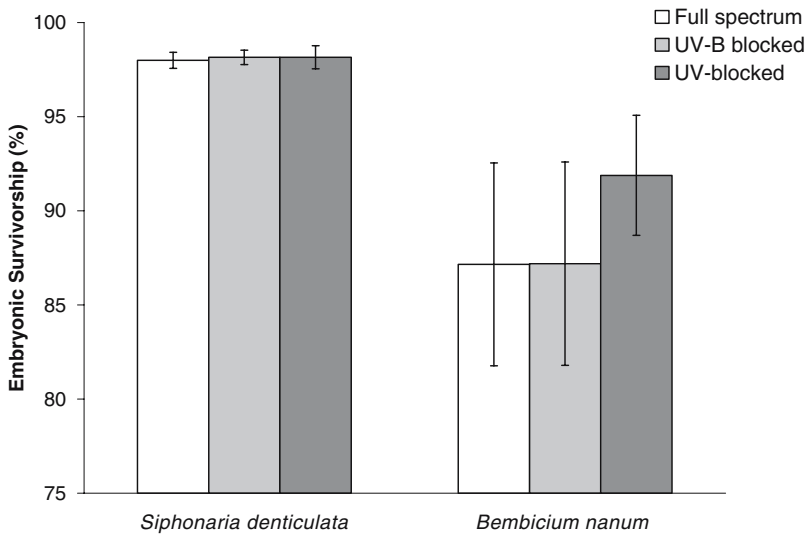


Fig. 2 Mean embryonic survivorship following exposure of each mollusk species to spectral treatments ($N = 26$). Error bars are standard error of the mean

Embryonic Survival

On average, *S. denticulata* had the highest proportion of surviving embryos in all spectral treatments with at least 98% survivorship across all treatments (Fig. 2). Furthermore, embryonic survivorship of this species showed little variation as evidenced by the relatively small error bars (Fig. 2). On average, *B. nanum* had lower embryonic survivorship (Fig. 2). An ANOVA confirmed that embryonic survivorship significantly varied between species ($F = 11.149$, $P < 0.002$) (Table 2). In contrast, spectral treatment did not significantly affect embryonic survival ($F = 1.581$, $P = 0.211$) (Fig. 2, Table 2).

MAA Concentration and Embryonic Survival

Data plots revealed several outliers that disproportionately affected correlations (Fig. 3, Table 3). Accordingly, these outliers were omitted from remaining analyses following the criteria outlined in Chatterjee and Price (1977). Embryonic survival of *B. nanum* was significantly positively correlated with total MAA concentration only

Table 2 Effects of species and spectral treatment on embryonic survivorship as determined by ANOVA

Effect	df	MS	F	P
Egg mass (species)	50	0.085	2.527	<0.001
Species	1	0.373	11.149	0.002
Spectral	2	0.053	1.581	0.211
Species \times Spectral	2	0.018	0.541	0.584
Residual	100	0.034		

An arcsin transformation was used on data. Italics denote a random factor.

under the full spectrum treatment ($R = 0.486$, $P = 0.016$) (Fig. 3a) (Table 3). Embryonic survival of *S. denticulata* was not correlated with MAA concentration in any treatment (Fig. 3, Table 3).

Discussion

We documented high survivorship for embryos of both species across all spectral treatments (Fig. 2), irrespective of MAA concentration. Contrary to our prediction, MAAs do not seem to be an effective sunscreen in embryos of either *Bembicium nanum* or *Siphonaria denticulata*. Rather, our results suggest that MAAs play only a minor photoprotective role for the embryos of *B. nanum* and provide no detectable photoprotection for embryos of *S. denticulata*. It therefore, seems likely that *B. nanum* and *S. denticulata* embryos use protective measures other than MAAs to minimize the deleterious effects of UV radiation.

The photoprotective role of MAAs in developing intertidal embryos varies across species as indicated by differences in the relationships between embryonic survivorship and MAA concentration under full spectrum treatments for *B. nanum* and *S. denticulata* (Fig. 3a). Moreover, a relatively large intraspecific variation in *S. denticulata* MAA complements (Fig. 1) suggests that MAAs are not of uniform importance to all encapsulated embryos of this species, despite all egg masses being laid in habitats exposed to direct sunlight. Factors such as diet and viability can affect MAA complements (Przeslawski et al., 2005a). These results highlight the importance of examining the interspecific variation in the photoprotective role of MAAs and emphasize the need to be cautious in drawing general conclusions regarding the role of MAAs in marine organisms from other studies that examine single species (Gleason and Wellington, 1995; Adams and Shick, 1996; Franklin et al., 1999).

The distinct MAA complements provide a possible explanation for the difference in photoprotective dependence between *B. nanum* and *S. denticulata*. In this study, the only MAA detected that absorbs maximally in the UV-B range is mycosporine-glycine ($\lambda_{\max} = 310$ nm). Mycosporine-glycine is also a moderate antioxidant and so may provide additional protection against damaging reactive oxygen species produced by UVR exposure (Dunlap and Yamamoto, 1995). Therefore, mycosporine-glycine may provide more protection to developing embryos against UV-B-induced damage than the other MAAs.

In addition, the distinct MAA complements within egg masses of both species could potentially be used as a taxonomic character. This result supports findings by Shick and co-workers (2002), who found that the MAA complements of four North American sea anemone species *Anthopleura* showed distinct phylogenetic differences. Phylogenetic factors were recently shown to affect MAA complements in intertidal egg masses (Przeslawski et al., 2005a).

Bembicium nanum embryonic survivorship was not significantly correlated to MAA concentration when UV-B was blocked (Fig. 3b), suggesting that it was primarily the damage associated with UV-B that was minimized. UV-B is absorbed by nucleic acids and proteins and can thus negatively affect the rapidly dividing cells of marine larvae. For example, UV-B can cause developmental retardation and abnormalities in planktonic urchin and coral larvae (Gleason and Wellington, 1995; Adams and Shick, 2001). Egg masses of *B. nanum* had the highest concentrations of

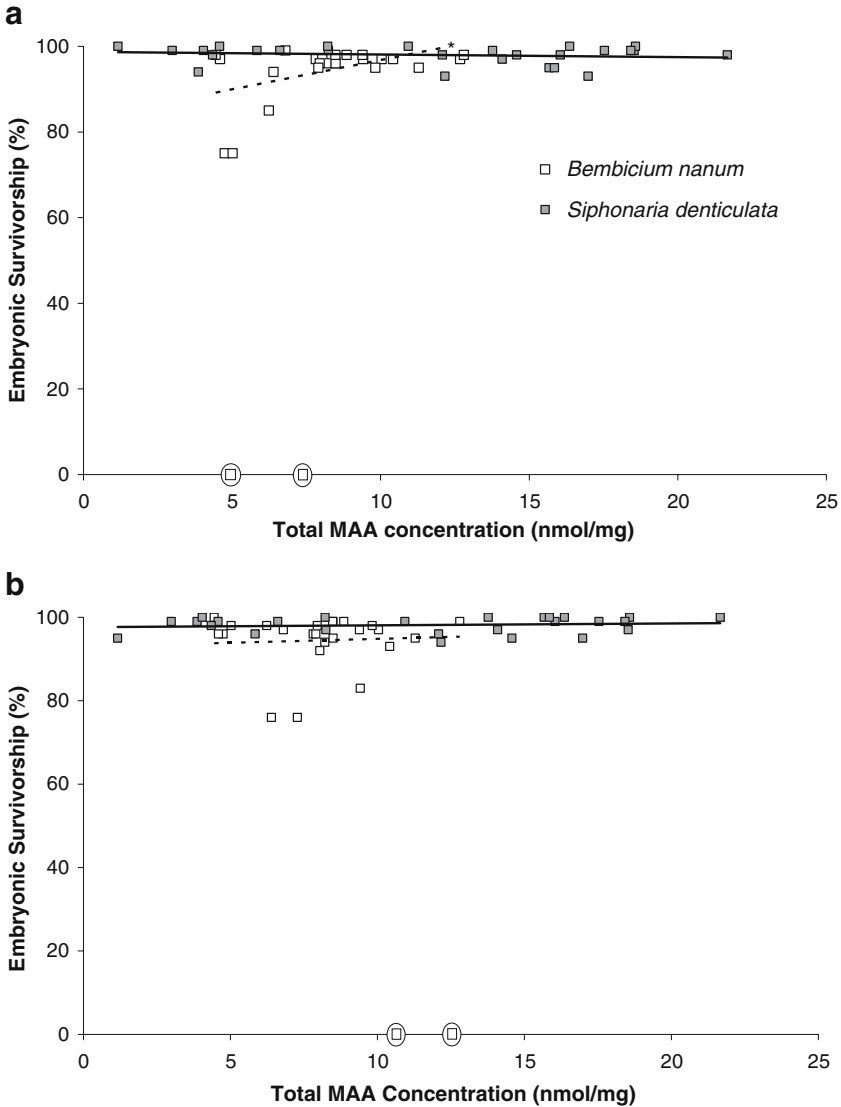


Fig. 3 Correlation between total concentration of mycosporine-like amino acids and embryonic survivorship for two species of mollusk under different spectral treatments: (a) full spectrum, (b) UV-B blocked, and (c) UV-blocked. See Table 3 for *N* values excluding outliers. Solid lines indicate best-fit lines for *Siphonaria Denticulata*, and dashed lines indicate best-fit lines for *Bembicium Nanum*. Outliers are circled and excluded from best fit lines. (*Significance at $A = 0.05$)

mycosporine–glycine (Table 1), and MAAs in egg masses of this species showed the clearest photoprotective function (Fig. 3a, Table 3).

High embryonic survivorship observed in this study across all spectral treatments, irrespective of MAA levels, suggests that neither species relies heavily on MAA's photoprotective qualities (Fig. 3a,b). *B. nanum* and *S. denticulata* may incorporate other photoprotective measures including UV-absorbing compounds such as

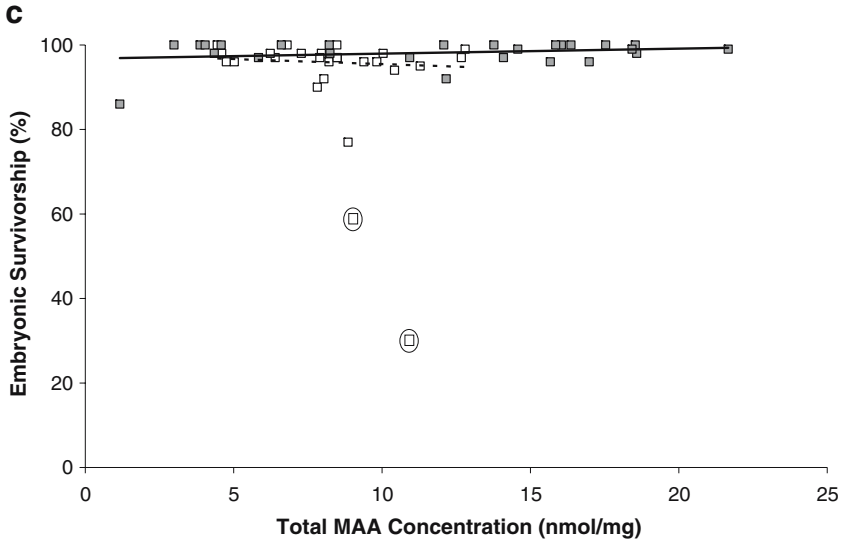


Fig. 3 (continued)

melanin (Cockell and Knowland, 1999; Karentz, 2001), which has been found in other mollusks (Prota, 1988), as natural photoprotection. Alternatively, these species may contain high levels of DNA repair enzymes to combat the potential negative effects of UV exposure. Photolyase, an inducible DNA repair enzyme, has been found in several adult mollusks (Carlini and Regan, 1995), but its presence and protective capabilities in molluskan embryos remains unknown. DNA repair enzymes are more effective at warmer temperatures (Macfadyen et al., 2004), and recent research has revealed that embryos of *S. denticulata* show reduced UV-induced mortality at warmer temperatures, a finding consistent with the presence of such enzymes (Przeslawski et al., 2005b). Moreover, embryos of *S. denticulata* show some evidence of UV-A inducible repair functions (Przeslawski, 2005). The consistently high survivorship of embryos of both species following UVR exposure, regardless of their MAA concentration, is striking and merits further investigation into other potential protective mechanisms.

Despite the unexpectedly low dependence for MAA photoprotection in embryos of both species, the presence and abundance of MAAs in all egg masses examined indicates that they may nonetheless be important to encapsulated embryos. This study only focused on UV-induced mortality, and it is possible that MAAs reduce

Table 3 Correlation analyses of embryonic survival and total MAA concentration for both species at each spectral treatment

	<i>Siphonaria denticulata</i>			<i>Bembicium nanum</i>		
	<i>N</i>	<i>R</i>	<i>P</i>	<i>N</i>	<i>R</i>	<i>P</i>
Full spectrum	26	0.167	0.414	24 (26)	0.486 (0.021)	0.016 (0.919)
UV-B blocked	26	0.136	0.508	24 (26)	0.062 (0.270)	0.776 (0.182)
UV blocked	26	0.227	0.180	24 (26)	0.120 (0.415)	0.578 (0.035)

Data in parentheses indicate correlation analyses including outliers.

the rates of abnormalities and other sublethal effects not detected in this study. Furthermore, MAAs have been considered as reproductive and osmotic regulators, although the case remains to be proven for marine invertebrates (reviewed by Shick and Dunlap, 2002). Therefore, MAAs may have such regulatory functions in encapsulated development for the intertidal species examined here.

It has been widely assumed that MAAs have a photoprotective role in marine organisms (Garcia-Pichel and Castenholz, 1993; Teai et al., 1997; Helbling et al., 2002); however, it is difficult to assign such a role unambiguously given the potential for other photoprotective mechanisms or physiological functions associated with MAAs (Cockell and Knowland, 1999). Previous research has shown that the accumulation of MAAs lowers the rates of UV-induced mortality and abnormalities in coral planulae and urchin embryos (Gleason and Wellington, 1995; Adams and Shick, 2001); however, these studies focused on single taxa. Our work shows that the photoprotective role of MAAs is species-specific in encapsulated intertidal embryos. Further research examining interspecific variation in planktonic or subtidal larvae may reveal similar species-specific effectiveness. Alternatively, the trend may be confined to intertidal encapsulated embryos. They are potentially exposed to more synchronous abiotic stressors than planktonic embryos and larvae (Przeslawski, 2004). These abiotic stressors can act synergistically with UVR to increase risk to embryos (Przeslawski et al., 2005b). Species that spawn in the intertidal zone, particularly those like *S. denticulata* and *B. nanum*, which spawn in habitats exposed to full sun, may have evolved multiple protective mechanisms to protect against damage from environmental stress, including UVR. Based on the results of this study, we conclude that MAAs do not represent the primary photoprotective strategy used by either species.

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Genetic Control of the Enantiomeric Composition of Ipsdienol in the Pine Engraver, *Ips pini*

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Abstract The genetic nature of pheromone variation within species has rarely been studied, and never for male-produced long-range pheromones. Males from western North American populations of *Ips pini* produce predominantly (*R*)-(-)-ipsdienol, whereas those from eastern North American populations produce higher proportions of (*S*)-(+)-ipsdienol. From a population in the hybrid zone, we divergently selected lines for the opposing pheromonal types and then created F1, F2, and backcross lines. We formed additional F1, F2, and backcross lines, first by using populations with low (+)-ipsdienol enantiomeric ratios near to and distant from the hybrid zone, and then by using populations with high (+)-ipsdienol enantiomeric ratios near to and distant from the hybrid zone. Three types of analysis were employed: (1) line means analysis; (2) Mendelian analysis of assigned high and low (+)-ipsdienol enantiomeric ratio phenotypes when applicable; and (3) Castle–Wright estimation of the number of effective factors. Dominance at one autosomal locus explains much of the variation in ipsdienol blend between the divergently selected lines. Thus, as in all previously studied female long-range pheromone systems, a major genetic element is implicated. The populations with low (+)-ipsdienol enantiomeric ratios near and distant to the hybrid zone differ negligibly for this trait. We confirmed previous studies showing slightly higher ratios of (+)-ipsdienol at the hybrid zone than in a distant eastern population and reveal a genetic basis for this difference.

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Introduction

An important consideration for the speciation theory is the number of genes required for reproductive isolation. Traditional population genetic theory suggests that evolution progresses by the accumulation of small genetic changes (Fisher, 1930; Dobzhansky, 1937). In the past two decades, this assumption has been challenged on theoretical and experimental grounds (Kimura, 1983; Orr and Coyne, 1992), and the list of cases where major genes are involved in premating isolation has grown (Coyne and Orr, 1998; Ritchie and Phillips, 1998).

While pheromones are involved in reproductive isolation across a wide range of taxa (Costa et al., 1997; Kotani et al., 2001; Miyanoshita and Tatsuki, 2001; Lemaster and Mason, 2003), inquiry into the genetic nature of such isolation has generally been limited to a few well-studied systems. Variation in *Drosophila* cuticular hydrocarbon profiles both between- (Coyne et al., 1994; Coyne, 1996) and within-species (Takahashi et al., 2001) has been shown to be controlled by relatively few loci, as are corresponding response preferences (McMahon et al., 2002). Major gene control of female-produced, long-range pheromones is also a well-established phenomenon in Lepidoptera (Löfstedt, 1990). Underscoring the degree to which major genetic shifts can occur in moth systems, the activation of long-dormant pseudogenes was recently implicated in differences between pheromone components of Asian vs. European corn borers (Roelofs et al., 2002). Major gene control of pheromone blend production and response have also been shown between pheromone races of moth species such as the European corn borer, *Ostrinia nubilalis* (Klun and Maini, 1979; Hansson et al., 1987; Roelofs et al., 1987), and the turnip moth, *Agrotis segetum* (LaForest et al., 1997).

Males of most species are more likely to experience strong sexual selection because females are usually the resource-limiting sex (Bateman, 1948; Williams 1966; Trivers, 1972; Clutton-Brock, 1988). Phelan (1992) argued that signaling systems involving pheromone production by females allow relatively high variation in pheromone production, but they place more stringent selection upon male response. This pattern, applicable to most of the well-studied lepidopteran systems, leads to the possibility of sudden large pheromone shifts by females that will inevitably be tracked by responding males. Conversely, Phelan (1992) proposed that changes in male signaling systems will more often involve runaway sexual selection (Fisher, 1930), leading to a building of linkage disequilibrium between signal and response.

The implications of such strong sexual selection in male signaling systems upon the genetics of pheromone blend variation remain largely unexplored. Indeed, variation in orthopteran male auditory signals, which perform a similar behavioral role, is usually controlled by multiple loci (Shaw, 1996, 2000). Alternatively, biochemical constraints may make major genetic changes likely for all pheromone systems, including those where males signal. Major gene control has been implicated in variation of male *Drosophila melanogaster* hydrocarbons (Ferveur and Jallon, 1996), but it is unclear if such hydrocarbons are actually used in mate discrimination (Scott, 1994; Cobb and Ferveur, 1996). Males of many bark beetle species (Coleoptera:

Scolytidae) produce aggregation pheromones that may be relevant to reproductive isolation. In scolytids, pheromone blend usage across multiple species is not affected by phylogenetic proximity (Symonds and Elgar, 2004a), or overlap in geographic range or host utilization (Symonds and Elgar, 2004b). Symonds and Elgar argue that these patterns suggest frequent saltational shifts in pheromone blends.

The pine engraver, *Ips pini* (Say), provides an ideal candidate for investigating some of the above-stated theoretical predictions on a microevolutionary scale. Eastern and western populations of this species are distinguished by morphological characteristics (Hopping, 1964) and pheromones, including ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) (Birch et al., 1980; Lanier et al., 1980), and lanierone (4,4,6-trimethyl-2-hydroxy-2,5-cyclohexadiene-1-one) (Teale et al., 1991). Populations from California to southern British Columbia are predominantly characterized by greater production of and preference for high ratios of the (*R*)-(-)-enantiomer of ipsdienol (Birch et al., 1980; Miller et al., 1989; Seybold et al., 1995a) (Fig. 1). We generally refer to these populations as “western.” Conversely, populations from the eastern United States to western Canada produce and respond to blends of ipsdienol that are racemic or have a higher ratio of the (*S*)-(+)-enantiomer (Lanier et al.,

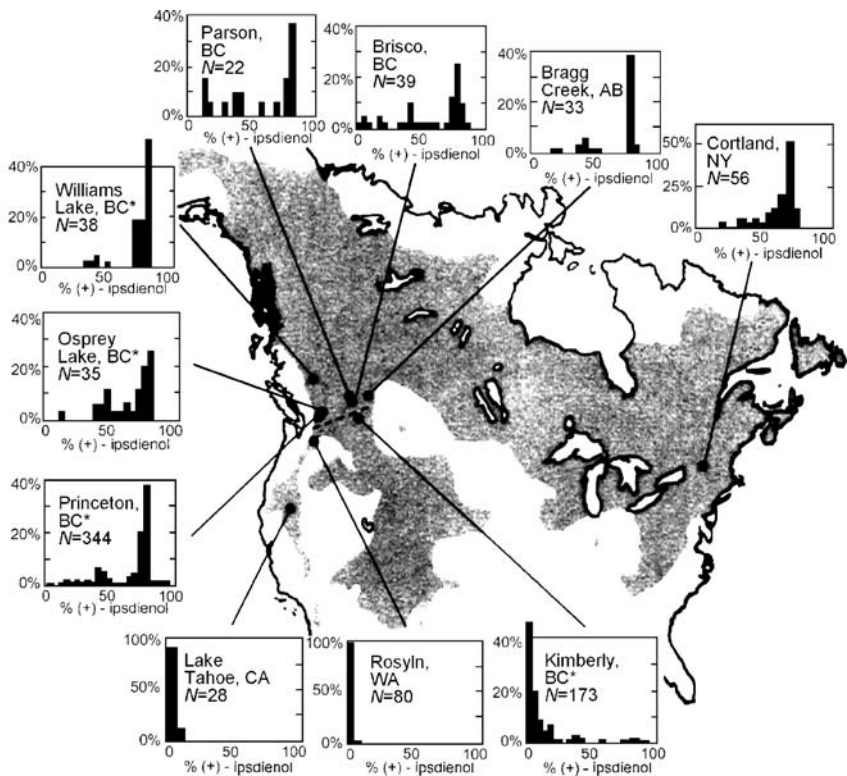


Fig. 1 Distribution of *Ips pini* pheromone types in North America based on the profiles of the enantiomeric composition of ipsdienol from individual males. Shaded area indicates range of *I. pini* (Lanier, 1972). A hybrid zone is depicted in southern British Columbia by a dashed line. Histograms indicated with an asterisk (*) have been reproduced from Miller et al. (1996), with the permission of the authors and publisher

1980; Miller et al., 1989; Seybold et al., 1995a). We refer to such contiguous populations as “eastern.”

This pattern of geographic variation also suggests that there is a hybrid zone in southern British Columbia (Fig. 1), lying on a north–south gradient (Miller et al., 1989, 1996; Seybold et al., 1995a). Additionally, British Columbia populations have many individual males that produce approximately 80% (+)-ipsdienol (Miller et al., 1989, 1996), while no more than 70% (+)-ipsdienol has been observed in individuals further east (Miller et al., 1989) (Fig. 1). Assortative mating based on ipsdienol enantiomeric blend has been demonstrated within a New York population (Teale et al., 1994).

Lanierone is strongly synergistic with ipsdienol in eastern populations of *I. pini* (Teale et al., 1991; Miller et al., 1997), but is rarely produced and weakly synergistic with ipsdienol in California (Seybold et al., 1992; Miller et al., 1997). Lanierone has been shown to increase trap captures in eastern Oregon (Zhou et al., 2001).

To investigate the genetic control of geographic variation in ipsdienol enantiomeric blend in *I. pini*, we performed three crossbreeding experiments. First, we obtained breeding lines that exhibit the extreme pheromone phenotypes from one population in the putative hybrid zone, and performed crosses between these lines. We also performed mirroring crosses between populations near to and distant from the hybrid zone, on the two opposing sides.

Methods and Materials

Field Populations

Ips pini was collected from two sites near the hybrid zone in British Columbia. The first site, near Brisco, British Columbia, Canada (hereafter referred to as BC) (Fig. 1), was approximately 30 km from Radium where Miller et al. (1996) observed high population diversity in pheromone enantiomeric composition. The Brisco site was characterized by a cluster of blown down trees of lodgepole pine, *Pinus contorta latifolia*, which had been colonized by *I. pini* within 5 d of our collection. All of the adult specimens that we collected had recently colonized the logs, and the females were in the early stages of egg gallery construction. Forty frass samples and the corresponding males were collected from the bark surface and phloem for pheromone analysis. These frass samples represented boring dust contributed by both males and females. An additional 30 males and 70 females were collected and stored at 5°C in fresh phloem. Twenty-one of these males were each paired with two females in logs of red pine, *Pinus resinosa*, to establish a laboratory colony in Syracuse, NY, USA. From a second site in Parson, BC, 40 males were excavated from infested logs of *P. contorta latifolia* and later sampled for pheromone production on *P. resinosa* in Syracuse.

From Roslyn, Washington, USA (hereafter referred to as WA), 10 males and 14 mated females were collected from logs of *P. contorta latifolia* and taken live to Syracuse. Females were provided with individual slabs of *P. resinosa* in separate containers to oviposit and progenies were allowed to emerge. Ipsdienol enantiomeric blend was measured (see below) on frass samples from all first- and second-generation males that had developed in *P. resinosa*. This population is proximate to

previously described populations with low (+)-ipsdienol enantiomeric ratios south of the hybrid zone (Seybold et al., 1995a).

From three sites near Lake Tahoe, California, USA (hereafter referred to as CA), 51 males and 47 females were collected from ponderosa pine, *P. ponderosa*, singleleaf pinyon pine, *P. monophylla*, or Sierra lodgepole pine, *P. contortamurrayana* and taken live to Syracuse to establish another colony of *I. pini* in a manner identical to that described above.

From Bragg Creek, Alberta, Canada (hereafter referred to as AB), eight males and 30 females were collected from *P. contorta latifolia*, taken live to Syracuse, and handled similarly to the WA and CA populations.

From two sites near Cortland, New York (hereafter referred to as NY), *P. resinosa* logs colonized by *I. pini* were collected and placed in 25-l emergence containers. Approximately 200 emerging beetles were collected and used to initiate a laboratory colony. Ipsdienol enantiomeric composition was not analyzed from males for three to four generations. At that time, males were randomly selected for analyses before line crosses were initiated.

Measurement of Pheromone Component Ratios

With the exception of the field-collected samples of frass from BC, pheromone samples were obtained by extraction of frass from single males by using the gelatin-pill-capsule technique (Borden, 1967). Laboratory-collected samples were obtained from males that had been secured for 48 h under gelatin capsules (Bioquip, Gardena, CA, USA) that covered holes drilled in the bark of *P. resinosa* logs. The accumulated frass was transferred to 500- μ l glass inserts in 1.5-ml autosampler vials with 200 μ l HPLC-grade pentane (Sigma-Aldrich). We stored pheromone extracts at -30°C for up to 6 mo before analysis.

Gas chromatography (GC) was used for analytical resolution of the enantiomers of ipsdienol (Seybold, 1992). A 1- μ l aliquot of each sample was injected into a Hewlett Packard gas chromatograph/mass spectrometer (GC-MS) (model 5890 series II/model 5971), equipped with a GC column with a chiral stationary phase [0.25 mm (internal diameter) \times 50 m] (Cydex-B; SGE Inc., Austin, TX, USA). A Hewlett Packard Automatic Liquid Sampler (model 7673) was used to increase the throughput of the GC-MS. Synthetic standards of racemic ipsdienol (10 ng/ μ l) were analyzed approximately every 50 samples.

The mass spectrometer was set to selective ion-monitoring mode to minimize signal from host volatiles and maximize signal from pheromones ($m/z = 85$ for ipsdienol, and $m/z = 109, 124, 137, 152$ for lanierone). Although we screened the samples for lanierone, we did not include it in our phenotypic analyses. A number of practical considerations precluded us from analyzing the quantities of ipsdienol and lanierone, and the presence or absence of lanierone. The large number of crude frass extracts injected into the GC-MS for the analyses resulted in the steady degradation of the injector and column in the analytical system. Ensuring reliable and low detection limits for ipsdienol and lanierone would have required an impractical allocation of effort for maintaining our analytical equipment. Thus, we focused our analysis on the measurement of ipsdienol enantiomeric blend ratio, which is highly reproducible in *I. pini* (Teale et al., 1994). If sample traces showed no ipsdienol, the corresponding beetles were not considered in further statistical analyses.

Initiating Crosses

Potential female mates were tested for virginity by securing them for 5–7 d under metal wire screens in holes drilled in the bark of *P. resinosa* and observing whether they oviposited. To initiate crosses, males were secured in holes drilled in the bark of separate *P. resinosa* slabs (~15 × ~25 cm). Gel capsules were used to secure the males if pheromone was to be collected; otherwise, wire screen was used. Females were introduced 48 h later into the male galleries and the gallery entrance holes were again covered with screen. Cylindrical metal containers [60 cm (length) × 25 cm (diameter)] with detachable glass vials were used to house each log and to collect offspring.

Divergent Selection of BC Population

For the BC population, we divergently selected lines for extreme ratios of (+)- to (–)-ipsdienol. From 189 first-generation males, those producing greater than 75% (+)-ipsdienol or less than 30% (+)-ipsdienol were randomly mated. In succeeding generations, males matching these criteria were mated to females, both of which originated from broods where at least half of the males met the appropriate criteria. Five generations were used to obtain lines for crossbreeding. Eight related lines of beetles with high (+)-ipsdienol enantiomeric ratios bred true for three successive generations, matching the selection target in all male progeny. Beetles from these lines will be referred to as P^+ . Males of two unrelated lines, P^{-a} and P^{-b} , were used for the opposite parental type. These lines did not yield broods with uniformly low (+)-ipsdienol enantiomeric ratios over multiple generations. We proceeded with the line crosses at this time because we feared that inbreeding depression might cause extinction of the P^{-a} and P^{-b} lines.

Line Crosses

Line crosses were performed on beetles derived from five locations with an emphasis on the divergent lines from BC (Table 1). Biases in the numbers of crosses performed were typically attributable to shortages of beetles of a particular sex or line. For example, there were no virgin P^{-b} females available for crossing to P^+ males. Additionally, crosses were performed within parental lines concurrent to the F1 generation. Twelve crosses were performed within and between the P^+ broods. Because the F1^{-b} results suggested that the P^{-b} line was heterozygous, only two crosses within the P^{-a} line were used for further breeding.

For the CA × WA line crosses, the parental lines are represented as P^- and P^+ , respectively (Table 1). Because these crosses were derived from large colonies, we were able to maintain a more balanced design. We performed 11 crosses of both of the F1 subtypes and five of each of the F2 and backcross subtypes.

We also created crosses between the AB and NY populations. In AB, we hoped to obtain a population that uniformly produced high ratios of (+)-ipsdienol. However, this population possessed similar variation in ipsdienol enantiomeric composition as that observed in eastern British Columbia (Fig. 1). We did not wish to select for this high (+)-ipsdienol ratio phenotype as we did in the divergent line crosses by using BC material. Such a practice may inadvertently remove epistatic

Table 1 Number of crosses of the pine engraver, *Ips pini*, in line cross experiment among populations from Alberta, British Columbia, California, New York, and Washington

Line name ^a	Paternal grandparents		Maternal grandparents		BC ^b crosses	CA/WA crosses	AB/NY crosses
	Male	Female	Male	Female			
P ⁺	+	+	+	+	8	c	c
P ⁻	-	-	-	-	1a, 1b	c	c
F1	+	+	-	-	6a, 0b	11	18
F1 _r	-	-	+	+	9a, 5b	11	0
F2	+	-	+	-	12a	5	19
F2	-	+	-	+	5a	5	0
F2	+	-	-	+	5a	5	0
F2	-	+	+	-	8a	5	0
B ⁺	+	+	+	-	6a	5	10
B ⁺	+	+	-	+	4a	5	0
B _r ⁺	+	-	+	+	5a	5	0
B _r ⁺	-	+	+	+	5a	5	0
B ⁻	-	-	+	-	3a	5	10
B ⁻	-	-	-	+	2a	5	0
B _r ⁻	+	-	-	-	3a	5	10
B _r ⁻	-	+	-	-	2a	5	0
P ⁻ × B ⁺	-	-	+	F1	0	0	16

^aThe subscript r indicates that X-chromosome inheritance is different despite similar autosomal inheritance.

^bPopulations: BC (P⁺, P⁻) is Brisco, British Columbia; CA (P⁻) is Lake Tahoe, California; WA (P⁺) is Roslyn, Washington; AB (P⁺) is Bragg Creek, Alberta; and NY (P⁻) is Cortland, New York. For BC, the crosses derived from the independently selected low (+)-ipsdienol enantiomeric ratio lines are noted separately as a and b.

^cFor pheromone analyses and subsequent line crosses, beetles were taken directly from colony populations, where parentage was unknown from amongst more than 30 males and females.

genetic variation that might influence the ipsdienol blend, which was not desired in this case.

To avoid this difficulty, we used only AB males that produced >80% (+)-ipsdienol and performed our F1 crosses in only one direction, between AB males (P⁺) and NY females (P⁻) (Table 1). These F1 offspring were used to create F2 crosses and backcrosses to the parental types with the exception that we did not perform backcrosses to AB females. Furthermore, all AB males used in backcrosses also produced greater than 80% (+)-ipsdienol (B⁺). Finally, we performed additional crosses between the B⁺ females and NY males. Crosses of this type help to increase our power in detecting X-linked genetic effects, being most similar to the reciprocal F1 that could not be performed.

Means Analysis

A genetic model based on the mean ipsdienol enantiomeric ratios of the line crosses was used to evaluate composite additive [d], dominance [h], and X-linked [d_X]

effects, and all possible digenic epistatic effects. The model was derived from groupings of the parental, F1, F2, and backcrosses that shared the same possible autosomal and X-linked genetic effects (Table 1), and included up to nine lines. We considered additive by additive [i], additive by dominance [j], dominance by dominance [l], additive by X-linked [$d-d_x$], and dominance by X-linked [$h-d_x$] as epistatic factors. The mean effect [m] was included in all models. The notation follows Mather and Jinks (1982).

The line cross data from each experiment did not generally meet the assumption of normality, due—in many cases—to pronounced bimodality in the phenotypic distribution of ipsdienol blend. Therefore, a resampling procedure was performed to estimate the mean pheromone enantiomeric ratio of each line. For 1000 replicates, data from each line were sampled with replacement N_i times. Here, N_i is the number of observations for the i th line (Simon, 1969).

The average resampled line means were used to estimate the parameters for each genetic effect contained in vector $\hat{\mathbf{y}}$, and their variances $\hat{\mathbf{S}}$ (Mather and Jinks, 1982; Lynch and Walsh, 1998):

$$\hat{\mathbf{y}} = (\mathbf{C}^T \mathbf{V}^{-1} \mathbf{C})^{-1} \mathbf{C}^T \mathbf{V}^{-1} \mathbf{x},$$

and

$$\hat{\mathbf{S}} = (\mathbf{C}^T \mathbf{V}^{-1} \mathbf{C})^{-1},$$

where \mathbf{C} is the coefficient matrix of genetic effects specific to each cross type, \mathbf{V} is a diagonal matrix of the variances of the resampled line means, and \mathbf{x} is the vector of the average resampled line means. Models were evaluated by a χ^2 test for goodness of fit (Hayman, 1958).

We used a stepwise procedure to decide which genetic effects to include in our model. We began with a null model by using the mean effect [m]. At each successive step, we tested all models that added a single appropriate genetic effect to the existing model. Additive [d] and X-linked [d_x] effects were added first. Dominance [h] was not tested until an additive effect was established in the model, nor were any interactions tested until multiple effects had been included in the model. We continued with this process until we arrived at a model that showed no significant improvement in the χ^2 statistic (Lynch and Walsh, 1998). This procedure, called joint-scaling, evaluates the difference between χ^2 values in successive models as a χ^2 distribution with 1 *df*. The significance levels of individual parameters (genetic effects) were determined by using the 1000 resampled estimates to construct two-tailed 95% confidence intervals.

The data also allowed us to expand the analyses to consider maternal and paternal effects as long as we further divided the lines to accommodate such effects. This division can be observed by noting that the four entries of F2 have different combinations of paternal grandfathers and maternal grandmothers (Table 1). We performed such analyses for the BC and the CA \times WA experiments. Because maternal and paternal effects were never included in these models, we do not provide details of these analyses or further consider the issue. Such an analysis was not possible for the AB \times NY crosses, because all these line crosses had AB paternal and NY maternal effects.

Mendelian Analysis

Pronounced bimodality appeared in the outcomes of many of the BC crosses. Furthermore, examination of the pooled distribution of phenotypes from all parental hybrid and backcross lines revealed a bimodal distribution with a sharp trough at 70% (+)-ipsdienol (Fig. 2). This observation led us to group all males in this experiment into categories of high or low (+)-ipsdienol enantiomeric ratio. We tested the categorical data for deviations from patterns predicted by Mendelian inheritance by using χ^2 analysis. This analysis was not performed for the other experiments because segregation was not clear (Fig. 2).

Number of Effective Factors

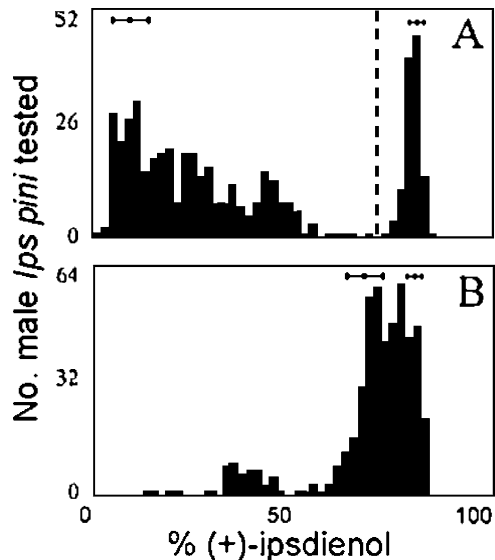
For all three line cross experiments, we calculated the Castle–Wright estimator (Castle, 1921; Wright, 1968) for the number of effective genetic factors, n_e , that cause differences in ipsdienol enantiomeric composition. We used two different methods for estimating one model component, the segregation variance, $\hat{\sigma}_s^2$. The first estimate employed a least-squares approach to minimize the residual variance in a model that simultaneously examines additive and segregation variance in all lines (Hayman, 1960; Lynch and Walsh, 1998). We also calculated segregation variance by using twice the variance in F2, minus the variance in each backcross. The latter estimate is more robust against biases caused by dominance (Lynch and Walsh, 1998).

Results

Enantiomeric Composition of Ipsdienol from Source Populations

Male *I. pini* from BC showed a highly variable distribution in the enantiomeric composition of ipsdienol (Fig. 1). Of the frass samples collected from *P. contorta latifolia* in the field from Brisco, 21 clustered between 71% and 84% (+)-ipsdienol.

Fig. 2 Frequency histograms of the enantiomeric composition of ipsdienol from male *Ips pini* based on pooled parental hybrid and back cross data from the Brisco, British Columbia (A) and Bragg Creek, Alberta \times Cortland, NY (B) line-cross experiments. The mean (\pm SD) enantiomeric compositions of ipsdienol of the two parental lines are indicated as horizontal bars above the histograms. In (B), the horizontal line at the lower percentage of (+)-ipsdienol represents the NY parental population; the horizontal line at the higher percentage represents the AB parental population. In (A), a vertical dotted line indicates the criterion for separating phenotypes for Mendelian analysis



The remaining 18 samples were distributed widely between 2.4% and 62% (+)-ipsdienol (39 of 40 samples were successfully analyzed). Of the frass samples collected from Brisco males in *P. resinosa* in the laboratory, 14 of 21 contained sufficient ipsdienol for enantiomeric determination. Half of the 14 samples contained 74–84% (+)-ipsdienol. The enantiomeric compositions in the remaining samples varied between 18% and 41% (+)-ipsdienol. Of the frass samples collected from Parson males in *P. resinosa* in the laboratory, 22 of 40 males contained sufficient ipsdienol for enantiomeric determination. In this case, 11 of the males produced greater than 70% (+)-ipsdienol (Fig. 1).

Male *I. pini* from WA and CA produced very low enantiomeric ratios of (+)-ipsdienol in *P. resinosa* logs in the laboratory. Of 51 WA males tested in the first laboratory generation, 49 produced less than 5% (+)-ipsdienol, and two produced between 5% and 10% (+)-ipsdienol (Fig. 1). Of 28 CA males tested in the first laboratory generation, 25 produced less than 5% (+)-ipsdienol, and three produced between 5% and 10% (+)-ipsdienol (Fig. 1).

Male *I. pini* from AB, similar to the BC populations, had a highly variable distribution of ipsdienol enantiomeric compositions (Fig. 1). In the first laboratory generation, 24 of 33 tested males produced greater than 75% (+)-ipsdienol. The enantiomeric composition of the remaining nine samples varied from 20% to 50% (+)-ipsdienol.

Divergent Selection of the BC Population

Twenty-two lines for the high (+)-ipsdienol trait (P^+) were established by using BC material that bred true for all male offspring for at least two generations. In our crosses, we conservatively used 14 males from eight of the lines that bred true for all male offspring for three generations. The phenotypic distribution of ipsdienol enantiomeric ratio in these lines was quite narrow (Fig. 3A). In generations 3–5, P^+ males produced 80.2–84.1% (+)-ipsdienol; P^{-a} males produced 9.6–53.4% (+)-ipsdienol; and P^{-b} males produced 14.1–43.1% (+)-ipsdienol (Table 2).

We did not obtain a line for very low (+)-ipsdienol enantiomeric ratio production that reached the selection target for all offspring for multiple generations. In earlier generations, broods with uniformly low (+)-ipsdienol blends were found, but were unsuccessfully propagated. Between the third and fifth generations, two such broods (P^{-a} and P^{-b}) showed a progressive decline in both (+)-ipsdienol enantiomeric ratio and variability in enantiomeric composition (Table 2). The phenotypic distributions of ipsdienol enantiomeric ratio were relatively broad in the fifth generations of P^{-a} and P^{-b} (Fig. 3B and C, respectively).

Divergent Line Crosses

The phenotypic distributions of (+)-ipsdienol produced by males in all line crosses resulted in a variety of patterns (Fig. 3). The $F1^a$ and $F1_r^a$ crosses generally show a range of phenotypes between 0% and 50% (+)-ipsdienol (Fig. 3D, E). The $F1^a$ distribution is more skewed to intermediate phenotypes than $F1_r^a$. The $F1^b$ hybrids showed a much different distribution, with approximately half of the individuals segregating to the P^+ parental type (Fig. 3F). The B^+ and B_r^+ backcrosses showed distinct bimodal distributions, with roughly half of offspring very similar to P^+ and the other half varying between 0% and 50% (+)-ipsdienol (Fig. 3G, H). The dis-

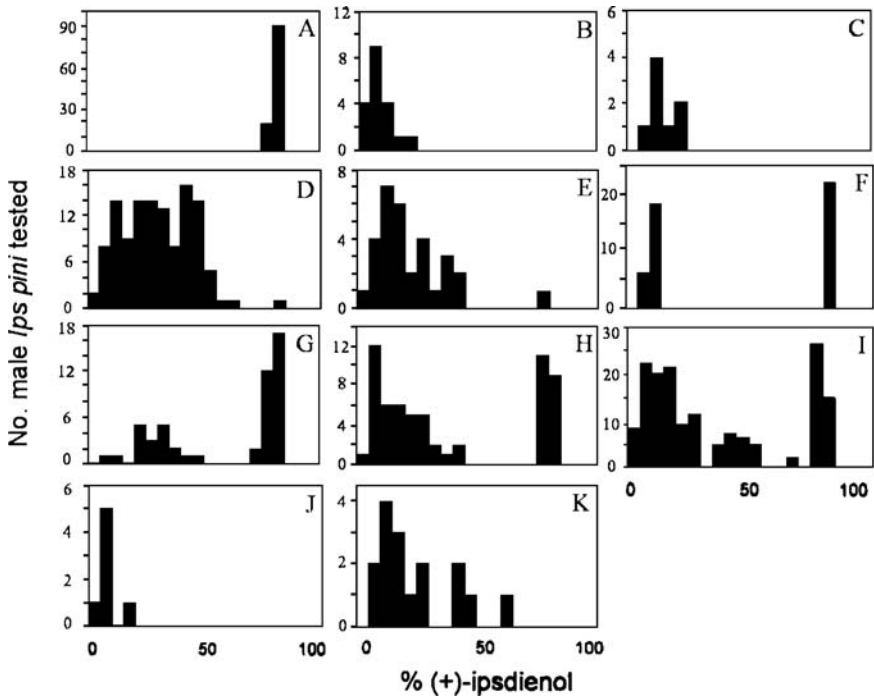


Fig. 3 Frequency histograms of the enantiomeric composition of ipsdienol from all experimental crosses with the Brisco, British Columbia population. Data include parental populations (A, B, and C = P⁺, P^{-a}, and P^{-b}, respectively); F1 generation (D, E, and F = F1^a, F1^a, and F1^{-b}, respectively); backcrosses of F1 to P⁺ (G, H = B⁺, B⁺); F2 generation (I = F2), and backcrosses of F1 to P^{-a} (J, K = B⁻, B⁻) for all male progeny from a given group

tribution of the B_r⁺ line was more skewed to the P⁻ phenotype. The F2 generation showed three peaks in distribution (Fig. 3I), one of which was very narrow at 80% (+)-ipsdienol and the others wider at 0–30% and 35–55% (+)-ipsdienol. The number of samples for B⁻ and B_r⁻ was small, but both distributions centered at low ratios of (+)-ipsdienol (Fig. 3J, K).

We omitted P^{-b} and F1^b from the BC means analysis, because of the high degree of segregation in F1^b, which suggests that P^{-b} was not homozygous. Six genetic effects [*m*], [*d*], [*h*], [*d_x*], [*j*], and [*h.d_x*] were included in the model that provided the

Table 2 Proportion of (+)-ipsdienol in the P⁺, P^{-a}, and P^{-b} lines of the pine engraver, *Ips pini*, for the three generations prior to crossing in the BC divergent selection experiment (mean ± sd, *n*)^a

Generation	P ⁺	P ^{-a}	P ^{-b}
3	80.2 ± 1.0, 33	53.4 ± 30.9, 24	43.1 ± 34.8, 40
4	84.1 ± 1.6, 136	31.2 ± 27.3, 5	28.4 ± 15.4, 10
5	81.3 ± 1.6, 24	9.6 ± 4.6, 19	14.1 ± 5.9, 8

^a Data include measurement of all male siblings in the broods used for further line propagation. P⁺ represents all broods containing individuals from eight lines, whereas P^{-a} and P^{-b} both represent one line.

Table 3 Estimates of genetic effects from optimal models based on the means of crosses of various lines of the pine engraver, *Ips pini*^a

Effect ^b	BC ⁺ × BC ⁻	CA × WA	AB × NY
<i>m</i>	45.061	1.978	73.938
<i>d</i>	72.086	0.425	6.960
<i>h</i>	-19.756		-2.437
<i>d_X</i>	-35.853	-0.235	-1.655
<i>j</i>	-35.470		
<i>h:d_X</i>	31.081		

^a For all effects, 95% confidence intervals (not shown) do not overlap zero.

^b Effects include mean (*m*), additive (*d*), dominance (*h*), X-linked (*d_X*), additive by dominance (*j*), and dominance by X-linked (*h:d_X*).

best fit to the line means (Table 3). Dominance [*h*], X-linked [*d_X*], and additive by dominance [*j*] effects were negative. All factors, including the interactions, [*j*] and [*h:d_X*], were relatively large in magnitude and capable of strongly affecting ipsdienol enantiomeric blend. The expected line mean values calculated by the model do not differ significantly from the resampled line means ($\chi^2 = 2.667$, $df = 3$, $P = 0.446$; Table 4). Joint-scaling tests were significant at $\alpha = 0.05$ for the addition of each factor up to the accepted model. No factor could be added to this model that significantly improved the fit as evaluated by joint-scaling. Because of the strong dominance and interaction factors, we repeated the analysis by using a square root transformation, which did not affect the results (details not shown).

CA × WA Crosses

All of the lines in the CA × WA experiment were represented by 26 or more samples of males, with the exception of the B_r⁺ line, which expired before sampling. Nearly all males produced <5% (+)-ipsdienol in every line (Fig. 4). However, with

Table 4 Averaged resampled line means, model-predicted means, and sample sizes for line cross experiments among populations of *Ips pini* from British Columbia, California, Washington, Alberta, and New York

Line	BC crosses			CA × WA crosses			AB × NY crosses		
	Mean	Model	<i>N</i>	Mean	Model	<i>N</i>	Mean	Model	<i>N</i>
P ⁺	81.309	81.295	24	2.097	1.434	51	80.131	80.102	77
P ⁻	8.990	8.827	19	2.149	2.252	55	67.921	68.156	79
F1	30.360	30.076	120	2.214	2.211	88	73.846	73.672	30
F1 _r	21.601	20.533	31	1.730	0.621	53	–	–	–
F2	36.397	35.183	157	2.653	3.115	69	72.834	73.045	67
B ⁺	60.162	62.358	50	3.374	3.935	42	74.367	76.887	30
B _r ⁺	38.370	42.047	60	–	–	–	–	–	–
B ⁻	7.686	8.007	7	1.765	0.562	33	70.724	69.204	54
B _r ⁻	24.704	28.319	16	1.834	0.751	26	71.289	70.914	101
B ⁺ × P ⁻	–	–	–	–	–	–	68.515	69.728	42
Joint scaling ^a	<i>P</i> = 0.446			<i>P</i> = 0.132			<i>P</i> = 0.139		

^a Test is based on χ^2 test for goodness of fit, where acceptable model is assumed when $P > 0.05$.

the exception of F1_r and B⁻ (Fig. 3D, F), all the lines had a few individuals that produced slightly higher ratios of (+)-ipsdienol. The resampled line means from these crosses were similar and ranged from 1.730 to 3.374 (Table 4). Line means analysis for the CA × WA crosses resulted in the addition of small, but significant, additive and X-linked effects to the model (Table 3). The expected line means derived from this model do not differ significantly from the resampled line means ($\chi^2 = 8.480, df = 5, P = 0.132$) (Tables 3 and 4). Joint-scaling tests were significant at $\alpha = 0.05$ for the addition of both factors. No factor could be added to this model that significantly improved the fit.

AB × NY Crosses

All of the planned lines in the AB × NY experiment had more than 30 samples each (Fig. 5). The AB (P⁺) line consisted of 77 males from the first two generations of laboratory breeding that all produced >75% (+)-ipsdienol (Fig. 5A). These samples were among the top 65% in (+)-ipsdienol enantiomeric ratio and were separated from those producing a lower ratio of (+)-ipsdienol by at least 20%. Seventy-nine males from the NY (P⁻) colony generally showed a range of phenotypes between 50% and 70% (+)-ipsdienol (Fig. 5B). This distribution was skewed toward a large peak near 70% (+)-ipsdienol. All the line crosses derived from the AB and NY lines had mean enantiomeric compositions ranging from 68.5% to 74% (+)-ipsdienol (Table 4). The frequency histograms of each generation showed phenotypes between approximately 50% and 85% (+)-ipsdienol, with varying degrees of skewness to higher percentages of (+)-ipsdienol (Fig. 5C–H).

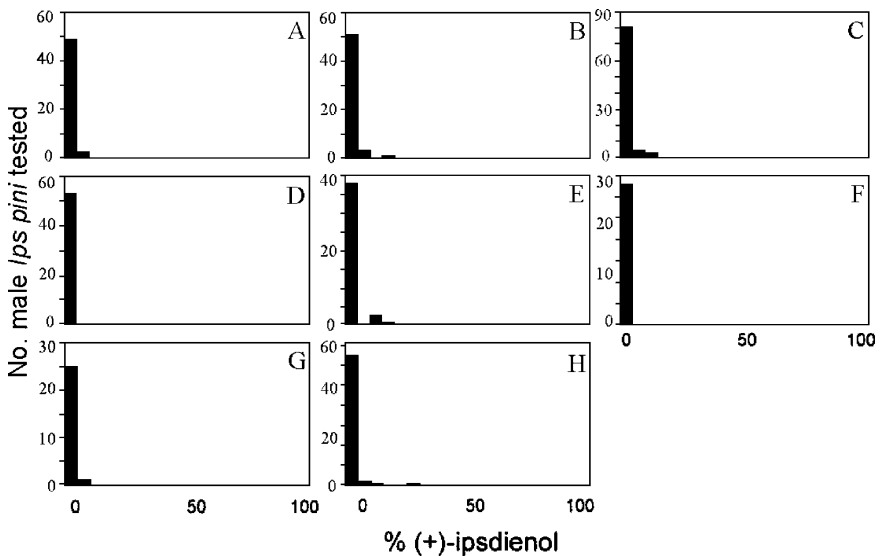


Fig. 4 Frequency histograms of the enantiomeric composition of ipsdienol from male *Ips pini* from all experimental crosses with the Lake Tahoe, CA and Roslyn, WA populations. Data include parental populations (A, B = WA:P⁺, CA:P⁻); F1 generation (C, D = F1, F1_r); backcross to WA (E = B⁺), backcrosses to CA (F, G = B⁻, B_r⁻), and F2 generation (H) for all male progeny from a given group

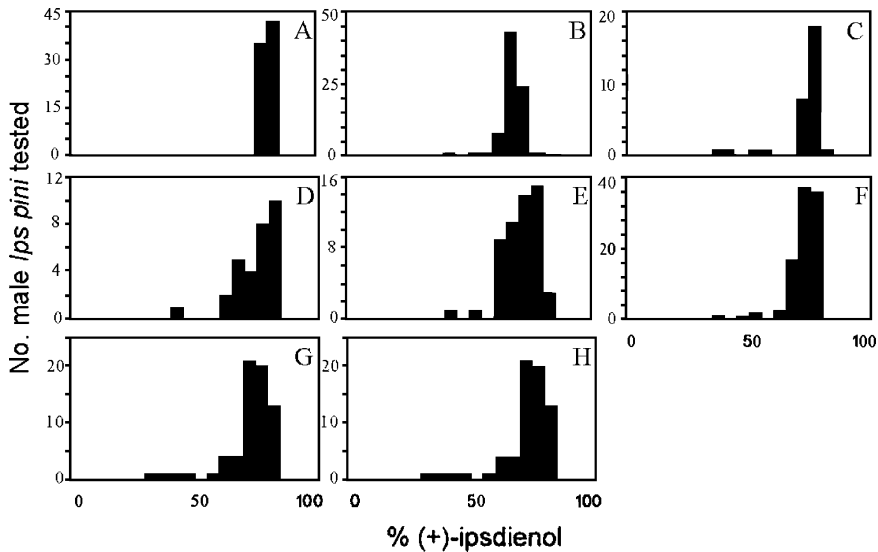


Fig. 5 Frequency histograms of the enantiomeric composition of ipsdienol from male *Ips pini* from all experimental crosses with the Bragg Creek, Alberta (AB) and Cortland, NY populations. Data include parental populations (A, B = AB:P⁺, NY:P⁻), F1 generation (C), backcross of F1 to AB (D = B_r⁺), backcrosses to NY (E, F = B⁻, B_r⁻), F2 generation (G), and B⁺ crossed to NY (H) for all male progeny from a given group

The line means analysis for the AB × NY crosses resulted in the addition of additive, X-linked, and dominance effects to the model (Table 3). While the additive effect [*d*] was positive, the other effects were negative and smaller in magnitude. The expected line means derived from this model do not differ significantly from the resampled line means ($\chi^2 = 6.938$, $df = 4$, $P = 0.140$) (Tables 3 and 4). Joint-scaling tests were significant at $\alpha = 0.05$ for the addition of all the factors.

Adding the dominance by X-linked effect [*h.d*_X] significantly improved the model ($P = 0.014$). However, we chose not to include this factor. With this interaction, the confidence interval for the dominance factor overlapped zero and the model became more difficult to interpret. Furthermore, the simpler model did not show a significant difference between the predicted and observed line means (Table 4).

Mendelian Analysis of Divergent Line Crosses

For the Mendelian analysis, we assumed a simple autosomal model and combined reciprocal lines (e.g., B⁺ = B⁺ and B_r⁺). From F1^a, 149 of 151 males produced <70%

Table 5 Comparisons of the observed to predicted Mendelian ratios of high/low (+)-ipsdienol phenotypes of *Ips pini* from British Columbia crosses

Cross	Ratio	3:1	1:1	1:3
F1 ^a	2:150	440.1 ^b	144.1 ^b	45.47 ^b
F2	41:116	200.1 ^b	35.83 ^b	0.1040
B ⁺	52:58	45.10 ^b	0.3273	29.10 ^b
B ⁻	0:23	69.00 ^b	23.00 ^b	7.667 ^a
F1 ^b	22:24	18.12 ^b	0.0870	12.78 ^b

^a $P < 0.01$ (χ^2 test).

^b $P < 0.005$ (χ^2 test).

Table 6 Castle–Wright estimators of number of effective segregating loci for the BC, CA × WA, and AB × NY line cross experiments

V(S) estimate	BC crosses	CA × WA crosses	AB × NY crosses
Least squares	0.662 ± 0.072	−0.002 ± 0.040	0.340 ± 0.071
2F2–B ⁺ –B [−]	1.383 ± 0.565	−0.004 ± 0.064	0.228 ± 0.086

(+)-ipsdienol. This low (+)-ipsdienol ratio differed significantly from a 3:1, 1:1, or 1:3 ratio (Table 5). The F1^b line yielded a bimodal distribution with 22 of 46 male offspring producing >70% (+)-ipsdienol, and this ratio did not differ significantly from a 1:1 phenotypic ratio (Table 5).

In the F2 generation, there were 41 high (+)-ipsdienol ratio males to 116 low (+)-ipsdienol ratio males. This ratio did not differ significantly from a 1:3 phenotypic ratio. The ratio was significantly different from 3:1 or 1:1 (Table 5). The backcrosses to the P⁺ lines (B⁺) exhibited a 52:58 high/low ratio, which was not significantly different from a 1:1 phenotypic ratio, but was significantly different from 3:1 and 1:3 phenotypic ratios (Table 5). All 23 offspring of backcrosses to P^{−a} (B[−]) were assigned the low (+)-ipsdienol ratio phenotype, which differed significantly from 3:1, 1:1, and 1:3 phenotypic ratios. An X-linked single gene model shows significant difference from expected ratios in each generation (comparisons not shown).

Number of Effective Factors

Significance of the estimates for number of effective factors cannot be estimated because of nonnormality and nonadditive effects. The estimates were low for each of the three experiments (Table 6). Both the least-squares and 2F2–B⁺–B[−] estimates for the segregation variance in the BC line crosses yielded estimates of n_e close to 1. The Castle–Wright estimator for the CA × WA crosses was near 0 for both segregation variance assumptions. The AB × NY crosses led to estimates of n_e that were less than 1.

Discussion

This study represents the first instance where the genetic control of a long-range pheromone signal is investigated at a natural hybrid zone. Our analyses of the BC experiment implicate a recessive allele that controls most of the variation in ipsdienol enantiomeric ratio at the hybrid zone. For the Mendelian model, only the F1^b line differed from such a pattern, because half of the beetles show the phenotype associated with P⁺. This result can easily be explained if all the P^{−b} parents of this line were heterozygous carriers of the recessive allele. The strong dominance effect in the line means analysis is also consistent with the action of this major gene. The Castle–Wright estimator is close to 1, which also supports our general conclusion that few genetic factors control the differences in pheromone blend at the hybrid zone. However, nonnormal data and inequality of genetic effects may bias this estimate.

Miller et al. (1989) speculated that there was quantitative variation in ipsdienol blend production because of the variety of phenotypes found in his samples. Despite

the major gene effect on pheromone production, there may be quantitative effects at other levels of genetic control. Additional loci for ipsdienol enantiomeric blend are implied in our means analysis by the interactions [j] and [$h.d_X$], and the X-linked factor [d_X]. Zhu et al. (1996a) similarly showed that there are two levels of control in pheromone production between variants of the European corn borer, but were unsure if the second level of control involves additional loci. Thus, despite the different ecological roles between *Ips* aggregation pheromones and lepidopteran sex pheromones, we do see a convergence.

Our experimental design and analyses assume that there are no postmating isolation mechanisms relevant to this system. Lanier (1972) found high egg hatchability in hybrid crosses between Ontario and California populations of *I. pini* in both directions. Throughout the five generations of our divergent selection experiment, we observed many broods with high phenotypic variation, suggesting that hybrids can produce viable offspring for several generations. Furthermore, by divergently selecting from one population in that experiment, we decreased the likelihood of encountering a factor such as *Wolbachia* infection, which has been reported in this family (Vega et al., 2002).

Although the knowledge of the biochemical mechanisms of insect pheromone production is increasing (Tillman et al., 1999), we do not have a full mechanistic understanding for how changes in isomer-specific pheromone blends occur. *I. pini* produces ipsdienol *de novo* in the midgut by using the mevalonate pathway (Seybold et al., 1995b; Tillman et al., 1998; Hall et al., 2002), but may also convert the host-produced compound myrcene to ipsdienol (Vanderwel, 1991). Seven mevalonate pathway genes have been identified from *I. pini* that are active in the midgut (Eigenheer et al., 2003), with two male-specific genes (Keeling et al., 2004), indicating involvement in pheromone production. Because enantiomers of ipsdienol are interconvertible in the California fivespined ips, *Ips paraconfusus* (Fish et al., 1979, 1984), the control of ipsdienol enantiomeric blend may occur relatively late in this pathway. Lepidopteran pheromones generally arise from fatty acid synthetic pathways, with important roles of desaturation and chain-shortening enzymes (Roelofs, 1995). In European corn borers, the reductase complex has been implicated in the production of the specific ratios of the pheromone isomers (Zhu et al., 1996b). However, the enzymes actually determining the isomer-specific pathway also remain unknown for European corn borers. Further knowledge in the biochemical pathways of pheromone production may reveal how the putative major genes operate. Adding a quantitative trait loci (QTL) approach to line crosses may be necessary to fully describe how genetic control of isomer blends is achieved, particularly if modifiers of normal biochemical pathways are involved.

Our experiment involving crosses between beetles from California and Washington indicates little difference in the genetic control of enantiomeric blend. In these parent populations, and all their line cross derivatives, most beetles produced close to 0% (+)-ipsdienol. However, we did uncover very small significant additive and X-linked effects that were likely due to rare alleles for slightly higher ratios of (+)-ipsdienol. Because the high (+)-ipsdienol ratio phenotypes appeared so rarely in our lines, we cannot argue that our model results accurately indicate any real population differences between the two geographic areas.

On the other side of the hybrid zone, we confirm that many *I. pini* males in British Columbia and Alberta produce proportions of (+)-ipsdienol that are higher than ever measured in eastern populations like New York (Miller et al., 1989, 1996).

The use of the same host material and rearing facility ensures that these differences are not attributable to environmental conditions, an explanation previously considered (Miller et al., 1989; Seybold et al., 1995a). Our study also provides a limited insight into the genetic nature of this difference. Our failure to observe transgressive segregation in the AB \times NY crosses (Table 5, Fig. 5) suggests that the NY population may possess a gene similar to that which distinguishes the P⁺ and P⁻ lines of the BC experiment. Moreover, there were no complicated genetic interactions in the AB \times NY crosses that suggest radically different genetic control of pheromone blend. However, without a full mechanistic understanding of the genes involved, our results cannot completely rule out such radically different genetic architectures for the high (+)-ipsdienol ratios observed in these populations. Additionally, our comparison only involves two populations from a large geographic continuum (Lanier, 1972). The enantiomeric composition of ipsdienol across this range has been estimated from groups of 20–230 males (Seybold et al., 1995a), but profiles from individual males do not exist for many populations over this range. Thus, additional genetic modes of enantiomeric blend production may exist.

The separation of eastern and western forests during Pleistocene glaciation has been cited as the likely cause for the divergence in pheromone biology between the corresponding populations of *I. pini* (Seybold et al., 1992, 1995a). However, a more complete evolutionary model for the system must also explain the variation in pheromone profiles within what is considered the “eastern type” population, which is consistent with character displacement at the hybrid zone. A review of the literature on *I. pini* suggests three possible causes for this current geographic pattern of ipsdienol enantiomeric ratios: (1) reinforcement, (2) strong natural selection, and (3) multiple allopatric separations.

Serious consideration of the reinforcement model would require much deeper knowledge on the geographic pattern of ipsdienol variation, strength of assortative mating at the hybrid zone, and amount of introgression between populations. While assortative mating based on ipsdienol enantiomeric blend has been described in New York (Teale et al., 1994), the matter has not been investigated at or near the hybrid zone. As a further caution, Miller et al. (1996) found that profiles of male pheromone production do not always match that of female response to lures in British Columbia.

Nevertheless, aspects of the genetic architecture of ipsdienol enantiomeric blend production uncovered here may be consistent with a process such as reinforcement. For multilocus reinforcement models, assortative mating will be difficult to maintain in the face of any gene flow. Single-locus reinforcement models are also problematic because narrow clines will limit the power of selection for further reproductive isolating methods (Butlin, 1989). A two-level genetic model of canalization of pheromone blend (Zhu et al., 1996a) could avoid these theoretical difficulties via the reinforcement hypothesis. Pheromone production and response may change in allopatry by minor incremental changes at multiple loci due to sexual selection (Fisher, 1930; Lande, 1981) and/or natural selection. A single locus encoding a major shift in the trait may arise, but remains rare until selected after secondary contact. Our characterization of two levels of control of pheromone blends in British Columbia supports the plausibility of this process. However, our lack of a complete mechanistic understanding of how such genetic factors operate, coupled with the unknown role of female preference, suggests caution.

Much research on North American *Ips* spp. has focused upon the intense interspecific competition and predation mediated by semiochemicals. Among competitors, resource partitioning might influence blends used. For example, *I. paraconfusus*, a species that occurs in California and Oregon, is interrupted by (–)-ipsdienol (Light and Birch, 1979). Conversely, this species produces (+)-ipsdienol and (–)-ipsenol, which interrupt aggregation by *I. pini* (Birch et al., 1977, 1980). However, the selective pressures from such competitors are often more complicated because competitors may also be synergistically attracted to semiochemical blends, where the components are derived from multiple species (Hedden et al., 1976; Ayres et al., 2001).

Predators can be a stronger selective factor than competitors in some populations of *I. pini* (Erbilgin et al., 2002). Furthermore, predators often exhibit preferences for certain pheromone phenotypes of *I. pini*, potentially resulting in selective feeding upon beetles (Raffa and Klepzig, 1989; Herms et al., 1991; Raffa, 1991; Raffa and Dahlsten, 1995). The pheromone lanierone tends to increase trap captures of predator species in western populations where it is not used by *I. pini* (Seybold et al., 1992; Miller et al., 1997; Dahlsten et al., 2003). Conversely, in Wisconsin, some predators are attracted most strongly to low (+)-ipsdienol enantiomeric blends with no lanierone (Aukema et al., 2000). Thus trapping both predators and competitors over a range of blend ratios at the hybrid zone might reveal if a selective advantage to the specific “80% (+)-ipsdienol phenotype” exists.

Finally, the differences in pheromone biology between the California, New York, and Alberta/British Columbia populations may reflect at least three recent allopatric separations. Both lodgepole pine and jack pine, *Pinus banksiana*, the two primary hosts of *I. pini* in Canada and Alaska, may have had multiple refugia at the last glacial maximum (Critchfield, 1985). Furthermore, Cognato et al. (1999) found three mitochondrial lineages of *I. pini*. The distributions of these lineages coincide roughly with the eastern (NY), Rocky Mountain (AB/BC), and southwestern (CA) populations of *I. pini*. Given such a model, the slight differences between the AB/BC and NY pheromone phenotypes may have evolved in allopatry due to genetic drift or convergent evolution.

In summary, our results help to define at least three distinct geographic populations with respect to the genetics of ipsdienol enantiomeric ratio. Additionally, a major gene controls most variation in crosses between the opposing pheromone types at the presumed hybrid zone. Although AB/BC and NY populations are genetically distinct with respect to ipsdienol enantiomeric ratio, crosses between these populations do not provide evidence that the major gene architecture is unique to the hybrid zone. A more complete understanding of the evolutionary system requires further inquiry into the biochemical details of enantiomeric blend production and the strength of assortative mating and natural selection in various populations. In a broader perspective, we provide the first description of the genetic architecture of intraspecific variation in a male long-range pheromone component. A major genetic change occurs, just as it has been often reported for female-produced sex pheromones (Löfstedt, 1990), and as predicted for scolytids by Symonds and Elgar (2004a).

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Cuticular Hydrocarbon Composition Reflects Genetic Relationship Among Colonies of the Introduced Termite *Reticulitermes santonensis* Feytaud

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Abstract Nestmate recognition plays a key role in kin selection to maintain colony integrity in social insects. Previous studies have demonstrated that nestmate recognition is dependent on detection of cuticular hydrocarbons. However, the absence of intraspecific aggression between some colonies of Isoptera and social Hymenoptera questions whether kin recognition must occur in social insects. The purpose of this study was to determine if cuticular hydrocarbon similarity and high genetic relatedness could explain the lack of intraspecific aggression among and within colonies of the introduced subterranean termite *Reticulitermes santonensis*. We performed both GC analysis of cuticular hydrocarbons and genotyping by using 10 DNA microsatellite loci on the same 10 workers from each of 14 Parisian colonies. Multivariate analyses demonstrated correspondence between cuticular hydrocarbon patterns and genetic variation. By using a redundancy analysis combining chemical and genetic data, we found that a few hydrocarbons (mainly short vs. long chains; saturated vs. unsaturated alkanes) were associated with most genetic variation. We also found a strong positive correlation between chemical and genetic distances between colonies, thus providing evidence of a genetic basis for cuticular hydrocarbon variation. However, genetic distance did not account for all chemical variation, thus suggesting that some hydrocarbon variation was environmentally derived. Investigation at the intracolony level indicated that cuticular hydrocarbons did not depend on colony social structure. Based on our findings, we speculate that the absence of intraspecific aggression in *R. santonensis* may result

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from a loss of diversity in genetically derived recognition compounds in this species that presumably descended from *R. flavipes* populations imported from North America.

Keywords Cuticular hydrocarbons · Microsatellite DNA · Subterranean termite · *Reticulitermes santonensis* · Multivariate analyses · Social structure

Introduction

In social insects, the ability to distinguish among individuals from different colonies of the same or different species is essential to prevent competition, predation, and parasitism (Wilson, 1971). Nestmate recognition is also required for kin selection that is essential for maintenance of eusocial behavior (Hamilton, 1964). In most cases, discrimination of unrelated individuals triggers aggressive behavior, but this may not always be the case. Aggressive behavior is not observed between colonies of arboreal nesting termite species (Leponce et al., 1996), dampwood *Zootermopsis* species (Haverty and Thorne, 1989), and subterranean species such as *Coptotermes formosanus* (Su and Haverty, 1991; Husseneder and Grace, 2001) and *Reticulitermes flavipes* (Grace, 1996; Bulmer and Traniello, 2002).

In France, colonies of the subterranean termite *R. santonensis* never display intraspecific aggression regardless of the geographic distances among populations (Clément, 1986; Clément and Bagnères, 1998; Vieau, 2001). Many studies support that *R. santonensis* descended from one or more populations of *R. flavipes* imported from North America (see Dronnet et al., 2004, 2005a; Austin et al., 2005). Based on this assumption, the nonaggressive behavior of *R. santonensis* is comparable to that of other imported species such as the Argentine ant, *Linepithema humile* (Hymenoptera: Formicidae), which forms competitively dominant supercolonies without territorial boundaries (Tsutsui et al., 2000; Giraud et al., 2002) and the tramp ant, *Wasmannia auropunctata* (Le Breton et al., 2004). Therefore, the understanding of mechanisms that explain the lack of intraspecific aggression can be tackled, at least partially, by investigating the compounds involved in recognition systems.

Cuticular hydrocarbons, i.e., epicuticle lipids that prevent desiccation, are reported to serve as chemical cues in the recognition process during interindividual interactions (Howard and Blomquist, 2005; Ozaki et al., 2005). Recognition processes based on genetically derived compounds are also essential for determining the degree of relatedness for kin selection (reviewed in Breed and Bennett, 1987). Hydrocarbon composition is often colony-specific (e.g., ants, Vander Meer and Morel, 1998; termites, Clément and Bagnères, 1998; bumblebees, Dronnet et al., 2005b). Although they can be influenced by the physical and social environment (e.g., Husseneder et al., 1997), cuticular hydrocarbons are thought to have a genetic basis that played a major role in the evolution of social behavior (Carlin and Hölldobler, 1986).

Study of chemical cues provides a powerful system for understanding the absence of intraspecific aggression. Since the hydrocarbon composition of related colonies can be expected to be similar, it should be possible to establish genetic relationships

among colonies by evaluating the potential for recognition on the basis of putative heritable compounds, as is the case in *Schedorhinotermes lamanianus* (Husseneder et al., 1998) and *Macrotermes subhyalinus* (Kaib et al., 2004). Within colonies, since the breeding system is fundamentally linked with colony genetic structure, it is essential to understand how it could influence cuticular hydrocarbon variation. In *Reticulitermes* species, the number and type of reproductives may vary from one single outbred primary pair to multiple inbred secondary reproductives (offspring of the primary reproductives) or to multiple unrelated reproductives (Thorne et al., 1999). Colonies with a number of unrelated reproductives may present a greater variety of derived compounds than highly inbred colonies. In this regard, it should be underlined that *R. santonensis* colonies consist mainly of families headed by inbred secondary reproductives (neotronics) (Dronnet et al., 2005a).

The aim of this study was to determine if the cuticular hydrocarbon composition of the nonaggressive introduced termite *R. santonensis* reflects the genetic relationships inferred from microsatellite molecular markers among and within colonies. We asked the following four questions. Can the apparent lack of conspecific recognition be explained by similarities in cuticular hydrocarbon composition between colonies? Is there sufficient genetic variation to distinguish colonies? Is there a correlation between the cuticular hydrocarbon composition and genetic distance among colonies? Are differences in cuticular hydrocarbon composition among nestmates within the same colony affected by colony social structure?

Methods and Materials

Termites

In 2003, 14 colonies of the subterranean termite *R. santonensis* were collected for gas chromatographic (GC) analysis of cuticular hydrocarbons and for genotyping analysis using microsatellite DNA. The sampling sites were selected within and around Paris, France (Fig. 1). Ten workers per colony ($N = 140$) were randomly taken either from mud tubes on tree bark or from inside buildings. Each termite was isolated into different conical glass vials (Wheaton, 0.3 ml) to avoid mixing of cuticular hydrocarbons, immediately killed by freezing, and stored at -20°C until the time of analyses.

Gas Chromatographic Analysis of Cuticular Hydrocarbons

Cuticular hydrocarbons were extracted by rinsing individuals in 500 μl pentane for 2 min. Following extraction, termites were retrieved with a fine wire (previously washed in pentane) and placed in 95% ethanol for later genetic analyses. After evaporation of pentane, the extracted hydrocarbons were redissolved in 50 μl pentane containing 0.1 $\mu\text{g}/\mu\text{l}$ *n*-eicosane as an internal standard. Samples were analyzed with a Perkin-Elmer Autosystem XL GC (Perkin-Elmer, Wellesley, MA, USA) equipped with a flame ionization detector (FID) and interfaced with Turbochrom workstation software. Analyses were carried out in splitless mode using a 25 m \times 0.32 mm \times 0.5 μm BP1 nonpolar capillary column (SGE, Austin, TX, USA) programmed at 150°C for 2 min, followed by $5^{\circ}\text{C}/\text{min}$ up to 300°C for 5 min.

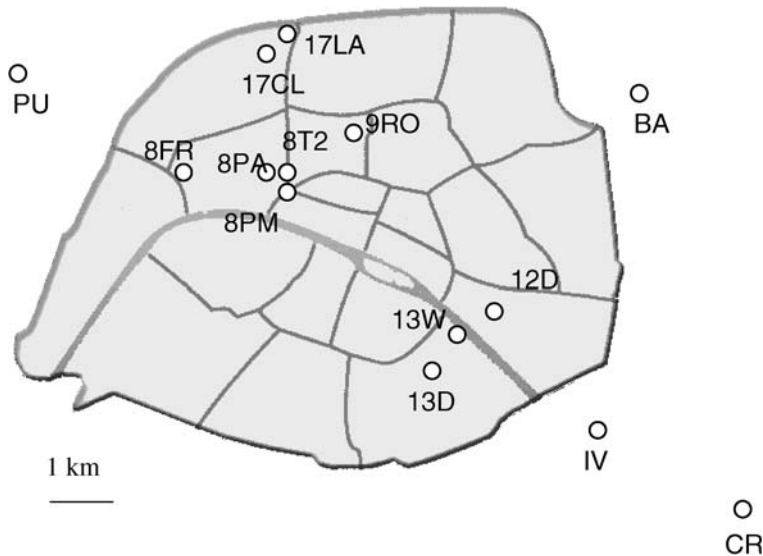


Fig. 1 Map of the city of Paris (France) showing the locations of the 14 *Reticulitermes santonensis* colonies. Ten colonies were collected within Paris: 8T2 [8th Arrondissement (=Ardt), Tronchet street], 8PA (8th Ardt, Pasquier street), 8PM (8th Ardt, Madeleine Place), 8FR (8th Ardt, Friedland avenue), 9RO (9th Ardt, Rochechouart street), 12D (12th Ardt, Daumesnil avenue), 13D (13th Ardt, Dunois street), 13W (13th Ardt, Louise Weiss street), 17CL (17th Ardt, Clichy avenue), 17LA (17th Ardt, Lantiez street). Four colonies came from cities around Paris: PU (Puteaux), BA (Bagnole), IV (Ivry-sur-Seine), and CR (Créteil)

The carrier gas was helium at 1 ml/min. Extracts from different colonies were injected alternatively to avoid the possible biasing effect of injecting extracts from the same colonies one after another. Compound identification was based on previous analyses of the cuticular hydrocarbons of *R. santonensis* by coupled GC-MS (Bagnères et al., 1990).

Chemical Analyses

To allow analysis of the 140 individual hydrocarbon profiles, areas of peaks were readjusted by using an FID correction coefficient (Bagnères et al., 1990). We selected cuticular hydrocarbons that represented >0.3% relative peak area and were present in all individuals. Cuticular hydrocarbons were coded following the notation used by Bagnères et al., 1990 (see also footnote “b” in Table 1): 9-C23:1 (*e1*), *x*-C23:1 (*e2*), *n*-C23 (*a3*), 11-MeC23 (*m4*), 4/2-MeC23 (*m5*), (*Z*)-9-C24:1 (*e6*), 3-MeC23 (*m7*), *n*-C24 (*a8*), 11+12-MeC24 (*m9*), 4/2-MeC24 (*m11*), (*Z*)-9-C25:1 (*e12*), *n*-C25 (*a14*), 11+13-MeC25 (*m16*), 7,9-C25:2 (*n17*), 4/2-MeC25 (*m18*), 3-MeC25 (*m19*). The relative areas of some cuticular hydrocarbon pairs were summed [9-C23:1 + *x*-C23:1 (i.e., *e1* + *e2*); (*Z*)-9-C24:1 + 3-MeC23 (i.e., *e6* + *m7*)], because they were most frequently combined (integrated together) by the GC software. To visualize patterns of chemical similarity among individuals from the same and different colonies, the relative areas of the 14 selected peaks were subjected to principal component analysis (PCA) using the XLSTAT 7.1 software

Table 1 Mean Percentages^a of Cuticular Hydrocarbons of 14 Colonies of *Reticulitermes santonensis* (Paris, France)

Hydrocarbons	Peaks ^b	8T2	8PA	8PM	8FR	9RO	12D	13D	13W	17CL	17LA	PU	BA	IV	CR
9-C23:1 + <i>i</i> -C23:1	<i>eI</i> + <i>e2</i>	1.93 (0.39)	0.68 (0.46)	0.72 (0.34)	1.67 (0.58)	1.17 (0.33)	1.17 (0.30)	1.15 (0.52)	2.09 (0.12)	3.86 (1.31)	1.21 (0.39)	1.10 (0.29)	1.42 (0.63)	4.33 (1.79)	3.17 (0.61)
<i>n</i> -C23	<i>a3</i>	13.07 (2.10)	7.50 (0.49)	6.64 (0.88)	9.07 (1.17)	9.63 (0.68)	6.58 (1.45)	9.45 (1.54)	6.22 (0.82)	8.91 (1.36)	7.48 (1.16)	13.61 (0.84)	11.09 (1.21)	15.62 (3.22)	8.05 (1.49)
11-MeC23	<i>m4</i>	0.81 (0.14)	0.70 (0.24)	1.08 (0.22)	0.93 (0.12)	1.95 (0.33)	1.11 (0.26)	1.95 (0.22)	2.37 (0.33)	1.32 (0.22)	0.99 (0.17)	0.76 (0.17)	1.73 (0.14)	1.57 (0.44)	3.16 (0.74)
4:2-MeC23	<i>m5</i>	2.70 (0.26)	1.81 (0.22)	1.62 (0.21)	1.71 (0.09)	2.14 (0.19)	1.94 (0.18)	1.78 (0.22)	1.57 (0.20)	1.86 (0.22)	1.63 (0.26)	1.84 (0.15)	1.32 (0.12)	0.74 (0.22)	1.90 (0.29)
(<i>Z</i>)-9-C24:1 + 3-MeC23	<i>e6</i> + <i>m7</i>	1.03 (0.09)	0.50 (0.05)	0.78 (0.10)	0.58 (0.06)	1.35 (0.19)	0.76 (0.03)	1.38 (0.10)	1.31 (0.09)	1.32 (0.18)	0.79 (0.08)	0.58 (0.12)	0.98 (0.09)	1.24 (0.14)	1.59 (0.13)
<i>n</i> -C24	<i>a8</i>	3.18 (0.44)	4.47 (0.96)	3.05 (0.70)	2.55 (0.40)	4.39 (0.17)	2.32 (0.29)	4.25 (0.54)	2.22 (0.35)	3.35 (0.14)	3.05 (0.69)	4.35 (0.41)	2.68 (0.26)	3.88 (0.43)	2.63 (0.63)
11+12-MeC24	<i>m9</i>	0.98 (0.23)	1.60 (0.15)	1.71 (0.16)	1.35 (0.25)	1.64 (0.15)	1.78 (0.51)	1.73 (0.22)	2.03 (0.12)	1.92 (0.13)	1.55 (0.24)	1.07 (0.23)	1.22 (0.12)	1.35 (0.17)	2.04 (0.21)
4:2-MeC24	<i>mI1</i>	18.27 (1.18)	16.15 (0.82)	14.64 (1.21)	18.73 (0.89)	13.14 (0.70)	15.58 (2.27)	13.87 (1.22)	11.07 (0.74)	11.94 (1.28)	15.64 (1.94)	18.69 (1.85)	13.02 (0.62)	6.55 (1.06)	11.20 (1.44)
(<i>Z</i>)-9-C25:1	<i>eI2</i>	14.51 (1.89)	8.96 (2.16)	15.38 (3.22)	7.88 (1.37)	9.56 (1.92)	9.22 (1.95)	6.92 (1.72)	21.77 (1.20)	21.22 (2.10)	13.47 (3.26)	7.96 (2.16)	13.99 (2.15)	16.59 (1.96)	22.90 (2.12)
<i>n</i> -C25	<i>aI4</i>	11.74 (1.07)	18.79 (1.07)	12.94 (1.07)	15.34 (0.96)	18.11 (1.47)	11.01 (1.99)	20.39 (2.81)	8.88 (0.78)	13.70 (1.16)	14.48 (1.59)	18.06 (0.98)	16.00 (1.19)	23.30 (2.68)	9.25 (2.16)
11+13-MeC25	<i>mI6</i>	17.45 (2.41)	25.49 (2.79)	27.47 (1.99)	22.01 (1.95)	21.92 (1.91)	31.27 (5.45)	20.65 (2.93)	28.99 (3.10)	20.59 (1.36)	25.72 (2.19)	16.97 (2.33)	20.50 (1.68)	16.47 (1.26)	24.10 (1.61)
7,9-C25:2	<i>nI7</i>	4.79 (0.60)	3.38 (0.34)	4.04 (0.99)	6.75 (1.05)	4.35 (0.59)	5.36 (0.95)	5.20 (0.66)	4.14 (0.20)	3.68 (0.46)	3.64 (0.17)	5.95 (0.51)	7.12 (1.03)	3.38 (0.43)	3.76 (0.49)
4:2-MeC25	<i>mI8</i>	2.93 (0.22)	3.39 (0.15)	3.09 (0.23)	3.93 (0.36)	2.96 (0.36)	3.83 (0.50)	3.08 (0.29)	2.33 (0.14)	2.33 (0.44)	3.42 (0.19)	3.37 (0.27)	2.75 (0.19)	1.66 (0.17)	2.05 (0.07)
3-MeC25	<i>mI9</i>	6.60 (0.44)	6.58 (0.47)	6.84 (0.44)	7.51 (0.41)	7.69 (2.11)	8.08 (1.40)	8.19 (0.87)	5.01 (0.42)	3.99 (0.52)	6.92 (0.70)	5.69 (0.41)	6.18 (0.59)	3.34 (0.50)	4.21 (0.21)

^a Means (SD) were calculated across the 10 individual profiles of workers analyzed per colony.

^b Codes for cuticular hydrocarbon peaks: a = *n*-alkane, e = monoene, n = diene, m = monomethyl-alkane.

(Addinsoft, NY, USA). The 95% confidence ellipses around workers from the same colony were obtained using STATGRAPHICS v.4.0 (StatPoint, Inc., Herndon, VA, USA) and UNIWIN PLUS v.3.0 (SIGMA PLUS, Levallois-Perret, France).

Microsatellite Genotyping

Microsatellite genotyping was performed on all 140 individuals that underwent chemical extraction. Genomic DNA extraction and PCR amplification were performed as described by Dronnet et al. (2004). The amplified products were separated by electrophoresis on 6% polyacrylamide gels with a Li-Cor 4000L sequencer. Genotyping was performed at 10 loci. These included four loci originally isolated from *R. flavipes* (*Rf1-3*, *Rf6-1*, *Rf11-1*, and *Rf15-2*; Vargo, 2000) and six loci characterized from *R. santonensis* (*RS10*, *RS15*, *RS68*, *RS76*, *RS78*, and *RS81*; Dronnet et al., 2004). For the description of statistical analyses, these loci will be numbered 1 through 10 in the order listed in the previous sentence. Alleles were scored using the computer program GENE PROFILER 4.03 (Scanalytics, Inc., Rockville, MD, USA).

Genetic Analyses

Patterns of genetic differentiation were initially ordinated by factorial correspondence analysis (FCA) to maximize the correspondence between allele and individual scores. The GENETIX 4.05.2 software used for FCA also codes the alleles by the presence/absence of each feature including homozygous state (Belkhir et al., 1996–2001). As a secondary approach to assess an unrooted network of the genetic distances between the 140 individuals, the proportion of shared allele distance, defined as one minus half the mean number of shared alleles per locus, was computed with the SHAREDST program (J. Brzustowski; available at <http://www2.biology.ualberta.ca/jbrzusto/sharedst.php>). A Fitch & Margoliash tree (Fitch and Margoliash, 1967) was constructed from the two distance matrixes by using the FITCH program in the PHYLIP package v.3.06 (Felsenstein, 1993). Conventional population genetic analyses were used to support the previous analyses. Genotypic frequencies between colonies were compared by means of a log-likelihood (*G*)-based test of differentiation using GENEPOP ON THE WEB (Raymond and Rousset, 1995). The overall significance was determined by using Fisher's combined probability test with Bonferroni's method for multiple comparisons.

Correlation Between Genetic and Chemical Data among Colonies

Redundancy Analysis

The relationship between cuticular hydrocarbon and microsatellite DNA data was tested via direct canonical ordination by using a redundancy analysis (RDA; Ter Braak, 1987) performed with Biplot and Singular Value Decomposition Macros for Excel (Lipkovich and Smith, 2002). The major difference between RDA and PCA is that RDA minimizes error by basing linear regression directly on *x* variables (environmental variables), thus forcing ordination vectors to be maximally related to combinations of the *x* variables (see Legendre and Legendre, 1998). In this way,

results can be expected to express only the part of variation that is significantly linked to the y variables (response variables). RDA was originally used for ecological applications, such as the analysis of community gradients to obtain constrained ordinations of species abundance frequency data in the function of environmental variables. In the present study, the table of y variables corresponded to the allele frequency data in each colony, and x variables corresponded to the mean relative area of each cuticular hydrocarbon peak calculated from the relative areas of the 10 individuals per colony. In addition, PCA was performed again on chemical data at the colony level. Spearman's rank correlation coefficients (r_s) between the colony scores were calculated from RDA and PCA to explore the cuticular hydrocarbon variables affecting colony ordination in multivariate analysis.

Matrix Correlations

Differences in hydrocarbon composition among colonies were quantified by modifying the standard genetic distance of Nei (1987) using the following formula, $D = -\ln \sum^n x_i y_i / \sqrt{(\sum^n x_i^2 \sum^n y_i^2)}$, where n is the number of selected cuticular hydrocarbons, x_i is the relative area of hydrocarbon i for colony x , and y_i is the relative area of hydrocarbon i for colony y . The dissimilarity matrix including all possible colony pairs was constructed from the previously calculated mean relative areas of cuticular hydrocarbons. Independent matrices coding genetic distances between colonies were then calculated from allele frequency data. Nei's standard genetic distance (D_S ; Nei, 1987) among colonies was calculated by using the GENDIST program in the PHYLIP package v.3.06. To test the robustness of our results, we used the Cavalli-Sforza and Edwards (1967) chord distance that makes no biological assumptions except that gene frequency changes can be attributed to genetic drift alone. For matrix correlations, we used XLSTAT 7.1 software to perform Mantel tests with a permutation test strategy (10,000 permutations), because the pairwise distance values among colonies were not independent of each other.

We investigated whether there was isolation by distance by plotting geographic distances vs. chemical and genetic distances, respectively. For genetic distance, F_{ST} among colonies was computed using FSTAT 2.9.3.2 (Goudet, 1995), and significance of the correlation between $F_{ST}/(1 - F_{ST})$ and the logarithm of geographical distances were tested. Mantel tests were computed using XLSTAT 7.1.

Correlation between Chemical Distance and Genetic Relatedness within Colonies

The breeding system was studied with hierarchical F -statistics using FSTAT, following the notation of Thorne et al. (1999), as explained in detail in Dronnet et al. (2005a). The 95% confidence intervals were obtained by bootstrapping over loci 15,000 times. It was assumed that the colony inbreeding coefficient, F_{IC} , was strongly negative in simple family colonies; approached zero as the number of neotenic reproductives increased; and became positive with assortative mating among multiple unrelated reproductives (Thorne et al., 1999).

Variation in chemical composition within colonies was investigated as a function of intracolony genetic relatedness. Genetic relationship between nestmate workers was assessed by measuring intracolony relatedness using RELATEDNESS v. 5.00

(Queller and Goodnight, 1989). Colonies were weighted equally. Mean Nei chemical distance within colonies was calculated from all possible pairs of individuals. Standard error of relatedness within single colonies was determined by jackknifing over loci. Spearman's rank correlation (r_s) was applied to test the relationship between these chemical and genetic variables within colonies.

Results

Chemical Analyses

Quantitatively, the major cuticular hydrocarbons were 11+13-MeC25, *n*-C25, 4/2-MeC24, (*Z*)-9-C25:1, and *n*-C23 (Table 1). The mean percentages of these cuticular hydrocarbons and others were used for statistical analyses *between* colonies (RDA, PCA among colonies, Nei's distance for matrix correlations). Principal component analysis of the quantitative variation in individual hydrocarbon data revealed that the first two principal components, PC-I and PC-II, accounted for 60.2% of total chemical variation (Fig. 2A). Nestmates were regrouped in their respective colonies, but variation also occurred among colonies ranging from low individual variability within some colonies (e.g., *I3W*) to more dispersed patterns (e.g., *I2D*). In the multivariate space, the distinction between colonies was relatively pronounced for *CR*, *IV*, *PU*, *I3W*, *I7CL*, and *I2D*. There was some overlap among the eight other colonies. Differences in colony profiles can be attributed to the effects of the cuticular hydrocarbons that contributed to the principal components. On the biplot (not shown), major contributions to PC-I (in brackets with the hydrocarbons) included two positive compounds, 4/2-MeC24 (*m11*, 14.2%) and 4/2-MeC25 (*m18*, 13.6%), and three negative compounds, (*Z*)-9-C25:1 (*e12*, -14%), (*Z*)-9-C24:1 + 3-MeC23 (*e6+m7*, -11.8%) and 11-MeC23 (*m4*, -10.9%). Major contributions to PC-II included one positive compound, 11+13-MeC25 (*m16*, 27.6%), and one negative, *n*-C23 (*a3*, -25.2%). Colonies *8PA* and *8T2* were more separate from the other colonies when PCA was performed by using PC-III that accounted for 11.4% of the variance (data not shown). Major contributions to PC-III included one positive compound, *n*-C24 (*a8*, 27.6%), and one negative compound, *n*-C25 (*a14*, -15.6%).

Genetic Analyses

Factorial correspondence analysis revealed that most colonies were distinct and plotted into three areas of the multivariate space (Fig. 2B). The two first factorial components (FC-I and FC-II) accounted for 25% of the total genetic variation. Three colonies (*CR*, *IV*, and *I3W*; group one) were completely separated from all the other colonies on the negative part of FC-I, whereas two other groups were distinct on FC-II. On FC-II, spatial distributions of the individual genotypes from four colonies (*9RO*, *I2D*, *I7CL*, *I7LA*) overlapped (group two), while individuals from the other colonies were mixed (group three) on the negative part of FC-II. Colony *PU* was clearly separated from all other colonies on FC-III (data not shown).

The unrooted tree based on allele-sharing distances among individuals provided some support to the results of FCA (Fig. 3). Individuals from colony *IV* clustered and formed a distinct subgroup from the mixed *I3W* and *CR* subgroups. Other

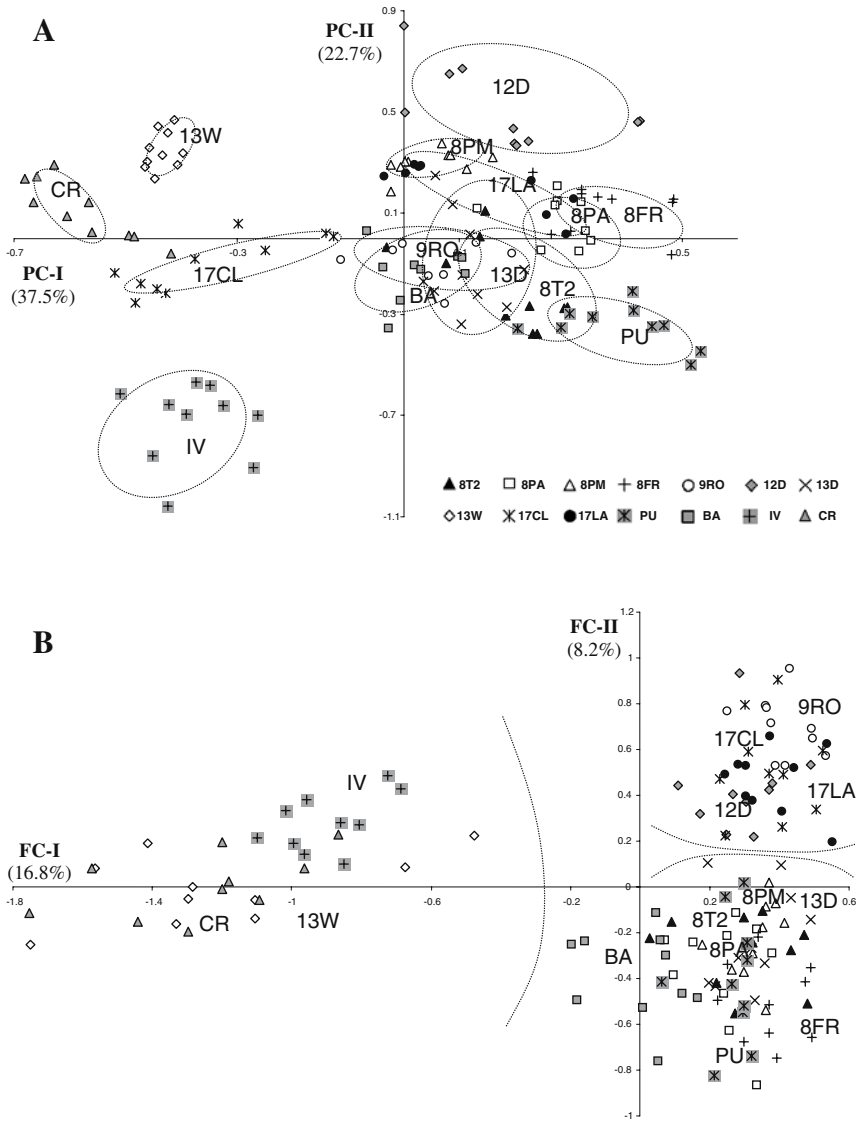


Fig. 2 Multivariate analyses showing the chemical and genetic relationships among the same 140 workers from *Reticulitermes santonensis* colonies. (A) Principal component analysis (PCA) of the cuticular hydrocarbon profiles. The 95% confidence ellipses are dotted. (B) Factorial correspondence analysis (FCA) of the microsatellite multilocus genotypes. The curves (dotted) were drawn by hand to delimit the distributions of the individuals belonging to the three distinct groups (see text). For both analyses, symbols of the same shape represent individuals from the same colony (see legend and Fig. 1)

distinct subgroups appeared including one formed by *8FR* and *PU*, and another by *17CL*, *17LA*, and *12D*.

Significant differentiation was observed among most of the colonies (*G* test: $P < 0.01$), except among colonies in the three groups defined by FCA.

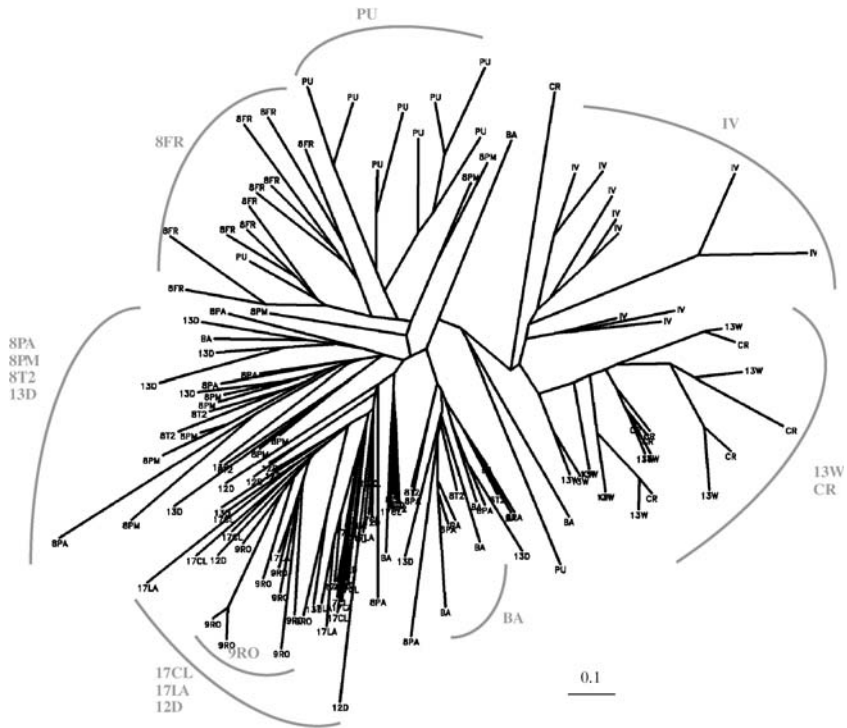


Fig. 3 Unrooted Fitch and Margoliash tree of genetic distances, based on the proportion of shared allele distance among individual microsatellite multilocus genotypes from the 14 *Reticulitermes santonensis* colonies

Correlation between Chemical and Genetic Data among Colonies

Redundancy Analysis

Redundancy analysis performed on allele frequency data for all 14 hydrocarbon variables revealed an ordination pattern of colonies (Fig. 4) that closely resembled the ordination pattern produced by FCA plotting of the individual genotypes (Fig. 2B). The first two axes (RD-I and RD-II) accounted for 57.6% of the total variation. The scores of the 14 colonies on RD-I were positively correlated (Spearman's rank test: $r_s = 0.54$, $P = 0.02$) with the scores on the first axis of a PCA performed on cuticular hydrocarbons among colonies (plot not shown), suggesting that these variables adequately explained the variation in the allele frequency data along this axis. Most of the variation on RD-I was explained by a combination of cuticular hydrocarbons strongly correlated with this axis (Fig. 4, long arrows). This correlation was positive mainly for long-chain methylbranched hydrocarbons [3-MeC25 (*m19*), 4/2-MeC25 (*m18*), 4/2-MeC24 (*m11*)] and negative mainly for short-chain hydrocarbons [mainly monoenes (*Z*)-9-C25:1 (*e12*), 9-C23:1 + *x*-C23:1 (*e1* + *e2*), (*Z*)-9-C24:1 (*e6*); shorter methylbranched hydrocarbons 11-MeC23 (*m4*), 3-MeC23 (*m7*)]. Again, CR, 13W, and IV appeared to be different

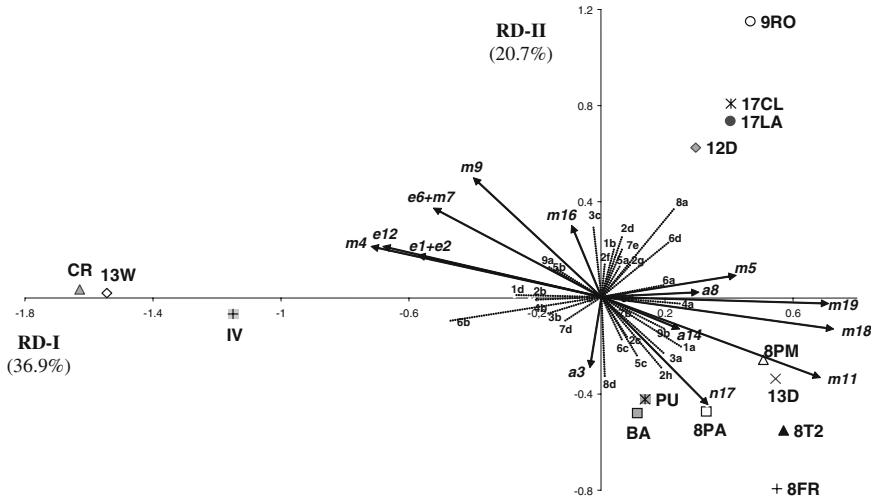


Fig. 4 Redundancy analysis (RDA) ordination triplot of the 14 *Reticulitermes santonensis* colonies (capitalized letters) with the microsatellite alleles from 10 loci (roman) indicated by dashed lines and the cuticular hydrocarbons (italic) indicated by arrows (environmental variables). Note that for clarity, only microsatellite alleles related to colony ordination are indicated. [For abbreviations of loci, see [Methods and Materials](#); the letters represent the alleles at each locus (not detailed in text)]

from the other colonies with regard to both allele frequency data and cuticular hydrocarbons. When colony *17CL*, whose position was relatively inconsistent with the results of the PCA, was removed from the datasets, the correlation coefficient slightly increased ($r_s = 0.63, P = 0.01$).

The scores of the 14 colonies on RD-II were not significantly correlated ($r_s = 0.25, P = 0.20$) with the scores on the second axis of PCA performed on colony hydrocarbons (without *17CL*, $r_s = 0.27, P = 0.19$). Therefore, cuticular hydrocarbons did not have an influence on the ordination among the colonies along the RD-II axis, as also revealed by the small size of the nearest arrows to this axis [11+13-MeC25 (*m16*), *n*-C23 (*a3*)]. These results suggested that ordination of the colonies along the RD-II axis was attributable mainly to genetic data, which would explain the congruence between the RDA and FCA plots. The main alleles involved in the variation in allele frequency data along the RD-II axis were from loci *RS76* (alleles 8a and 8d), *RS15* (6d), *Rf11-1* (3c), and *Rf6-1* (2d and 2h) (Fig. 4).

Matrix Correlations

Hydrocarbon distances among the 14 colonies were strongly and positively correlated with microsatellite genetic distances; Nei distance (Mantel test, $r_s = 0.40, P = 0.005$); and Cavalli-Sforza and Edward’s chord distance (Mantel test, $r_s = 0.42, P = 0.004$). Colonies showing more similar hydrocarbon profiles were genetically closer.

There was a positive correlation between geographic distance and genetic differentiation between colonies (Mantel test: $r = 0.49, P = 0.005$), but not between geographic and chemical distances (Mantel test: $r = 0.25, P = 0.072$).

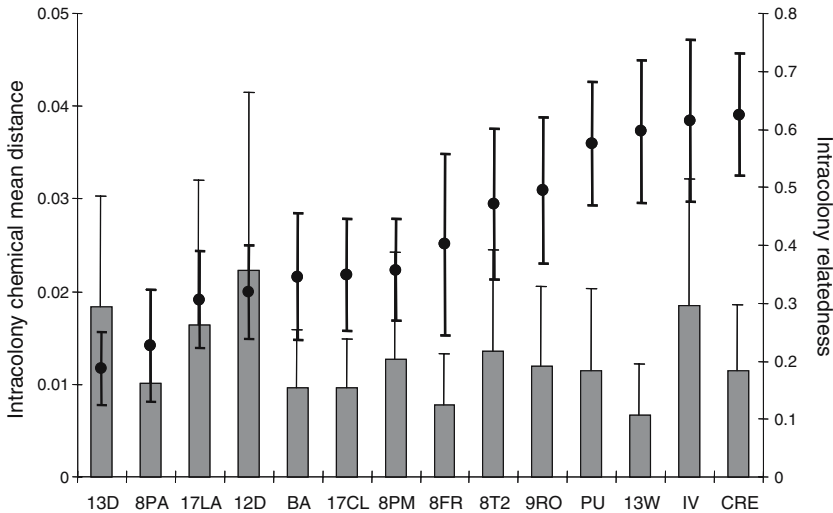


Fig. 5 Mean (+SD) chemical distances among nestmates within the 14 *Reticulitermes santonensis* colonies (histogram bars) and intracolony genetic relatedness (data points). Standard errors for genetic relatedness obtained by jackknifing over loci are in bold. The codes for the colonies are indicated on the horizontal axis

Correlation between Chemical Distances and Genetic Relatedness within Colonies

Based on examination of genotype arrays, all the studied colonies appeared to have more than one primary reproductive pair since all had more than four genotypes at one or more loci. Interestingly, the F_{IC} estimate was close to and not significantly different from zero; $F_{IC} = -0.037$ (-0.107 to 0.049).

Mean intracolony chemical distance was variable among colonies with large standard deviations (Fig. 5). Intracolony genetic relatedness ranged from 0.19 to 0.63 (mean \pm SE = 0.44 ± 0.05). We arbitrarily ordered colonies according to increasing values of relatedness (Fig. 5). The correlation between intracolony chemical distance and relatedness was not significant (Spearman's rank test, $r_s = -0.20$, $P = 0.24$).

Discussion

Our cuticular hydrocarbon data indicated that there were chemical differences among most colonies. The PCA performed on individuals showed strong differences in five colonies, more specifically along the first axis, i.e., the axis that accounts for most of the variation. The other colonies had overlapping 95% confidence ellipses, and so could not be clearly distinguished from each other. These findings were unexpected considering the lack of intraspecific aggressive behavior in *R. santonensis*, but would not have been surprising in the other *Reticulitermes* species distributed in Europe. Indeed, intraspecific aggression recorded in *R. grassei* and *R. banyulensis* has provided evidence that these species are able to perform nestmate recognition, most likely on the basis of cuticular hydrocarbons (Clément and Bagnères, 1998). Results

similar to ours have been reported on imported populations of *C. formosanus* from Florida and Hawaii insofar as their cuticular hydrocarbon patterns were not correlated with agonistic responses (Su and Haverty, 1991). Aggressive behavior was demonstrated to be low or absent among Hawaiian colonies (Husseneder and Grace, 2001). However, it must be emphasized that absence of overt aggression does not necessarily mean that no nestmate recognition takes place. Signs of recognition can be observed by examining subtle differences in behavioral interactions and by various ethological tests (Cornelius and Osbrink, 2003; Bagnères and collaborators, unpublished data).

Our genetic data demonstrated almost the same ordination pattern as the cuticular hydrocarbon data. Three colonies were strongly separated along one FCA axis, whereas the other 11 colonies were distributed in two genetically distinct groups. Since the method of factorial analysis used maximized the correspondence between allele and individual scores, it can be assumed that individuals within each group shared common alleles. The unrooted tree based on allele-sharing distance not only confirmed this assumption, but also yielded better resolution among a few colonies within groups. There was sufficient variation to measure genetic distances among most colonies.

Our findings showing general variation in both cuticular hydrocarbon and microsatellite compositions suggest a genetic basis for cuticular hydrocarbons. The multivariate approach that uses a redundancy analysis of chemical and genetic data simultaneously allowed us to isolate the cuticular hydrocarbons that specifically explained the observed variation. The combination of six major cuticular hydrocarbons highly correlated with the first canonical axis appeared to account for genetic variation among colonies. Interestingly, long- vs. short-chain hydrocarbons and saturated vs. unsaturated alkanes were found to correlate positively and negatively, respectively, with the axis. Mantel tests provided a strong positive correlation between chemical and genetic distances among colonies. Other studies in ants and termites have demonstrated a correlation between the level of intercolonial aggression during recognition processes and genetic distances between colonies (Beye et al., 1998; Husseneder et al., 1998). In the mound-building termite, *M. subhyalinus*, direct analyses showed that intercolonial differences in cuticular hydrocarbons were also negatively correlated with genetic similarity among colonies (Kaib et al., 2004).

However, several findings indicated that some chemical compounds may not be genetically derived. Some colonies, e.g., *I7CL*, were genetically similar and yet showed different ordination based on hydrocarbon profiles. Observation of significant isolation by distance among colonies indicated that genetic differentiation increases with geographic distance but, as previously shown in *M. subhyalinus* (Kaib et al., 2004), hydrocarbon differences did not correspond to the geographic population structure (geographic distance). Cue origin could account for the differences between chemical data in the genetic or geographical datasets. Cuticular hydrocarbons may also depend on environmental factors. Previous studies in other social insects such as the wood ant, *Formica pratensis*, have shown that environmental compounds not only are important for nestmate recognition, but they can also override genetically based cues (Pirk et al., 2001).

The presumed imported origin of *R. santonensis* could explain the lack of intra-specific aggression in *R. santonensis* despite the chemical differences observed be-

tween colonies. Although *R. santonensis* does not display the extraordinary supercolony structure of the imported populations of the Argentine ant, *L. humile*, it may have undergone changes in its breeding system. Unlike *R. flavipes* colonies, which are mainly headed by primary reproductive pairs (Vargo, 2003), *R. santonensis* colonies tend to be large with high numbers of neotenic reproductives (Dronnet et al., 2005a). Introduction to Europe may have been accompanied by a loss of genetic diversity at neutral markers in *R. santonensis* resulting in fewer alleles and a lower level of heterozygosity in comparison with *R. flavipes* (Dronnet et al., 2004). One of the hypotheses used to explain the absence of intraspecific aggression in *L. humile* may also explain how introduction events affected the mechanisms underlying the recognition process in *R. santonensis* (Tsutsui et al., 2000; Giraud et al., 2002). A founder event accompanying initial introduction into Europe may have reduced genomic diversity, including both microsatellite loci and genes controlling hydrocarbon expression. The resulting fixation of most recognition alleles could have caused a breakdown of intraspecific recognition ability. However, it should be emphasized that colonies of *R. santonensis* have maintained the ability to discriminate among allospecifics since they sometimes behave aggressively against other species.

Investigation at the intracolony level allowed us to study the influence of the social structure inferred from worker genotypes on hydrocarbon composition. This was especially important insofar as many colonies were headed by many breeders (neotenic reproductives) in each colony, which was revealed by the low F_{IC} value close to zero. These results were consistent with our previous data on other *R. santonensis* populations (Dronnet et al., 2005a) as mating among multiple neotenic resulted in significant inbreeding in each colony. Although extensive variation in intracolony relatedness (from low to high) could have influenced the diversity in cuticular hydrocarbons within colonies, no significant correlation was found between intracolony chemical distance and relatedness. This suggests that cuticular hydrocarbon composition did not depend on social structure. The supposed loss of genetic diversity during introduction may also have affected the recognition process within colonies. However, other factors such as intestinal flora (*R. speratus*; Matsuura, 2001) or diet-derived chemicals (*C. formosanus*, Florane et al., 2004) may influence recognition among individuals within colonies.

Based on our findings, we conclude that genetic relationships among colonies are well reflected by cuticular hydrocarbon variation. This conclusion supports a genetic component to these chemical compounds. Because this species does not show intraspecific aggression, i.e., a common trait for invasive species, it is possible that the supposed loss of genetic diversity also affected the ability to discriminate among more or less related individuals. Further studies focusing on other *Reticulitermes* species showing various degrees of intraspecific aggression will be needed to investigate the influence of cuticular hydrocarbons on recognition.

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Virgin Queen Mandibular Gland Signals of *Apis mellifera capensis* Change with Age and Affect Honeybee Worker Responses

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Abstract The mandibular gland secretions of *Apis mellifera capensis* virgin queens were analyzed by gas chromatography–mass spectroscopy. Changes in the patterns of the mandibular gland volatiles of *A. m. capensis* virgin queens were followed from emergence until 14-d-old. Ontogenetic changes in the mandibular gland secretions were largely quantitative in nature, delineating the age categories (global $R = 0.612$, $P = 0.001$), except for 7- and 14-d-old queens, which cannot be separated on their mandibular gland profiles ($P = 0.2$). (*E*)-9-Oxodec-2-enoic acid (9ODA) contributes most and most consistently to the dissimilarity between groups as well as the similarity within groups. Worker reactions to introduced virgin queens of various ages were recorded. Workers showed a significant increase in hostile reactions as queens aged ($r = 0.615$, $N = 20$, $P < 0.05$). Consequently, worker reactions and relative 9ODA production exhibit a positive queen age-dependent response.

Keywords *Apis mellifera capensis* · Mandibular gland secretions ·
(*E*)-9-ketodec-2-enoic acid · Virgin queen pheromones · Signal ontogeny

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Introduction

Age-related changes in the development of honeybees are well documented. The study of signal variation over time was first investigated in the 1960s and focused on the variation of (*E*)-9-oxodec-2-enoic acid (9ODA) in queens and (*E*)-10-hydroxydec-2-enoic acid (10HDA) in workers (Barbier and Pain, 1960; Pain et al., 1960, 1967). Boch and Shearer (1982) were the first to investigate whether the relative composition or the total quality of the mandibular gland secretion varied with the age of a worker bee. They found an increase in the amount of acids with age, which reached a plateau at 17 d. Subsequent studies have further documented the changes in the chemical composition of the extracts of exocrine glands during ontogenetic development (Allsopp, 1988; Whiffler et al., 1988; Crewe and Moritz, 1989; Slessor et al., 1990; Engels et al., 1997). The ultrastructure of the mandibular glands of queens has been found to develop in conjunction with pheromonal activity during queen aging (De Hazan et al., 1989b). Similar age-related changes in the fine structure and secretory activity of the mandibular glands have also been demonstrated for honeybee drones (Lensky et al., 1985). In addition, ontogenetic changes in the composition and quantity of the defense pheromones produced by workers in their sting and mandibular glands have also been shown (Whiffler et al., 1988).

The attractiveness of queens to workers and drones increases as the queens develop and are mated, with the mandibular gland secretion being primarily responsible for queen attractiveness (Butler, 1960; Gary, 1961). The components and blends in virgin queens' signals change with age and become dominated by 9ODA and (*E*)-9-hydroxydec-2-enoic acid (9HDA; Crewe, 1982; De Grandi-Hoffman and Martin, 1993; Plettner et al., 1995; Pankiw et al., 1996). Butler and Paton (1962) found that the quantity of 9ODA in queen heads increased considerably (7.2–132.5 µg) as queens aged from 1–2 d to 5–10 d. Mating induces additional changes to the signal with increases in aromatic compounds and the appearance of 4-hydroxy-3-methoxyphenylethanol (HVA; Slessor et al., 1990; Pankiw et al., 1996). The progressive change in mandibular gland secretions with age is evident in *Apis mellifera scutellata*, *A. m. mellifera*, and *A. m. intermissa* virgin queens (Pain et al., 1960, 1967; Pain and Roger, 1976) but not *A. m. capensis* queens (Crewe, 1988; Crewe and Moritz, 1989), which have been suggested to produce large amounts of 9ODA at emergence as a necessity in establishing queen–worker relationships (Crewe, 1982, 1988).

Detailed analyses of the mandibular gland extracts of honeybee queens have revealed that ontogenetic changes in the chemical composition of these glands are more complex than a simple increase in quantity. Most of the variation in secretions hinges on a quantitative difference in the relative proportions making up the mixture. The relative proportions of certain compounds, such as 10HDA, decrease in the mandibular gland signal, whereas those of the queen mandibular pheromones (QMP), 9ODA and 9HDA, increase as the queens age and are mated (Crewe and Velthuis, 1980; Crewe, 1982). Of the compounds comprising the QMP complex as defined by Slessor et al. (1988), 1-d-old *A. m. intermissa* queen head extracts are dominated by 9HDA with a significant contribution of 9ODA. In 4-d-old queens, the contribution of these two dominant components is reversed, with 9ODA dominating the signal (Crewe and Moritz, 1989; Slessor et al., 1990). Of those compounds not comprising the QMP complex, oleic acid has been shown to be the main component of recently emerged queens. It has been suggested that the

persistent levels of this acid in young queens, which decreases only with egg-laying activity, could label gynes (Engels et al., 1997).

This change in pheromone composition with queen age is an important factor for beekeepers to consider when replacing old queens with young vigorous virgin queens. The introduction of a new queen into an established colony often results in the workers behaving aggressively toward such queens by balling them (Yadava and Smith, 1971a,b). This worker aggression is related to the pheromonal attractiveness of a particular virgin queen (Yadava and Smith, 1971c). It is well documented that the response of workers to a queen changes during her maturation and subsequent aging, especially as related to changes in the mandibular gland pheromones (Gary, 1961). Besides the mandibular glands, several other sources of pheromonal secretions have been implicated in initiating worker aggression toward introduced queens (Boch and Morse, 1974; Ambrose, 1975; Lensky et al., 1991; Pettis et al., 1998).

The first objective of this study was to investigate ontogenetic patterns in the mandibular gland secretion profiles of *A. m. capensis* virgin queens. The second was to relate these profiles to the reactions of *A. m. capensis* workers exposed to virgin queens of different ages.

Methods and Materials

Samples and Sample Preparation

Queens were reared from established *A. m. capensis* colonies in Stellenbosch, South Africa (33°56'S, 18°52'E). Upon emergence, the virgin queens were placed alone in 40-ml bottles in an incubator at 30–32°C. Queens were supplied with honey-water and water *ad libitum*. They were aged and removed at 1, 3, 7, and 14 d old for mandibular secretion analyses and behavioral observations.

Behavioral Bioassay

Behavioral observations were conducted in the Department of Zoology and Entomology, Rhodes University, Grahamstown, South Africa. Three-frame free-flight queen-right observation hives ($N = 3$) were used to monitor colony behavior toward introduced virgin queens of different ages. Queens were introduced and removed through portals in the walls situated on either side of each frame. The colonies were established in the observation hives for a minimum of 2 wk prior to testing.

Five virgin queens from each age category were introduced to the top frame as far removed from the resident queen as possible, on the side of the frame opposite to that on which the mated queen was observed. A voice-activated tape recorder was used by the observer to record behavioral interactions. During continuous observations, the reactions of the workers to the introduced queens were analyzed in terms of those reactions during the initial 15 min after introduction. All interactions occurring between the introduced queens and the workers were noted. Specific reactions, such as licking and palpating, were considered nonhostile or investigative behaviors. Mounting, biting, pulling, and stinging were considered aggressive or hostile behaviors (Pettis et al., 1998).

A Spearman's correlation was carried out to determine whether virgin queen age affected the reactions of workers to introduced queens. The number of nonhostile

and hostile reactions observed during the 15-min observation periods was calculated as a percentage of the total number of reactions observed during that time period.

Chemical Analysis

The mandibular glands were removed from individual queens ($N = 10$ virgin queens per age category and four mated queens) and stored individually in 100 μl dichloromethane for spectroscopy (Merck, Uvasol). The extracts were evaporated just to dryness under a stream of N_2 , and the acids in the extracts were derivatized with bis-(trimethylsilyl)-trifluoroacetamide (Merck) and quantified according to the method of Gehrke and Leimer (1971). Extracts were analyzed on a Hewlett-Packard 5890 gas chromatograph (GC) fitted with a split-splitless injector, a flame ionization detector, and an HP-1 column (25 m \times 0.32 mm \times 0.52 μm film), temperature programmed from 60°C (1 min), 50°C/min to 100°C, and 3°C/min to 220°C (10 min). The injection port was set at 230°C, and the detector temperature was 280°C. Compounds were identified by comparing their electron impact mass spectra with those in the Wiley MS database and verified using previously published reports (see Table 1 for details) or by comparison with authentic standards (Sigma; Wessler and Crewe, 1999).

The 10 major compounds with possible biological activity were used in the analysis. The exact quantities and percentage compositions of the major biologically active compounds were calculated for queens of various ages. Multivariate analyses were performed by using PRIMER (Plymouth Routines in Multivariate Ecological Research, version 5.2.9, 2004; Plymouth Marine Laboratory, UK). The composite signal, as well as the percentage composition of the major compounds, was transformed by using double square root transformation to give an equal weighting to compounds with high or low representation in the profiles. The similarity matrix was calculated with Bray–Curtis coefficients (Bray and Curtis, 1957). Multidimensional scaling (MDS) was used to provide a visual representation of the pattern of proximities (i.e., similarities or distances) among the mandibular gland secretions of queens in various age classes (Kruskal and Wish, 1978). The MDS was rerun 10 times to ensure that the stress value was at the global minimum (Clarke and Warwick, 2001). Analysis of similarity (ANOSIM) was used to test for differences in queen mandibular gland secretion profiles between the various age classes (Clarke and Green, 1988). PRIMER'S SIMPER (similarity percentage) procedure was used to determine the percentage contribution of those compounds in the mandibular gland secretions responsible for the differences between each pair of test groups (Clarke, 1993). This method also computes the consistency of each of the compounds to the similarity within a test group, as well as to the differences between test groups. This is calculated as the ratio of the mean contribution to the standard deviation of the contribution; thus, a high ratio implies that the compound contributes consistently to the similarity/differences within and between groups.

Results

Chemical Analysis

The major component of the mandibular gland secretions was 9ODA for all test groups except 1-d-old virgin queens, in which the minor aliphatic acids dominated,

Table 1 Amounts (μg ; mean \pm SE) of selected compounds present in mandibular glands of *A. m. capensis* mated and virgin queens of different ages^a

Compound	Age (days)		Mated queen (<i>N</i> = 4)	Compound identifications
	1 (<i>N</i> = 10)	3 (<i>N</i> = 9)		
HOB	0.09 \pm 0.06	t	30.8 \pm 12.8 (8%)	Crewe and Moritz, 1989; Crewe and Velthuis, 1980; Slessor et al., 1988, 1990; Engels et al., 1997; Keeling et al., 2003
7HOA	0.21 \pm 0.07	0.82 \pm 0.5	1.45 \pm 0.54	Crewe 1982, 1988; Crewe et al., 1990
4HBA	t	t	0.01 \pm 0.01	Standard (Sigma)
8HOA	1.2 \pm 0.07 (1%)	1.74 \pm 0.35	2.93 \pm 0.68	Crewe 1982, 1988; Crewe et al., 1990
9ODA	24.7 \pm 7.0 (11%)	158.5 \pm 25.8 (56%)	421.6 \pm 53.6 (73%)	Barbier and Lederer, 1960; Butler et al., 1961; Crewe, 1982; Slessor et al., 1988; Plettner et al., 1997; Engels et al., 1997; Keeling et al., 2003
HVA	0.49 \pm 0.14	1.38 \pm 0.3	3.34 \pm 0.57	Crewe and Moritz, 1989; Crewe and Velthuis, 1980; Slessor et al., 1988, 1990; Engels et al., 1997; Keeling et al., 2003
9HDA	0.17 \pm 0.09	0.63 \pm 0.09	1.19 \pm 0.14	Callow et al., 1964; Engels et al., 1997
9HDA	3.44 \pm 0.51 (2%)	19.5 \pm 5.4 (7%)	35.2 \pm 5.9 (7%)	Crewe 1982, 1988; Slessor et al., 1988, 1990; Engels et al., 1997
10HDA	1.34 \pm 0.33	0.24 \pm 0.07	0.54 \pm 0.08	Standard (Sigma)
10HDA	1.04 \pm 0.22	2.01 \pm 0.84	12.4 \pm 4.5 (2%)	Crewe, 1982
Palmitic acid	17.3 \pm 3.2 (8%)	11.5 \pm 1.7 (4%)	0.41 \pm 0.08	Standard (Sigma)
Stearic acid	31.2 \pm 4.1 (15%)	17.3 \pm 2.6 (6%)	21.4 \pm 2.3 (4%)	Standard (Sigma); Plettner et al., 1996
Oleic acid	131.1 \pm 4.1 (62%)	70.7 \pm 12.3 (25%)	81.2 \pm 10.0 (14%)	Standard (Sigma)

^a Abbreviations for the compounds are as follows: 7HOA = 7-Hydroxyoctanoic acid; 8HOA = 8-hydroxyoctanoic acid; 9ODA = (*E*)-9-oxodec-2-enoic acid; 9HDA = 9-hydroxydecanoic acid; 9HDA = (*E*)-9-hydroxydec-2-enoic acid; 10HDA = 10-hydroxydecanoic acid; 10HDA = (*E*)-10-hydroxydec-2-enoic acid; HOB = methyl-*p*-hydroxybenzoate; 4HBA = 4-hydroxybenzoic acid; HVA = 4-hydroxy-3-methoxyphenylethanol. The percentage composition ($\geq 1\%$) of compounds relative to the other 12 compounds is given in parenthesis. Compounds in very low concentrations are denoted by a “t”.

particularly oleic acid (Table 1). The observed increase in 9ODA corresponded with an increase in 8HOA (end-point by-product of 9ODA biosynthesis) and a decrease in palmitic and stearic acids (precursors to 9ODA biosynthesis; Table 1; see Plettner et al., 1995). Increased levels of 9ODA, HVA, and 9HDA, the components of the QMP complex, were found in virgin queens aged 7- and 14-d (Table 1).

The percentage composition of those compounds generally considered as biologically active in the honeybee mandibular gland signal, the major aliphatic acids, and the phenols, are presented in Table 2. The profile of the overall signal changed as the queens aged, with significant quantitative differences found in the mandibular gland secretions of virgin queens from all different age categories (global $R = 0.61$, $P = 0.001$, permutations = 999, random sample from total possible number of permutations; Fig. 1 and Table 3), except for 7- and 14-d-old virgin *A. m. capensis* queens, which cannot be separated on their mandibular gland profiles (Table 3). These results are consistent for absolute amounts of compounds found in the signal as well as for the ratios of those compounds considered to be the major constituents of mandibular gland signals (global $R = 0.47$, $P = 0.001$, permutations = 999, random sample from total possible number of permutations). However, on pairwise comparisons (using the Bonferroni sequential correction; Rice, 1989), queens aged 3-, 7-, and 14-d-old cannot be delineated accurately from each other when ratios (qualitative variation) and not absolute amounts (quantitative variation) are considered. A multivariate approach of a more composite mandibular profile incorporated additional mandibular gland compounds in the analyses [compounds listed in Table 1, as well as other identified compounds in the secretion, namely, propanoic acid, 2-propenoic acid, 3-phenyl-2-propenoic acid, dodecanoic acid, decanoic acid, (*Z*)-9-hexadecenoic acid, (*E*)-9-hexadecenoic acid, 10-undecenoic acid, benzoic acid, and 4-hydroxy-3-methoxybenzoic acid], revealed the same results (global $R = 0.63$, $P = 0.001$, permutations = 999, random sample from total possible number of permutations), and similarly, 7- and 14-d-old queens could not be delineated on their mandibular profiles.

The PRIMER'S SIMPER procedure (Clarke, 1993) indicated which compounds contribute most, and most consistently, to the average dissimilarity between the

Table 2 Percentage (mean \pm SE) composition of selected aliphatic acids and phenols present in mandibular glands of *A. m. capensis* queens of different ages^a

Compound	Age (days)				Mated ($N = 4$)
	1 ($N = 10$)	3 ($N = 9$)	7 ($N = 10$)	14 ($N = 10$)	
HOB	0.2 \pm 0.1	t	t	t	9.8 \pm 2.8
7HOA	0.7 \pm 0.3	0.6 \pm 0.3	0.1 \pm 0.1	0.2 \pm 0.0	0.5 \pm 0.1
8HOA	4.4 \pm 0.8	1.0 \pm 0.2	0.8 \pm 0.2	1.6 \pm 0.2	5.2 \pm 0.4
9ODA	72.0 \pm 3.0	86.6 \pm 1.9	86.5 \pm 2.3	89.3 \pm 1.0	51.7 \pm 3.2
HVA	1.4 \pm 0.4	0.7 \pm 0.1	0.8 \pm 0.1	1.1 \pm 0.4	2.3 \pm 0.7
9HDAA	0.4 \pm 0.2	0.4 \pm 0.1	0.3 \pm 0.1	0.2 \pm 0.0	t
9HDA	12.0 \pm 1.2	9.5 \pm 1.6	8.5 \pm 1.4	5.9 \pm 0.4	16.9 \pm 1.0
10HDAA	5.3 \pm 1.6	0.1 \pm 0.0	0.2 \pm 0.1	0.1 \pm 0.0	4.9 \pm 0.8
10HDA	3.6 \pm 0.7	1.2 \pm 0.4	2.8 \pm 0.8	1.6 \pm 0.4	8.8 \pm 0.9

Compound abbreviations as in Table 1.

^a Compounds in very low concentrations are denoted by a "t."

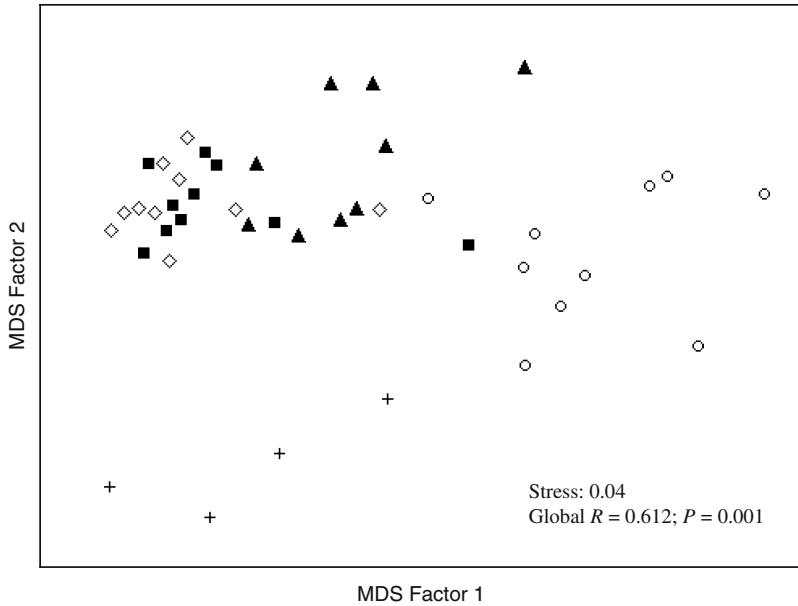


Fig. 1 Multidimensional scaling ordination for the mandibular gland secretion profiles of the 10 major compounds known to be biologically active for *A. m. capensis* queens of different ages. Queens of different ages, except 7- and 14-d-old queens, can be delineated on their mandibular gland profiles. The age groups are as follows: 1-d-old = ○; 3-d-old = ▲; 7-d-old = ■; 14-d-old = ◇; mated queen = +. See Clarke and Warwick (2001) for MDS methods

various test groups, in relation to absolute amounts. In all age classes of queen, 9ODA contributes most to the dissimilarity, followed by 9HDA (Table 4). Mated queens were different from the other test groups, not only because of 9ODA, but also because of the presence of methyl-*p*-hydroxybenzoate (HOB). Besides 9ODA being responsible for the dissimilarity between groups, it also made the greatest contribution within a group and was the compound most responsible for the similarity of the mandibular gland profiles within each of the groups (Table 4). 9HDA also made a major contribution to the mandibular profiles and contributed

Table 3 Comparisons of the probabilities similarity (ANOSIM) of the major aliphatic acids and phenols (µg) present in the mandibular gland secretions of the various test groups^a

Test group	<i>P</i>
1-d-old/3-d-old	0.001
1-d-old/7-d-old	0.001
1-d-old/14-d-old	0.001
1-d-old/mated queens	0.002
3-d-old/7-d-old	0.002
3-d-old/14-d-old	0.002
3-d-old/mated queens	0.001
7-d-old/14-d-old	0.21
7-d-old/mated queens	0.004
14-d-old/mated queens	0.002

^a Probability values significant at the table-wide α value of $P = 0.05$ using the Bonferroni sequential correction (Rice, 1989) are indicated in bold. The comparison of the probabilities similarity (ANOSIM) of a more composite signal (including the major and minor aliphatic acids, phenols, aromatic acids, and diacids) yield precisely the same results.

Table 4 The average percentage contribution (average) of the mandibular gland major aliphatic acids and phenols (μg quantities) that contributed most (*) and most consistently (ratio, ●) to the similarity (SIMPER) of the mandibular gland profiles of each queen test group

Compound	1-d-old		3-d-old		7-d-old		14-d-old		Mated	
	Average	Ratio	Average	Ratio	Average	Ratio	Average	Ratio	Average	Ratio
HOB	0.38	0.65	0.09	2.09	0.1	1.64	0.07	1.85	8.55	2.44
7HOA	1.66	0.83	1.54	1.23	1.7	5.43	2.3	7.35●	2.07	2.82
4HBA	0.4	4.57	0.18	5.96●	0.31	3.29	0.27	3.79	0.1	4.85
8HOA	8.06	5.23●	4.5	4.24	3.85	5.48	6.04	5.5	7.17	7.84●
9ODA	33.5*	5.94●	48.3*	8.28●	47.9*	3.57	50.3*	6.16●	23.5*	8.02●
HVA	2.93	1.05	4.08	5.43	4.18	4.22	3.9	3.23	3.78	1.97
9HDA	0.81	0.86	3.16	7.17●	2.76	8.05●	2.03	2.31	0.04	4.85
9HDA	14.2*	6.5●	13.72*	3.28	13.76*	5.72●	12.54*	7.91●	12.85*	6.83●
10HDA	7.61	3.1	1.14	1.14	1.83	5.73●	1.2	1.4	6.8	4.36
10HDA	6.18	1.94	2.66	1.08	5.87	2.71	4.66	2.07	9.12	4.7
Average similarity	75.7		79.4		82.29		83.32		73.99	

Compound abbreviations as in Table 1.

consistently to the similarity within the test groups, except for 3- and 7-d-old virgin queens, where 9HDAA had a low average contribution to the similarity within the groups; however, it contributed consistently to that similarity. The compounds contributing most to the similarity within the mated queen group were 9ODA, 9HDA, HOB, and 10HDA; of these, only 9ODA and 9HDA made consistent contributions, together with 8HOA (Table 4).

Behavioral Bioassay

The response of workers toward introduced virgin queens showed a significant increase in hostile reactions as queens aged ($r = 0.62$, $N = 20$, $P < 0.05$; Fig. 2). This consistent increase in aggression corresponded with the relative increase in 9ODA as queens aged (Table 2), suggesting a positive relationship between the level of aggression and the relative proportion of 9ODA. With regard to the major aliphatic acids and phenols, the mandibular gland secretions of 1-d-old virgin queens possessed 72% 9ODA (Table 2), and they received $55.8 \pm 6.5\%$ hostile reactions from workers, whereas queens 3 d and older possessed 87% 9ODA and elicit $88.6 \pm 7.9\%$ hostile responses from workers. Upon introduction into the observation hives, virgin queens attracted the immediate attention of workers. Workers generally reacted to 1-d-old virgin queens with a combination of investigative behaviors, including licking and palpating (44.2% investigative reactions), followed by hostile reactions prior to balling. Virgin queens, 3 d and older, elicited substantially more hostile reactions from workers than 1-d-old queens ($P < 0.05$; Kruskal–Wallis one-way analysis of variance by ranks), with few investigative reactions being detected prior to balling.

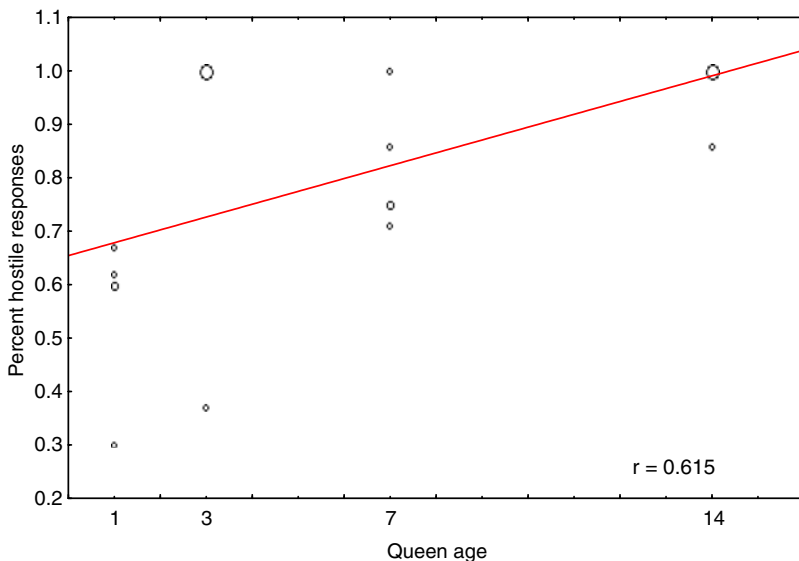


Fig. 2 Graphic representation of the percentage aggressive reactions by workers to virgin queens of various ages. The size of the circles indicates sample overlap for a given level of aggression

Discussion

It is well documented that the amount and composition of the compounds produced in queen mandibular glands change with age, with young virgin queens producing smaller quantities compared to older and mated queens (Crewe, 1982; Crewe and Moritz, 1989; De Hazan et al., 1989a; Slessor et al., 1990; Pankiw et al., 1996; Engels et al., 1997), as well as the proportions of these components differing from mated queens (Pankiw et al., 1996). This age-dependent change in the quantities and blends of chemicals produced in the mandibular glands is also evident for *A. m. capensis* virgin queens (Tables 1 and 2), with virgin queens of the different age classes being readily distinguishable on the basis of their mandibular signal. The only age classes that cannot be separated on the basis of signal (Table 3) are 7- and 14-d-old virgin queens, suggesting that “fully mature virgin queen” status has been reached when queens are ≥ 7 -d-old. This is to be expected because Cape virgin queens typically have mated by 5–6 d (Allsopp, unpublished data). It further suggests 7-d-old as an appropriate age class for fully developed Cape virgin queens for any subsequent studies.

The major factors in the maturity of the mandibular gland signals of *A. m. capensis* virgin queens are the increasing prominence of 9ODA and 9HDA as the queens age (Table 4). The observed increase in 9ODA production as *A. m. capensis* queens age corresponds with an increase in 8HOA and a decrease in palmitic and stearic acids (Table 1). These results concur with the biosynthetic pathways proposed by Plettner et al. (1998) for the synthesis of 9ODA in queens. Palmitic and stearic acids are precursors of the pathway, whereas 8HOA is considered a by-product in the synthesis of 9ODA (Plettner et al., 1995). Plettner et al. (1998) suggested that the stearic acid required for the biosynthesis of 9ODA is produced in the mandibular gland. The data presented here provide quantitative evidence to support this suggestion. The elevated levels of the precursors and their associated isomers found in 1-d-old queens (which decreased as 9ODA production increased as the queens aged) indicated that these compounds are produced in the mandibular gland and are utilized in 9ODA synthesis, resulting in the decreased levels observed in older queens producing increased levels of 9ODA.

The 1-d-old Cape queens analyzed in this study had a signal in which all of the identified volatiles, albeit in small or trace quantities, were present. Subsequent to our analyses, a further active compound, coniferyl alcohol, was reported in the mandibular glands of mated but not virgin *A. m. ligustica* queens (Keeling et al., 2003). Qualitative changes as described for other species of *Apis* (Plettner et al., 1997) and other races of *A. mellifera* (Crewe, 1982; Pankiw et al., 1996) were not as pronounced. Although *A. m. capensis* appears to produce a mandibular gland signal in young virgin queens that is unique to the race, recently emerged queens have a signal dominated by oleic acid, consistent with the findings of Engels et al. (1997). Oleic acid concentrations decrease with age in *A. m. capensis* virgin queens and again upon mating (Table 1); however in *A. m. carnica* virgin queens, oleic acid remains constant during the 2-wk postemergence and decreases only slightly after mating (Engels et al., 1997). With respect to the QMP compounds, 9HDA is the only compound in young Californian queens (<24 hr old; Slessor et al., 1990). The ontogenetic composition of the QMP compounds of *A. m. capensis* is more complex, with detectable quantities of 9ODA, 9HDA, and HVA found in all 1-d-old queens analyzed in this study. HOB was also detected in trace quantities in the queens

analyzed. Whereas the signal of *A. m. capensis* virgin queens more closely resembles that of the other southern African honeybee, *A. m. scutellata*, than it does that of Africanized and European queens, significant differences in the percentage composition of the major compounds, as identified by Crewe (1982), are apparent. Young (1–2-d-old) *A. m. scutellata* queen signals are dominated by 10HDA with a significant contribution by 9ODA (Crewe, 1982). In comparison, when considering the percentage of the major compounds of the mandibular gland secretions (Table 2), the 1-d-old queens analyzed had a signal in which 9ODA predominated (72%), with 10HDA making only a small contribution (3.56%) to the signal. It has been suggested that the significance of the uniqueness of the signal of young *A. m. capensis* queens is necessary for the establishment of appropriate queen–worker relationships in a race where workers readily produce queen-like signals (Crewe, 1988).

Comparisons of the relative proportions of 9ODA in the mandibular gland signals of virgin queens having reached mating age of the different races are difficult because many of the studies only provide data for the four compounds comprising the QMP complex (Slessor et al., 1990; Pankiw et al., 1996; Engels et al., 1997). These studies all found that 9ODA predominated in the QMP complex of receptive Africanized and European virgin queens. Data provided by Crewe (1982) and Plettner et al. (1997) suggest that the data of these other studies are not a true reflection of the signal of receptive queens. Their data indicate that when the “worker substance” 10HDA is incorporated into relative calculations of the contribution of the major compounds in mandibular gland extracts, a different pattern emerges. They found that receptive virgins of *A. mellifera* (Plettner et al., 1997) and *A. m. mellifera* (Crewe, 1982) had a signal dominated by 10HDA with a significant contribution of 9ODA. This is not the case in *A. m. capensis* virgin queens in which 10HDA contributes minimally to the mandibular signal.

Mating induces additional changes to the signal bouquet with increases in the aromatic compounds and the appearance of HVA in European queens (Slessor et al., 1990; Pankiw et al., 1996). Interestingly, *A. m. capensis* virgin queens produce detectable quantities of HVA at emergence and, by 14 d, are producing approximately 5.5 µg (Table 1), considerably higher than even mated queens of European origin, which produce between 1.5 and 4.2 µg (Slessor et al., 1990; Engels et al., 1997; Plettner et al., 1997). In contrast, HOB is found in lower concentrations in virgin *A. m. capensis* queens compared to virgin queens of European origin (trace quantities compared to 0.4 µg; Slessor et al., 1990), but queens of both origins produce a significant increase on mating. The change in mandibular gland secretions with mating is less pronounced in *A. m. capensis* queens than in *A. m. scutellata* queens and queens of European origin (Crewe, 1988; Slessor et al., 1990; Plettner et al., 1997).

Worker aggression toward *A. m. capensis* virgin queens increased with age (Fig. 2), with workers balling the introduced queens. Whereas the quantities of all the major compounds of the mandibular gland extract increased with age, the relative proportion of the queen substance 9ODA exhibited a positive age-dependent aggressive response by workers, as previously reported by Yadava and Smith (1971c). One-day-old virgin queens produced proportionally less 9ODA and elicited less aggressive behavior from workers than queens 3 d and older. Pettis et al. (1998) suggested that the lower levels of QMP in virgin queens might facilitate their acceptance by a colony. They illustrated that the acid component, and not the aromatic components, of the QMP elicits aggressive behavior by workers. We tested

the absolute amounts of compounds present in the mandibular gland secretions, and our results support the notion that the acid components, particularly 9ODA, correlate to aggressiveness. These findings partially support the suggestion that the reaction of workers to foreign queens is dependent on pheromone cues and qualitative as well as quantitative differences in mandibular gland volatiles (Engels et al., 1997; Pettis et al., 1998).

This study has not only shown ontogenetic changes in absolute amounts of various mandibular gland components but also that the pattern of the signal changes with age. These age-dependent changes in mandibular gland secretions are positively correlated to worker responses with respect to their levels of aggression.

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Mate Location Mechanism and Phase-Related Mate Preferences in Solitarius Desert Locust, *Schistocerca gregaria*

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Abstract Mate location responses of male and female solitary-reared locusts that had either experienced no crowding or that had been crowded for varying periods were studied in a flatbed wind tunnel. Two hypotheses were explored: that both sexes of this phase of the locust participate in locating the other by using a combination of chemical and visual signals, and that individuals that experience some crowding (i.e., undergo varying levels of phase shift) can compete effectively with their solitary counterparts in mate location and mating. Our results confirm that both male and female solitarius locusts actively participate in mate location, although the former is the more aggressive partner. The responses of the insects are stronger when a visual cue is provided with the olfactory signal. Crowding of solitary-reared adults enhances their responsiveness to the other sex in the absence and presence of the visual cue. This phenomenon may constitute one of several mechanisms that are involved in recruiting solitary individuals into gregarizing groups and facilitating the spread of gregarious characters across a reproductively active solitarius population.

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Introduction

Acridids are known to use acoustic, visual, tactile, and chemical signals, singly or in combination, in mate location and recognition (Whitman, 1990). Although pheromone communication has been recognized to play a role in the mating behavior of many orthopteroid groups, to date, the mediation of sex pheromones in acridids has been demonstrated in only a few species. In *Hieroglyphus nigrorepletus*, males are attracted to a pheromone of medium volatility associated with female excreta (Siddiqui and Khan, 1981). In *Taeniopoda eques*, a contact pheromone of low volatility present on the cuticle of female locusts and in their defensive exudates elicits copulatory attempts by males (Whitman, 1982). Behavioral observations have also implicated the mediation of sex pheromones in the neotropical grasshoppers *Elaeochlora trilineata* and *Chromacris speciosa* (Riede, 1987). None of these pheromones has been characterized.

In locusts, two extreme situations occur. High population densities ($>1.5 \times 10^4 \text{ ha}^{-1}$) in the gregarious phase (El Bashir et al., 1993) reduce mate finding to close-range recognition, probably mediated by visual cues and short-range or contact chemical signals (Inayatullah et al., 1994; Hassanali et al., 2005). In high-density populations, males move toward females, but females do not appear to display similar behavior (Inayatullah et al., 1994). On the other hand, solitarious-phase locusts occur in low densities ($<5 \text{ ha}^{-1}$) and may use both long- and short-range recognition cues. Moreover, both sexes appear to be active in the mate location process (Uvarov, 1977; Byer, 1991). In a detailed study of the responses of solitary-reared *Schistocerca gregaria* (Forskål) males in a wind tunnel to solitary-reared females, solitary-reared females with glued wings (to restrict their vibration and any acoustic signal), and female decoys, the responses of males to females with glued wings were not significantly different from those toward normal solitary-reared females, thus ruling out the possibility of involvement of an acoustic cue from the female (Inayatullah et al., 1994). Solitarious males oriented to live solitary-reared females upwind but not to female decoys, suggesting the mediation of a chemical signal. On the other hand, blinded males did not move upwind toward virgin females, suggesting that vision may be essential for predisposing individuals to sexual activities (Inayatullah et al., 1994).

In the solitarious population at the onset of phase shift, solitarious and gregarious locusts also encounter one another (Roffey and Popov, 1968). The present study attempts to understand the relationship between mating patterns and phase dynamics at the early stages of gregarization, to determine how phase shifts influence mating, and to clarify the relative importance of chemical and visual signals from the two sexes of solitarious individuals in mate location. We tested two hypotheses: (1) Solitarious individuals of both sexes use olfactory and visual cues for mate location and (2) insects that experience some crowding (and, therefore, are shifting to the gregarious phase) may compete effectively with solitary individuals in mate location and mating.

Methods and Materials

Insects

Locusts were from the ICIPE colony, propagated from a stock that originated from the Desert Locust Control Organization for Eastern Africa (DLCO-EA) in Addis Ababa, Ethiopia, in 1989, with repeated inclusion of collections from the field. Gregarious insects of mixed sexes were reared under crowded conditions (300–400) in aluminum cages (50 × 50 × 50 cm) in a well-aerated room (4.5 × 4.5 m) with a duct system (10–15 air changes/hr) that maintained a negative pressure (Ochieng-Odero et al., 1994). Solitary males and females were reared separately, each in an aluminum compartment cage (10 × 10 × 24 cm), arranged side by side in banks of eight with no visual contact between them (Ochieng-Odero et al., 1994). The top was made of wire screen for ventilation and the front of Plexiglas for visibility. Solitary rearing rooms (1.5 × 4.5 m) were maintained at 30 ± 4°C, 40–50% relative humidity, and 12:12 L/D photoperiod. Fresh wheat seedlings and bran were provided daily.

Treatment of Test Insects

Crowd-reared mature males (24-d-old) and females (23- to 26-d-old) were used from the appropriate colony without further treatment. Unmated (virgin) solitary-reared

Table 1 Test insects used to study behavioral responses to olfactory or combination of olfactory and visual cues derived from different sources

Test insect type	Pretreatments	Signal source	Signal type	
			o	o+v
♂s	Isolated	♀s	✓	
♂s		♀s		✓
♂s		None		
♀s	Isolated	♂s	✓	
♀s		♂s		✓
♀s		None		
♂s	Crowded 8 d	♀s	✓	
♂s		♀s		✓
♂s		None		
♂s	Crowded 16 d	♀s	✓	
♂s	Crowded 24 d	♀s	✓	
♂s	Crowded 24 d	None		
♂g	Crowded	♀s	✓	
♂g	Crowded			
♀g	Crowded	♂s	✓	
♀g	Crowded			
♀s	Crowded 8 d	♂s	✓	
♀s	Crowded 24 d	♂s	✓	
♀s	Crowded 24 d	None		

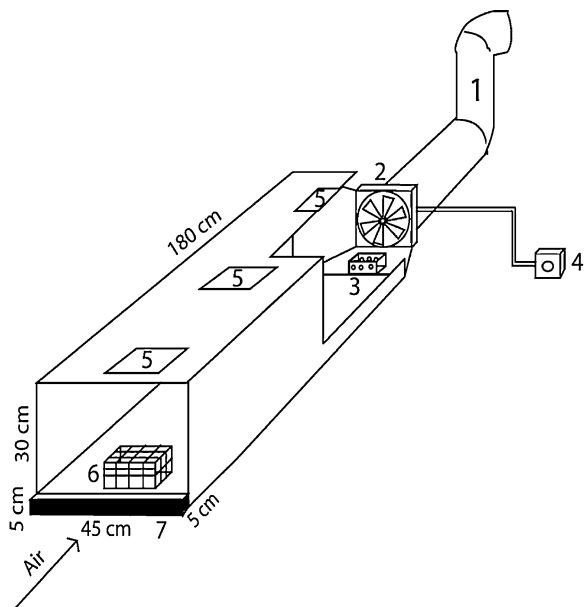
♂s and ♀s, solitary-reared males and females, respectively; ♂g and ♀g, crowd-reared males and females, respectively; o, olfactory cue; o+v, olfactory and visual cues.

mature locusts (24-d-old males, 23- to 26-d-old females) were used after varying periods of pretreatment as follows (Table 1): (i) crowded for the last 8 d (days 17 to 24) in mixed sex groups (1:1) of 8 per cage ($20 \times 20 \times 20$ cm), (ii) crowded for the last 16 d (days 9 to 24) as in (i), (iii) crowded for the whole period (fledgling to d 24) as in (i), and (iv) left uncrowded for the whole period. During these treatments, wheat seedling and bran were also provided daily.

Wind Tunnel

Experiments were performed in a Plexiglas flatbed tunnel, 180 cm long \times 45 cm wide \times 30 cm high (Fig. 1), similar to that described by Inayatullah et al. (1994). An exhaust fan sucked air through a Plexiglas charcoal filter container ($45 \times 5 \times 5$ cm, with inlet and outlet sides made of wire mesh). Air from the tunnel was vented outside the room through a chimney. The downwind end of the tunnel had a small wire mesh chamber ($14 \times 7 \times 7$ cm) for prerelease holding of the test insect. In the upwind side, 150 cm apart, was placed a small wire mesh cage ($20 \times 20 \times 5$ cm) that could be covered with black muslin to hide a locust placed therein. Air passed through activated granular charcoal filter (4–14 mesh; Sigma Chemical Co., St Louis, MO, USA) previously cleaned with dichloromethane and activated under nitrogen for 2 hr. The airspeed inside the tunnel was about 20 cm/sec. Smoke was initially used to confirm laminar airflow and to reveal the path of the odor plume through the tunnel. The floor of the working area of the tunnel was covered with thick gray manila paper marked with six stripes (30 cm apart) to facilitate the monitoring of the test insect's movement. Five bulbs (60 W each) were placed 70 cm above the tunnel equally spaced

Fig. 1 The flatbed Plexiglas wind tunnel. 1, PCV air duct; 2, exhaust fan; 3, test insect prerelease box; 4, fan speed controller; 5, doors for introduction and removal of insects; 6, insect signal source wire mesh box; 7, activated charcoal filter



along its length for uniform lighting. The temperature inside the tunnel during the experiments was $25 \pm 1^\circ\text{C}$.

Long-range Behavioral Observations

We studied the responses of virgin individual insects to one of the following sets of signals derived also from virgin individuals: (i) olfactory signals alone (wire mesh cage covered), (ii) combination of olfactory and visual signals, and (iii) control (empty cage upwind) (Table 1). For treatments (i) and (ii), one adult insect was placed in the upwind cage. Insects were initially observed in photo- and scotophase, but as there were no significant differences, all subsequent observations were made in the photophase. Each observation cycle in the wind tunnel was undertaken for 30 min. The Observer 3.0 software (Noldus, Wageningen, the Netherlands) was used to record (i) the number of scans (movement of the front part of body, $4\text{--}6^\circ$, to either side (Wallace, 1959); (ii) time spent walking/running vs. resting (no movement, abdomen touching the floor); (iii) number of jumps, flights, or abortive flights (in the limited wind tunnel space, abortive flights could not be distinguished from jumping); (iv) distance to signal source traversed in 30 min; and (v) number of individuals that crossed 100 cm and/or arrived at signal source.

Close-range Behavioral Observations

The close-range behaviors of solitary-reared males (24-d-old) and females (23- to 26-d-old) in response to the solitary-reared opposite sex were compared by moving the release cage within 30 cm of the upwind cage. The number of males and females that moved to the target in the presence and absence of olfactory and visual cues from the opposite sex over 30-min observation periods were noted.

Statistical Analyses

Mean distance traversed by test insects, as well as behavioral frequencies recorded during observations, were transformed to $\log_{10}(x + 10)$ to stabilize the variance and analyzed using analysis of variance (ANOVA) followed by least significant difference (LSD) test at $P < 0.05$ (The SAS System 8.02, SAS Institute, Inc., Cary, NC, USA). The percentage of test insects that reached the source of signal(s) in different treatments was compared by Student *t* test.

Results

Responses of Males to Female Olfactory Cues

Figure 2 summarizes the average behavioral responses of individual males in different physiological states to a putative olfactory cue from solitary-reared individual females upwind. Although solitary-reared males appeared more responsive in the presence of the olfactory signal than in its absence, none of

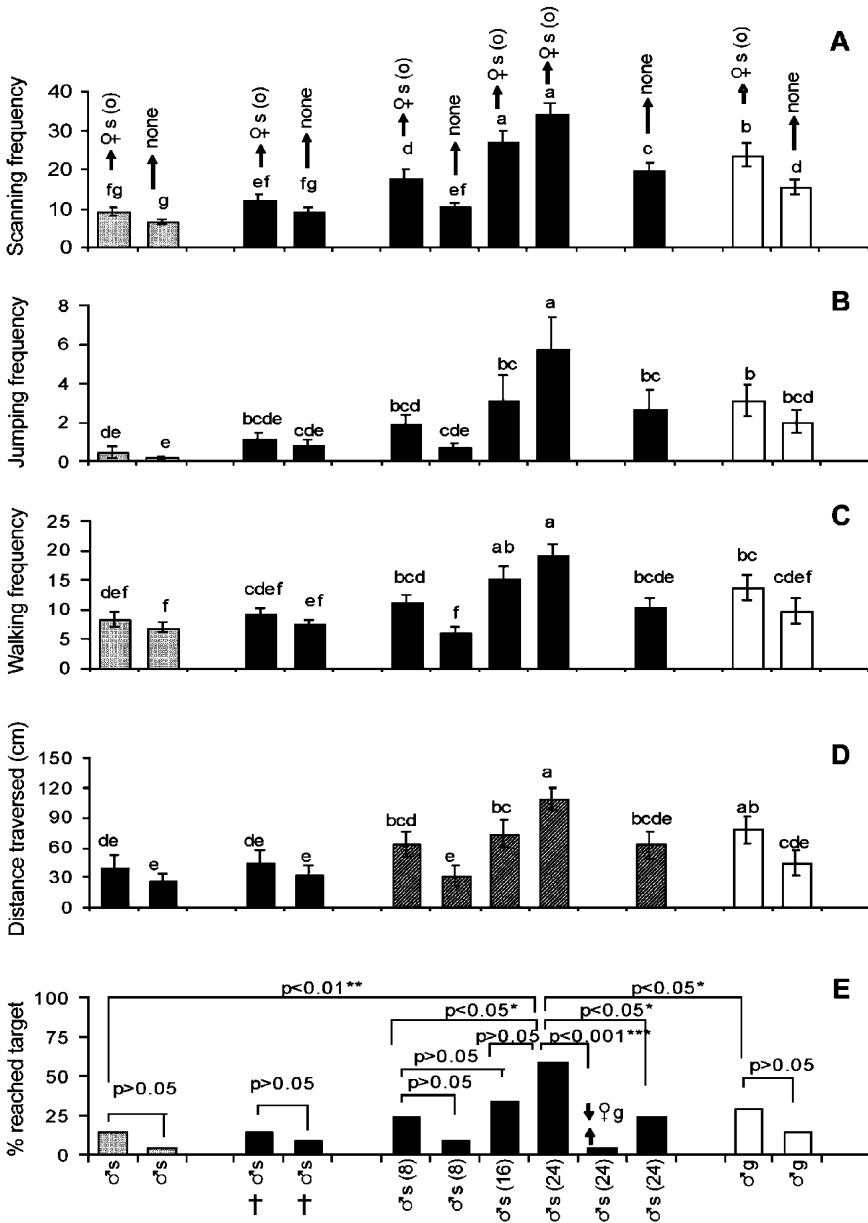


Fig. 2 Responses of mature solitary-reared males, uncrowded and crowded for varying periods, and crowd-reared (gregarious) males to hidden solitary-reared females (except where indicated). Status of males released downwind is indicated on the x axis of E: ♂s, solitary male; †, experiment in scotophase; ♂s (8), solitary male crowded for 8 d; ♂s (16), solitary male crowded for 16 d; ♂s (24), solitary male crowded for 24 d; ♂g, gregarious male. Signal sources are indicated in A: ♀s, solitary female; ♀g, gregarious female; (O), olfactory signal. Columns with the same letter are not significantly different at $P < 0.05$ (ANOVA, LSD test)

the behavioral elements quantified was statistically significant. There were no differences between insects in their photophase or scotophase. Males that were crowded for 8 d were significantly more active in the presence of the putative olfactory cue from uncrowded solitary-reared females than in its absence as reflected in scanning and walking frequencies and in the mean distance traversed (Fig. 2A, C, and D). Solitary-reared males that were crowded from fledgling to maturity (24 d) were most responsive. All behaviors that were quantified were significantly more pronounced in the presence than absence of the solitary female, and significantly more insects arrived at the target female when compared to the control ($t=2.39$, $df=38$, $P<0.05$). Interestingly, the 24-d crowded solitary-reared males were more responsive than male counterparts reared under crowded conditions continuously. Crowd-reared (gregarious) females elicited no similar responses from males, as illustrated by the large and significant ($P<0.001$) difference in the proportion of 24-d crowded solitary-reared males that arrived at the cage with solitary- and crowd-reared females, respectively (Fig. 2E).

Responses of Females to Male Olfactory Cues

Figure 3 summarizes the average responses of females in different physiological states to a putative odor signal from solitary-reared male. Comparison of these with those of males (Fig. 2) indicate some similarities, as well as differences between the sexes. The level of responses of females that experienced no crowding was, by and large, comparable to that of corresponding males, and there were no differences between insects in their photophase or scotophase. On the other hand, females that experienced crowding did not demonstrate levels of enhancement of the different behaviors comparable to those observed in the corresponding males. However, two significant differences in the behavioral responses of females (scanning and walking frequencies, Fig. 3A, C) in the presence and absence of the putative olfactory signal 150 cm upwind indicate that solitary females detect the presence of their male counterparts in the absence of the visual cue, although their response activities are relatively subdued.

Responses of Males and Females to Combinations of Olfactory and Visual Cues

Figure 4 summarizes average response data of uncrowded solitary-reared males, solitary-reared males that were crowded for 8 d, and uncrowded solitary-reared females to combinations of olfactory and visual cues of solitary-reared individuals of the opposite sex. The presence of the visual cue enhanced the responses of uncrowded and 8-d crowded solitary-reared males. This is reflected in significant increase in scanning and jumping frequencies (Fig. 4A, B), as well as in the mean distance traversed by the insects (Fig. 4D), in the proportion that traversed >100 cm, and that arrived at the source of the cues (Fig. 4E, F). Incremental effect of crowding is also reflected in higher relative responses of the crowd-reared individuals compared to those of the uncrowded ones. Solitary-reared females also responded more strongly when both visual and olfactory signals were present. This is illustrated in significantly higher responses of released individuals compared to those of insects exposed to only the olfactory signal (Fig. 4B, D) and/or to control (Fig. 4A–F).

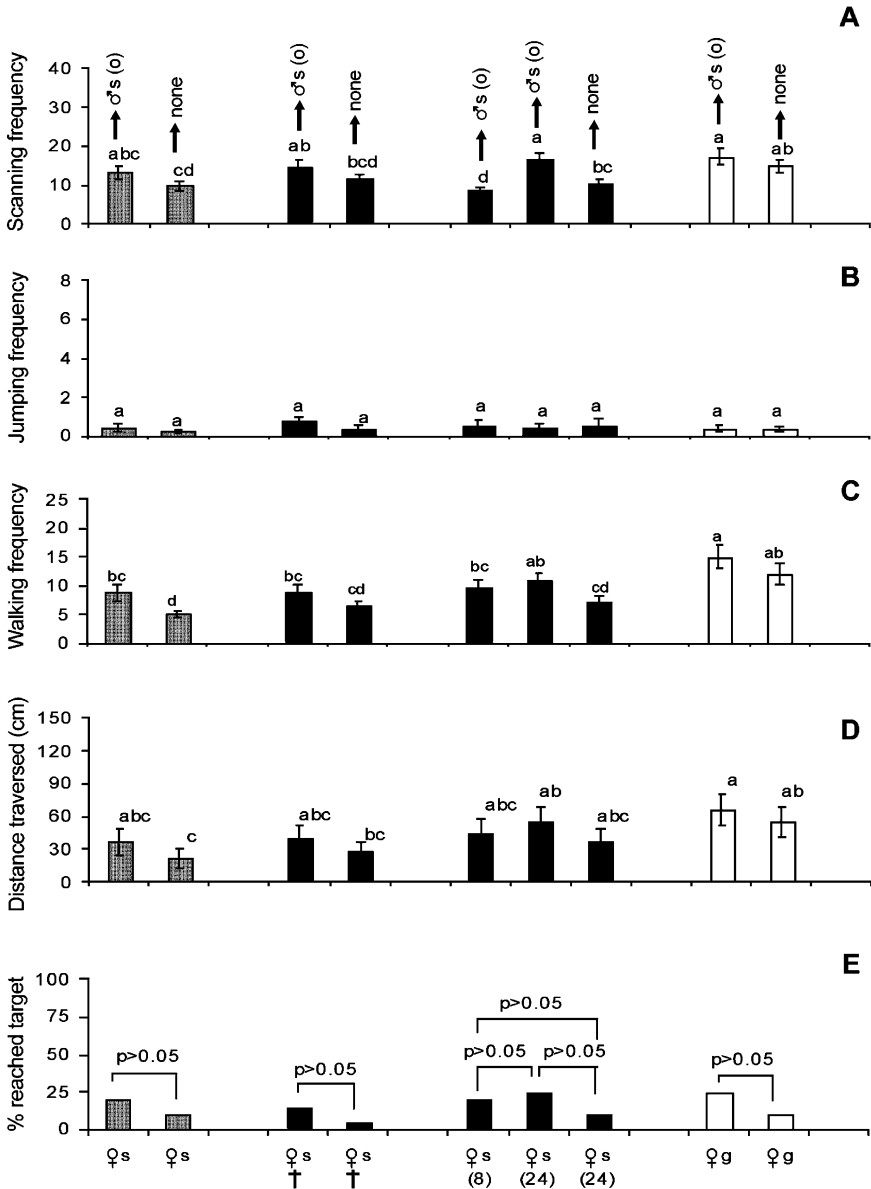


Fig. 3 Responses of mature solitary-reared females, uncrowded and crowded for different periods, and crowd-reared females to hidden solitary-reared males (except where indicated). Status of females released downwind is indicated on the x axis of E: ♀s, solitary female; †, experiment in scotophase; ♀s (8), solitary female crowded for 8 d; ♀s (24), solitary female crowded for 24 d; ♀g, gregarious female. Signal source is indicated in A: ♂s, solitary male; (O), olfactory signal. Columns with the same letter are not significantly different at $P < 0.05$ (ANOVA, LSD test)

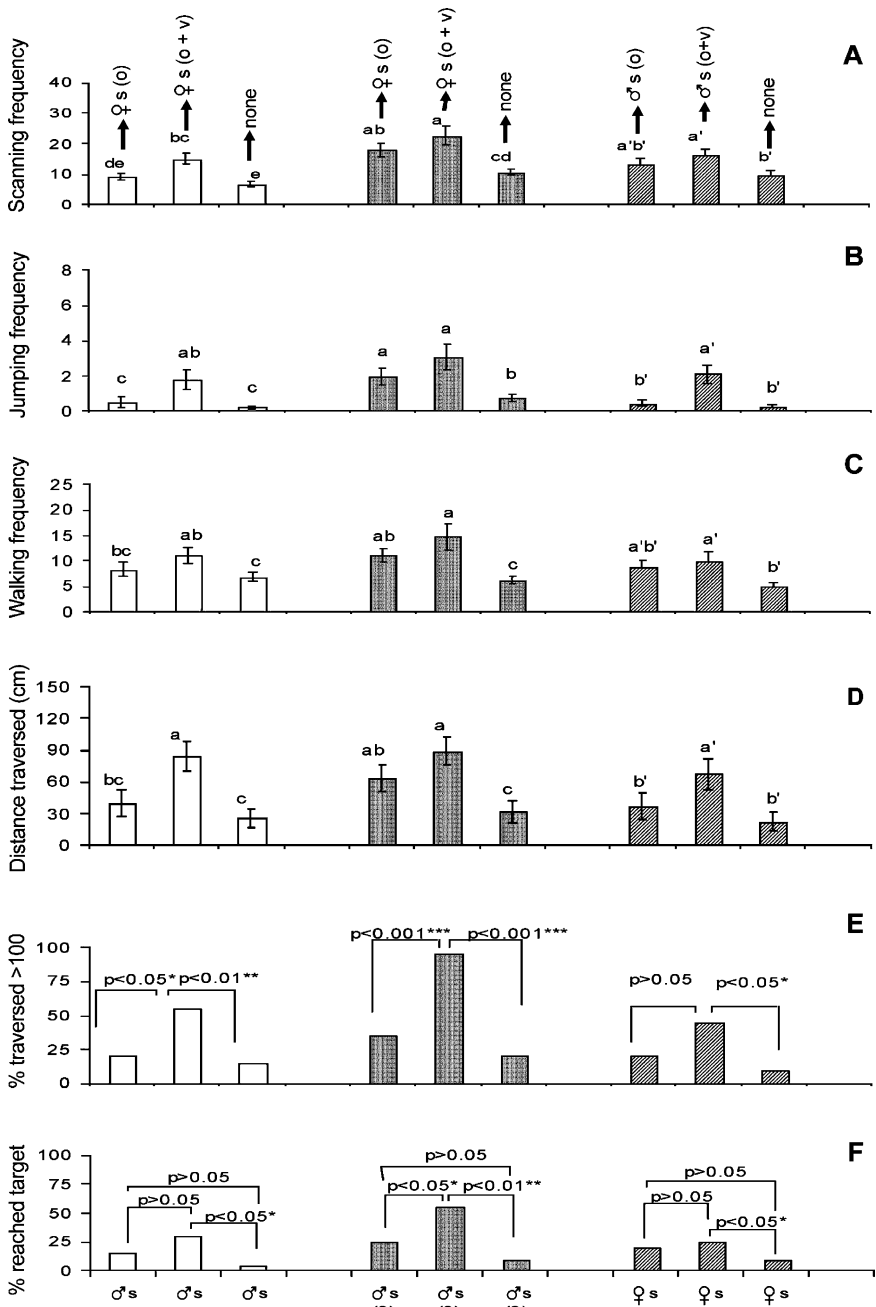


Fig. 4 Responses of mature solitary-reared males, uncrowded and crowded for 8 d, and solitary females to visual ± odor cues from solitary-reared females or males (except where indicated). Status of males released downwind is indicated on the x axis of F: ♂s, solitary male; ♂s (8), solitary male crowded for 8 d; ♀s, solitary female. Signal source is indicated in A: ♀s, solitary female; ♂s, solitary male; (O), olfactory signal; (O + V), olfactory + visual signals. Columns with the same letter are not significantly different at $P < 0.05$ (ANOVA, LSD test)

Close-range Behavioral Observations

The behavior of solitary-reared males and females closer to the insect-cage upwind, with both visual and olfactory cues of the opposite sex, differed. A significant percentage (60%) of the males arrived close to and physically contacted the cage and climbed on it compared to 10% in the control ($t = -3.89$, $df = 18$, $P < 0.001$). Within ~5 cm of the cage, males typically spent several minutes in a resting position, scanning and massaging/cleaning antennae and eyes, before swiftly approaching the caged female. The female moved away from the approaching male, and this was followed by successive attempts by the male to get to the female, with the latter moving away each time. On the other hand, although 80% of the test females came within 5- to 10-cm range of the caged male ($t = -4.48$, $df = 18$, $P < 0.001$), none contacted the cage. Most stood scanning in the direction of the caged male. However, 30% of the females approached the cage several times and groomed their eyes, antennae, or forewings.

Discussion

Our results indicate that, in the presence of olfactory signal alone, the responses of solitary-reared males to females are relatively subdued (Fig. 2). When a visual cue was included, there was significant increase in the attraction of the males (as reflected in scanning and jumping frequencies, and in the distance traversed upwind to the females). Similarly, solitary-reared females also responded more strongly when a visual cue associated with the male augmented the olfactory signal, although, in this case, the increases in female responses were somewhat weaker (Fig. 4). These results confirm our expectation based on observations in a greenhouse arena and in the field that both male and female solitary insects actively participate in mutually locating one another. Close-range observations also show that, on finding each other, they demonstrate sex-specific stereospecific behaviors, with the male as the more aggressive partner.

Previous investigations by Ochieng' et al. (1998) found that solitary adults had more antennal olfactory sensilla than their gregarious counterparts. There was no difference between sexes. Greenwood and Chapman (1984) obtained similar results in *Locusta migratoria*. The mutual responses of solitary-reared male and female desert locusts to the olfactory cues of each other are consistent with these findings and suggest that other locust species may also demonstrate similar mate location mechanism. Interestingly, two types of olfactory sensilla have been found on the antenna of *L. migratoria*, (Ameismeier, 1987). Type A has between 20 and 30 neurons, which probably function in recognition of plant odors. Type B has only three neurons that are more typical of pheromone sensilla (Ameismeier, 1987).

Our study also demonstrates that solitary-reared males and females that experience crowding exhibit enhanced responses to olfactory cues (Fig. 2). Indeed, solitary-reared males that were crowded from the fledgling stage were more successful in locating solitary-reared females than their counterparts that had been continuously crowd-reared. Solitary-reared females that were crowded also showed small increases in some of their responses (e.g., scanning and walking frequencies) to the olfactory signal from solitary-reared males, although, in this case, these were

not significantly different from those of continuously crowd-reared females (Fig. 3). These responses became more pronounced in the presence of the visual signal (Fig. 4). These results provide an additional insight into the mechanism that underlies the genesis and spread of gregarization in a reproductively active solitary population. Although the density of such a population is low, habitat heterogeneity, such as patchy distribution of food plants and perching sites, may promote crowding in isolated microenvironments (Bouaichi et al., 1996; Deng et al., 1996). Some phase-related traits, such as aggregation behavior (Roessingh and Simpson, 1994; Bouaichi et al., 1995) and emission of gregarious-phase aggregation pheromones (Deng et al., 1996; Hassanali et al., 2005), change rapidly with crowding. The results of our present study indicate that, in such situations, gregarizing adult males and females are likely to seek out and mate with their solitary counterparts, and thereby facilitate their recruitment into the gregarizing population.

Previously, two other processes were identified that may also contribute to horizontal spread of gregarious traits across a solitary population. Njagi et al. (1996) compared the electroantennographic (EAG) and behavioral responses of adults of solitary and crowd-reared desert locusts to the aggregation pheromone produced by gregarious males and its constituents. The general response patterns of the two phases of the insect were found to be similar, suggesting that solitary desert locusts are predisposed to respond positively to the aggregating effect of the pheromone and to join the crowd when they encounter it. Thus, clusters of pheromone-emitting gregarizing locusts in appropriate microenvironments may provide nuclei for recruitment of solitary individuals and spread of gregarious traits in the population (Njagi et al., 1996).

Another interesting process that involves active recruitment of solitary individuals relates to the oviposition preferences of gravid solitary females in the absence and presence of egg pods laid by gregarious females. In surveys in successive breeding seasons at desert locust-breeding habitats around Port Sudan in the Red Sea area, Bashir et al. (2000) found that incoming solitary females oviposited predominantly in the vicinity of annual species of *Heliotropium* and, to a lesser extent, near millet plants. Follow-up experiments in cage arenas in the absence of egg pods from crowd-reared gregarious locusts confirmed this preference for these plants. However, solitary females oviposited preferentially near *gregaria* egg pods, when these were present, like their gregarious counterparts and those *solitaria* that experienced some crowding. Communal oviposition, which is characteristic of gregarious-phase locusts (Popov, 1958; Stower et al., 1958; Uvarov, 1977), ensures temporal and spatial cohesiveness of the succeeding hopper generation. A primer gregarization pheromone associated with egg pods derived from gregarious females and solitary females that experience crowding induces the transformation of developing embryos to the gregarious phase (McCaffery et al., 1998; Malual et al., 2001) and thus facilitates transmission and accumulation of gregarious characters across generations. The preferential attraction of ovipositing solitary females to the *gregaria* egg pods then represents another mechanism exploited by the species to recruit *solitaria* into the gregarizing or gregarious population.

In summation, both male and female solitary-phase *S. gregaria* are attracted to each other by both odor and visual cues. Of special interest is the finding that solitary males and females that experience crowding are more effective in locating solitary mates. This constitutes yet another mechanism of recruiting

solitaria adults into gregarious groups and of facilitating the spread of gregarious characters across a solitary population.

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Pheromone Antagonism in the European Corn Borer Moth *Ostrinia nubilalis*

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Abstract Mixing the sex pheromones of the Mediterranean corn borer, *Sesamia nonagrioides*, and the European corn borer, *Ostrinia nubilalis*, results in significantly lower captures of *O. nubilalis* when compared to traps loaded with its pheromone alone. Rubber septa loaded with a constant concentration of the pheromone of *O. nubilalis* and different percentages of the *S. nonagrioides* pheromone (from 1 to 100%) causes dose-dependent antagonism in the field. Electroantennograms of *O. nubilalis* males showed high antennal responses to its own pheromone components, followed by smaller responses to the major, [(Z)-11-hexadecenyl acetate (Z11-16:Ac)], and two minor components [dodecyl acetate (12:Ac) and (Z)-11-hexadecenal (Z11-16:Ald)] of the *S. nonagrioides* pheromone. There was almost no response to the *S. nonagrioides* minor component (Z)-11-hexadecenol (Z11-16:OH). Field tests that used traps baited with the *O. nubilalis* pheromone plus individual components of *S. nonagrioides* showed that Z11-16:Ald causes the antagonism. Adding 1% Z11-16:Ald to the pheromone of *O. nubilalis* reduced oriented flight and pheromone source contact in the wind tunnel by 26% and 83%, respectively, and trap captures in the field by 90%. The other three pheromone components of *S. nonagrioides* inhibited pheromone source contact but not oriented flight of *O. nubilalis* males and did not inhibit capture in the field. Cross-adaptation electroantennogram suggests that Z11-16:Ald stimulates a different odor receptor neuron than the pheromone components of *O. nubilalis*. We conclude that Z11-16:Ald is a potent antagonist of the behavioral response of *O. nubilalis*.

Keywords Behavioral antagonism · Inhibition · Sex pheromone · Wind tunnel · Cross-adaptation EAG · *Sesamia nonagrioides* · *Ostrinia nubilalis*

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Introduction

Most moth species use sex pheromones for mate finding, with females normally being the signalers and males the responders (Cardé and Baker, 1984). Specificity of sex pheromone composition and response maintains reproductive isolation and prevents costly reproductive mistakes (Löfstedt, 1993). The identity of the pheromone blend is determined by the chemical structure of its components and by their absolute and relative quantities (Jurenka, 2003). Signal specificity is matched by specificity in male response, ensuring correct intraspecific attraction and mating (Birch and Haynes, 1982). The integrity of the female pheromone blend is essential to stimulate upwind responses in conspecific males. An incomplete blend, or a blend lacking the species-specific proportion of components, will fail to attract males of the same species, and may instead attract males of a different species (Cardé and Haynes, 2004). Similarly, the type of pheromone receptor neurons present on the male antennae and the integration of the pheromone information in the brain will determine the pheromone blend to which the males will respond (Todd and Baker, 1999; Hansson, 2002).

Typically, each pheromone receptor neuron type in the male antennae responds maximally to just one pheromone component, so there are as many specific neurons types as there are pheromone components in the blend (Todd and Baker, 1999; Hansson, 2002), although response of a single olfactory neuron to more than one pheromone compound can also occur (Cossé et al., 1998; Baker et al., 2004). Males of some species have olfactory receptor neurons attuned to pheromone components produced not by their own females but by those of other species. In some cases, the addition of heterospecific components to the conspecific blend causes inhibition of male upwind flight. These so-called pheromone (or behavioral) antagonists play a role in maintaining reproductive isolation of closely related species (same genus) sharing common pheromone components, and, therefore, susceptible of mating mistakes (Leal, 1996; Borden, 1997; Mustaparta, 1997; Cardé and Haynes, 2004). In addition, there are cases where pheromone antagonism occurs between species that are not closely related (different genera). For example, baiting individual traps, two components at a time, with all possible combinations of the sex pheromones of *Helicoverpa zea*, *Spodoptera frugiperda*, *Pseudaletia unipuncta*, and *Agrotis ipsilon*, causes a reduction in at least one of the species involved compared with traps baited with the pheromone of just one species (Lopez et al., 1990). Similarly, attraction of *Tetanolita mynesalis* to its own pheromone is inhibited by the presence of the sex pheromone of *Lacinipolia renigera* (Haynes et al., 2002), and the pheromone of *Adoxophyes orana* inhibits the attraction of *Cydia pomonella* (Potting et al., 1999).

The European corn borer *Ostrinia nubilalis* (Crambidae) and the Mediterranean corn borer *Sesamia nonagrioides* (Noctuidae), sympatric moth species in the Mediterranean region, both feed on corn stalks, causing important economic losses (Albajes et al., 2002). Each species uses a unique pheromone blend to attract the other sex, and no cross-attraction exists between the two species. On the Iberian peninsula, the predominant *O. nubilalis* pheromone phenotype is the so-called Z strain which has a 97:3 blend of (*Z*)-11-tetradecenyl acetate (Z11-14:Ac) and (*E*)-11-tetradecenyl acetate (E11-14:Ac) (Sans, 1997). The sex pheromone of *S. nonagrioides* has (*Z*)-11-hexadecenyl acetate (Z11-16:Ac) as the major component and smaller quantities of (*Z*)-11-hexadecenal (Z11-16:Ald), (*Z*)-11-hexadecenol (Z11-16:OH) and dodecyl acetate (12:Ac) (Mazomenos, 1989; Sans et al., 1997; Krokos

et al., 2002). In mating disruption tests against *S. nonagrioides*, we found that the number of larvae of both the target species and *O. nubilalis* decreased in treated fields (Eizaguirre et al., 2002). Combining the pheromones of both species in the same septum revealed the existence of mutual pheromone antagonism: Each species is inhibited by the pheromone of the other species (Eizaguirre et al., 2002).

Pheromone antagonists have potential application as tools in pest control programs (Bengtsson et al., 1994; Borden, 1997; Renou and Guerrero, 2000). The purpose of the present study was to determine which pheromone component(s) of *S. nonagrioides* reduces the response of *O. nubilalis* to its own pheromone. We also wanted to determine if the antagonist compound(s) is (are) perceived by the same or different pheromone receptor neurons as those attuned to the pheromone blend components of the species.

Methods and Materials

Colony Maintenance

The colony of *O. nubilalis* was started with 12 mated females collected in the field (Lleida, Spain, 2003). They were initially held as iso-female lines until 6 to 12 female progeny from each female were analyzed by GC-FID to confirm they were the Z strain. The colony was maintained in an environmental chamber under a 16:8 (light/dark) photoregime at $25 \pm 1^\circ\text{C}$, following the rearing procedure described in Sans (1997).

Chemicals

Z11-14:Ac and E11-14:Ac were purchased from Sigma-Aldrich Química, S.A. (Madrid, Spain). Z11-16:Ac, Z11-16:Ald, Z11-16:OH, and 12:Ac were purchased from Sociedad Española de Desarrollos Químicos, S.A. (SEDQ, Barcelona, Spain). (Z)-9-tetradecenyl acetate (Z9-14:Ac) was purchased from Pherobank (Wageningen, the Netherlands). Compounds were found to be 96–99% chemically pure and >99% isomerically pure by GC analysis.

Field Tests

For the dose–response experiment, varying amounts (0, 1, 10, 50, and 100%) of the *S. nonagrioides* pheromone were added to the *O. nubilalis* pheromone (Table 1A). For the effect of the individual components of the *S. nonagrioides* pheromone, each one of them was added individually to the sex pheromone of *O. nubilalis* in the same proportion at which they occur in the *S. nonagrioides* blend (Table 1B). Trapping experiments were carried out in 2002 (dose–response and individual components) and 2003 (dose–response) in corn (*Zea mays*) fields in Lleida (Spain). There were four plots in 2002 (two per field) and in 2003 (two in one field and one each in two other fields). Each plot consisted of a linear arrangement of traps placed 20 m apart. Plots in the same field were ≥ 20 m apart, and fields were ≥ 2 km apart. Traps were hung 1.5 m from the ground on wood stakes between corn plants at the edge of the fields from June 7 to October 10, 2002, and from July 18 to September 9, 2003. Trap position within a plot was randomized at the start of the experiment. Each week,

Table 1 Quantity (micrograms) of *S. nonagrioides* sex pheromone components in *O. nubilalis* pheromone lures used in field tests to determine their antagonist effect on *O. nubilalis*

(A) Dose response

Compound	Treatment name ^a				
	On	1%Sn	10%Sn	50%Sn	100%Sn
Z11-14:Ac	97	97	97	97	97
E11-14:Ac	3	3	3	3	3
Z11-16:Ac	–	0.77	7.7	38.5	77
Z11-16:Ald	–	0.1	1	5	10
Z11-16:OH	–	0.08	0.8	4	8
12:Ac	–	0.05	0.5	2.5	5

(B) Individual compounds

Compound	Treatment name					
	On	Ac	Ald	OH	12	Sn
Z11-14:Ac	97	97	97	97	97	97
E11-14:Ac	3	3	3	3	3	3
Z11-16:Ac	–	77	–	–	–	77
Z11-16:Ald	–	–	10	–	–	10
Z11-16:OH	–	–	–	8	–	8
12:Ac	–	–	–	–	5	5

^a Septa were loaded with 100 µg of the pheromone of *O. nubilalis* (On) plus (A) the complete *S. nonagrioides* pheromone (Sn) in varying concentrations or (B) the individual components of the *S. nonagrioides* pheromone: Z11-16:Ac (Ac), Z11-16:Ald (Ald), Z11-16:OH (OH) and 12:Ac (12). 100%Sn and Sn have the same composition. Septa loaded just with hexane served as negative controls.

counts were taken and traps were rotated to the contiguous position to avoid position effects, but were not re-randomized. Septa were replaced with new ones in week 14 in 2002, but were not replaced in 2003.

Rubber septa (Sigma-Aldrich Química) were rinsed with hexane for 1 hr, let dry overnight, and stored until used. Septa were pierced with an acetone-rinsed 5-cm-long wire. Each septum received 50 µl of the treatment solutions after which it was let dry for 30 min in a laboratory hood. Each septum was wrapped in aluminum foil and septa from the same treatment were placed in the same ziplock plastic bags and put in a cooler with ice. In the field, septa were taped by the wire to the underside of lids of green, white, and yellow universal moth traps (Biagro, Valencia, Spain). A different pair of rubber gloves was used to handle each treatment septa. A piece of plastic material loaded with insecticide (Dichlorvos[®]) was placed in the bottom of each trap to kill the trapped males.

The data were analyzed with ANOVA after being transformed ($\sqrt{x+1}$). For the test of concentrations, the terms in the model were lure, year, and lure × year. For the test of the effect of individual compounds, lure was the only term in the model. Differences among means were analyzed with Tukey's means separation test. In this and all other tests, except for those in the wind tunnel, the analyses were performed with the SAS statistical package (SAS Institute, 2000).

Wind Tunnel

The wind tunnel was a $2 \times 0.5 \times 0.5$ m ($l \times w \times h$) aluminum frame with glass walls on all four sides. A fan blowing air into the tunnel was placed in one of the two open ends (upwind end), and an extractor was connected to the other end (downwind end). Mosquito screen sealed both ends to prevent moths from escaping. One of the lateral glass walls had two sliding doors to access the inside of the tunnel. Regulating the speed of fan and extractor created a laminar flow inside of the tunnel of 0.3 m/sec throughout. Incoming air passed through a 1-cm-thick glass wool filter before entering into the tunnel. The glass bottom of the cage rested on black cardboard. Illumination, which was kept at the minimum necessary to see the moths, was provided by a 36-W fluorescent red light placed in the ceiling of the room and covered with a white cloth so that its intensity was 1.80 and 1.48 lx at the top and bottom of the tunnel, respectively. Temperature in the wind tunnel room was maintained at $24 \pm 1^\circ\text{C}$ by means of a heating/cooling unit, whereas relative humidity was ambient. A hood extracted air continuously, thus minimizing contamination of the room air with pheromone.

Treatment blends (Table 2) were loaded onto hexane-rinsed rubber septa, the solvent was allowed to evaporate, and the septa were kept individually in hexane-rinsed vials and stored at -20°C until used. At least 8 hr before the scotophase of observation, 3-d-old males were placed in individual 3×6 cm ($w \times h$) metal mosquito-screen cages and provided with 10% sugar water on a cotton pad. They were brought to the darkened wind tunnel room at least 15 min before the test started. Male response was observed between the third and the fifth hour of the scotophase. At the time of the test, a treatment septum was placed on a 10×10 cm galvanized metal top plate of a 16×6 cm ($h \times \text{diam}$) 1-cm-grid galvanized-wire platform that was centered on the floor at the upwind end of the tunnel. The caged males were placed individually on a similar metal platform 1.4 m downwind from the pheromone source. Each treatment blend was presented consecutively to two to three individual males before changing to a new treatment. Forty males were tested

Table 2 Quantity (micrograms) of *S. nonagrioides* sex pheromone components in *O. nubilalis* pheromone lures used in wind tunnel tests to determine their antagonist effect on *O. nubilalis*

Compound	Treatment name ^a									
	On	Ac	OH	12	0.1% Ald	0.3% Ald	1% Ald	3% Ald	10% Ald	Sn
Z11-14:Ac	38.8	38.8	38.8	38.8	38.8	38.8	38.8	38.8	38.8	38.8
E11-14:Ac	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Z11-16:Ac	–	30.8	–	–	–	–	–	–	–	30.8
Z11-16:Ald	–	–	–	–	0.048	0.144	0.48	1.44	4.8	4
Z11-16:OH	–	–	3.2	–	–	–	–	–	–	3.2
12:Ac	–	–	–	2	–	–	–	–	–	2

^aSepta were loaded with 40 μg of the *O. nubilalis* pheromone alone (On), with individual components of the *S. nonagrioides*: pheromone [Z11-16: Ac (Ac), Z11-16:Ald (Ald), Z11-16:OH (OH) and 12:Ac (12)], or with the sex pheromones of *S. nonagrioides* (Sn).

for each treatment and each male was tested only once. The treatment septa were replaced for new ones after 20 insects were tested.

After release, male behavior was observed for 2 min. Behaviors recorded included oriented flight (upwind zigzagging flight on the pheromone plume), contacting the pheromone source, and attempting copulation with the pheromone dispenser. Ryan's multiple range test for proportions (Ryan, 1960) was used to compare the treatments within each response category.

Electrophysiology

Males (2- to 4-d-old) were taken out of the rearing chamber during the scotophase shortly before being tested. They were immobilized with an insect pin and one of their antennae was detached with fine forceps. The proximal end of the antenna was placed in the narrow end of a Pasteur pipette filled with saline solution (0.2 M KCl). Several terminal segments of the distal end of the antenna were excised, and the blunt end was placed in a second capillary. Gold wires, 1-mm diam, connected the saline-filled capillaries to an electroantennogram (EAG) probe/preamplifier (PR-05, Syntech, Hilversum, the Netherlands) and IDAC-02 (Syntech) signal interface box. The antennal preparation (inside a steel mesh screen wired to the ground socket of an electric outlet) was introduced into a 7-mm-diam Teflon[®] tube, which carried humidified room air continuously at 1 l/min. Puffs of test samples were delivered through a hole in the tube with an air compressor that produced a flow of 0.3 l/min. The samples (10 μ l) were loaded onto hexane-rinsed and twice-folded rectangular filter paper pieces (1.5 cm²) placed into the wide section of a Pasteur pipette. The electric signal generated by the antenna was acquired in a PC that was equipped with a data acquisition board (Syntech) and EAG 2000 (Syntech) data analysis software.

EAG Calibration Curve

The calibration curve started with the blank (hexane), followed by the positive control (1 μ g pheromone of *O. nubilalis*). Then 0.1 μ g of each of the pheromone components of *S. nonagrioides* and *O. nubilalis*, followed by 1 and 10 μ g of the same components. Solvent and positive control were puffed every 3-4 test samples and at the end of the run. The order of the treatments was randomized between antennae. Each sample was puffed three times (puff duration ca 0.25 sec), and their average constituted the experimental unit. The Teflon[®] tube was rinsed with acetone at the end of each day. The experiment was replicated on 20 antennae using two different pieces of filter paper with a given test sample. Pipettes were stored at -20°C between uses. To control for variation among antennae, the average amplitude of each set of three EAG responses was divided by the average EAG amplitude of the nearest set of three puffs of the positive control. Comparison among treatments was performed on transformed data ($\sqrt{x+1}$) using a general linear model (GLM) with compound, concentration, and compound \times concentration as the terms of the model. Following GLM, a least square means analysis and the slice option (SAS Institute, 2000) were used to determine the significant differences between the means of all the treatment combinations.

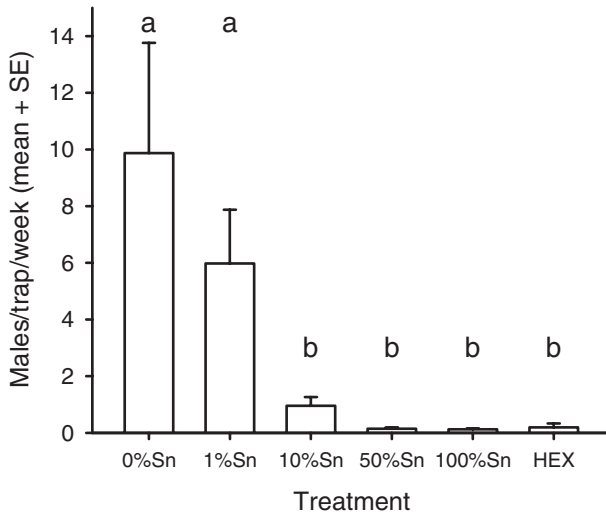


Fig. 1 The number of *O. nubilalis* males captured in traps baited with 100 µg of its own pheromone alone (0%Sn) or with increasing percentages of the *S. nonagrioides* pheromone (1%Sn, 10%Sn, 50%Sn, and 100%Sn). Control traps (HEX) were loaded with hexane. Different letters indicate differences among treatments after Tukey’s mean separation test ($P < 0.05$)

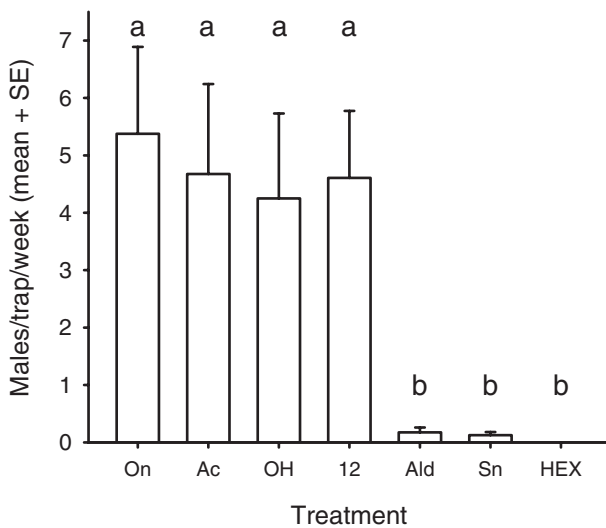


Fig. 2 The number of *O. nubilalis* males captured in traps baited with 100 µg of its own pheromone alone (On), its own pheromone plus individual components [Z11-16:Ac (Ac), Z11-16:OH (OH), 12:Ac (12), and Z11-16:Ald (Ald)] or the complete pheromone blend of *S. nonagrioides* (Sn). Control traps (HEX) were loaded with hexane. Different letters indicate differences among treatments after Tukey’s mean separation test ($P < 0.05$)

Cross-Adaptation EAG

The experiment consisted of two parts. In the first, a 1.5-cm long, 5-mm internal diam section of Teflon[®] tube containing a piece of filter paper loaded with 10 μ l of hexane was introduced into the humidified airstream passing over the antenna, upstream from the point where the puff would be applied. The response of a fresh antenna to the pheromone components of *O. nubilalis*, Z11-14:Ac and E11-14:Ac, a known antagonist (Z9-14:Ac) (Glover et al., 1989), Z11-16:Ald, and hexane was recorded by applying three puffs 20 sec apart of each treatment, with 1 min between treatments. In the second part of the experiment, the short section of tube containing the hexane filter paper was replaced by a new one containing one of the adapting stimuli. At 2 to 3 min after the adapting stimulus was introduced, the response of the same antenna to the same sample puffs was measured again, maintaining the same treatment order as before. The antenna was then discarded. Each antenna was considered a replicate of an adaptation treatment. The order of the puff treatments for each replicate was randomized among antennae. The section of the pipette exposed to the adapting stimulus was rinsed with acetone after each set of three puffs. We used concentrations of adapting odors that, as determined in preliminary tests, caused some adaptation but did not impair antennal response (0.01 μ g Z11-14:Ac, 0.1 μ g E11-14:Ac, 0.1 μ g Z9-14:Ac, 1 μ g Z11-16:Ald), and concentrations of test stimuli that were 10 times higher to obtain discernible EAG responses in the adaptation conditions. To estimate the amount of adaptation the mean of the three puffs of a given treatment was divided by the mean of the three puffs of hexane. This normalized EAG response after adaptation was divided by the equivalent response before adaptation (= adaptation index). The lower the index value, the higher the effect of the adapting stimulus. Ideally, a value of 1 means no adaptation. The data were transformed [$\log(x + 1)$] and analyzed with ANOVA followed by a planned means comparison test (Tukey's) ($N = 18$).

Results

Field Tests

Year had no effect on the captures ($df = 1, 47, F = 3.20, P = 0.083$) and there was no significant lure \times year interaction ($df = 5, 47, F = 1.07, P = 0.394$) in the data that were pooled (Fig. 1). Captures of *O. nubilalis* in traps loaded with its pheromone were reduced to a level similar to that of hexane traps when 10% or more of the complete *S. nonagrioides* pheromone was added to the blend ($df = 5, 47, F = 15.38, P < 0.001$). The only pheromone component of *S. nonagrioides* that decreased significantly the captures of *O. nubilalis* in the field, to a level comparable to the hexane traps, was Z11-16:Ald ($df = 6, 27, F = 18.94, P < 0.01$) (Fig. 2).

Wind Tunnel Tests

As in the field test, Z11-16:Ald (at 1% or more) was the only pheromone component of *S. nonagrioides* that caused a significant reduction in the percentage of *O. nubilalis* orientating to its own pheromone ($P < 0.05$) (Fig. 3). All the pheromone components of *S. nonagrioides*, at the same concentration that they

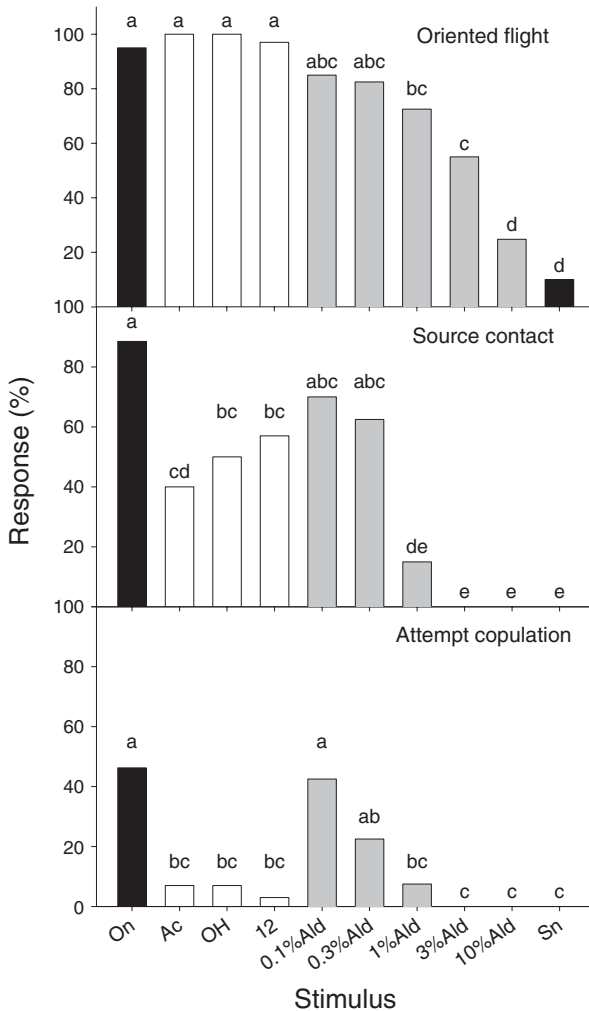


Fig. 3 The different behavioral responses (oriented flight, contact, and attempted copulation with the pheromone source) of *O. nubilalis* males in the wind tunnel to its own pheromone alone (On), its own pheromone plus individual components [Z11-16:Ac (Ac), Z11-16:OH (OH), 12:Ac (12), and Z11-16:Ald (Ald)] or the complete pheromone blend of *S. nonagrioides* (Sn). For Z11-16:Ald, five concentrations (0.1–10%) were used. Different letters indicate significant differences among treatments within each behavioral category (Ryan’s test, $P < 0.05$)

occur in the blend, caused reduction in the percentage of males that contacted ($P < 0.05$) or attempted copulation ($P < 0.05$) with the lure. As with oriented flight a minimum of 1% of Z11-16:Ald was necessary to reduce both contact and attempted copulation with the septum.

EAG Calibration Curve

Both concentration and compound had an effect on EAG peak amplitude ($df = 2, 359, F = 252.45, P < 0.01$, and $df = 5, 359, F = 143.27, P < 0.01$, respectively). The

Table 3 Mean (\pm SE) and means separation of EAG responses of *O. nubilalis* male antennae to three concentrations of its own pheromone components and those of *S. nonagrioides*

Compound ^a	Dose ^b		
	0.1 μ g	1 μ g	10 μ g
Z11-14:Ac	0.45 \pm 0.02 aC	0.82 \pm 0.03 aB	1.19 \pm 0.09 aA
E11-14:Ac	0.47 \pm 0.02 aB	0.68 \pm 0.02 aA	0.82 \pm 0.02 bA
Z11-16:Ac	0.28 \pm 0.02 bB	0.34 \pm 0.02 bB	0.57 \pm 0.03 cA
Z11-16:Ald	0.32 \pm 0.02 abB	0.35 \pm 0.02 bB	0.61 \pm 0.03 cA
Z11-16:OH	0.24 \pm 0.02 b	0.24 \pm 0.02 b	0.34 \pm 0.02 d
12:Ac	0.25 \pm 0.02 bB	0.39 \pm 0.02 bB	0.69 \pm 0.02 bcA

^aDifferent small letters in a column indicate significant differences among compounds.

^bDifferent capital letters within a line indicate differences among concentrations. Mean separation by least square analysis after ANOVA ($P < 0.01$).

interaction was significant ($df = 10, 359, F = 11.35, P < 0.01$) due to the much higher response of some of the treatments at the highest concentration (Table 3). The highest EAG responses were to the main components of the pheromone, followed by 12:Ac and Z11-16:Ald, depending on the concentration. At the lowest 0.1 μ g

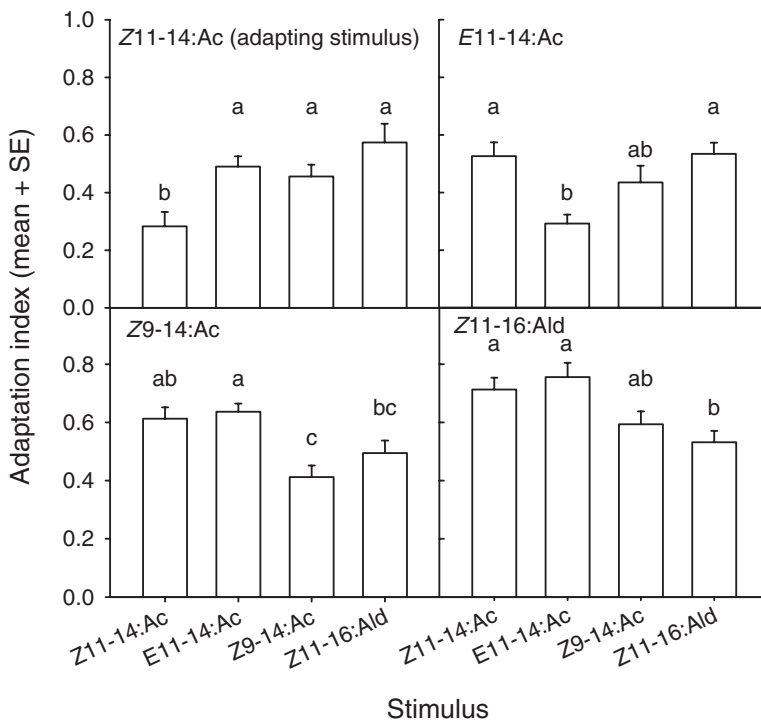


Fig. 4 Effect of adaptation of *O. nubilalis* male antennae on its response to conspecific pheromone components and behavioral antagonists. The adaptation index reflects the decrease of the antennal response after it has been exposed to the adapting stimulus, shown on the top of each graph. Different letters within each graph indicate differences among adaptation indexes using Tukey's mean separation test ($P < 0.05$)

pheromone concentration the difference among treatments was not as marked as at the 10 μg concentration. The lowest response was obtained with Z11-16:OH at any concentration.

Cross-Adaptation EAG

Exposing the antenna to a continuous flow of a pheromone compound resulted in a significant lower response to a puff of this compound than to a puff of most of the other components (Fig. 4). Z11-14:Ac adapted the antenna more to itself than any other compound ($df = 3$, $F = 6.71$, $P < 0.001$), whereas E11-14:Ac ($df = 3$, $F = 6.73$, $P < 0.001$), Z9-14:Ac ($df = 3$, $F = 7.89$, $P < 0.001$), and Z11-16:Ald ($df = 3$, $F = 5.63$, $P < 0.001$) caused greater adaptation to themselves than to some of the other pheromone components (Table 3).

Discussion

Z11-16:Ald is the only pheromone component of *S. nonagrioides* that reduces the attraction of *O. nubilalis* to its own pheromone. The antagonism in the field probably occurs at long range, as Z11-16:Ald was the only pheromone component of *S. nonagrioides* that reduced both oriented flight in the wind tunnel and trap captures in the field. However, the other *S. nonagrioides* components reduced source contact in the wind tunnel, although less strongly than Z11-16:Ald. The small antagonistic effect of these compounds could explain why the 1% Z11-16:Ald field blend (treatment 10%Sn), which included them, reduced trap captures by 90%, whereas the 1% Z11-16:Ald tunnel blend (treatment 1% Ald), which did not include them, reduced oriented flight only by 26%. This finding explains why mating disruption against *S. nonagrioides* using different blends of its sex pheromone components caused a reduction in the population of *O. nubilalis* only when Z11-16:Ald was present in the blend (Eizaguirre et al., 2002).

In addition to Z11-16:Ald several other compounds are known to antagonize the response of *O. nubilalis*. For instance, the minor component of the Z strain, E11-14:Ac, when present in a larger than natural proportion, and (*E*)-9-tetradecenyl acetate (E9-14:Ac) act as antagonists (Klun and Robinson, 1971; Klun et al., 1979). A 0.1% mixture of (*Z*)-9-dodecenyl acetate (Z9-12:Ac) or Z9-14:Ac added to a 100:3 blend of Z11-14:Ac and E11-14:Ac reduces captures of *O. nubilalis* in the field by 50% (Struble et al., 1987). Further tests with these two compounds and their *E* isomers in the wind tunnel showed that just 1% of any of them in the blend reduced oriented flight from 97 to 26–42% compared to the pheromone alone, and contact with the source from 95 to 2–61% (Glover et al., 1989). The strongest antagonist in these studies was Z9-14:Ac, and its antagonistic strength, both in the field (Struble et al., 1987) and in the wind tunnel (Glover et al., 1989), was similar to the level we have found for Z11-16:Ald.

Single-sensillum recording (SSR) has revealed three types of pheromone receptor neurons in the antenna of *O. nubilalis* males, two that respond, respectively, to the pheromone components Z11-14:Ac and E11-14:Ac, and a third type that responds to the behavioral antagonist Z9-14:Ac (Hansson et al., 1987). Our cross-adaptation EAG test showed that each of these compounds really only adapted the antenna to that specific compound, validating the method, which has been used in previous

insect pheromone studies (Gemeno et al., 2003). The new antagonist, Z11-16:Ald, one of the minor pheromone components of *S. nonagrioides*, also caused a significantly higher adaptation to this compound than to the pheromone components of *O. nubilalis*, suggesting that Z11-16:Ald is perceived by a specific odor receptor neuron that is different from those tuned to the pheromone components of *O. nubilalis*. There also was moderate cross-adaptation between the two antagonists, Z11-16:Ald and Z9-14:Ac, which suggests both might be detected by the same olfactory receptor neuron. However, this seems unlikely given that, with some exceptions, pheromone receptor neurons are narrow in their specificity of response (Todd and Baker, 1999), and these two compounds have different functional groups. Clearly, in order to resolve this question additional SSR tests need to be performed.

Although there are many examples of pheromone antagonism among pairs or groups of species, the adaptive significance has only been demonstrated in a few cases. For example, *H. zea* males have neurons that respond to Z11-16:Ald and Z9-16:Ald that are produced by conspecific females, as well as one attuned to Z9-14:Ald, a compound that is produced by *H. virescens*. Z9-14:Ald acts as an antagonist to *H. zea*, which benefits both species as cross-attraction and mating is possible, given they both have Z11-16:Ald as the major compound in their respective pheromone blends (reviewed by Mustaparta, 1997). Similar cases of adaptive antagonism have been described in other moths (Cardé and Haynes, 2004) and beetles (Leal, 1996; Borden, 1997).

Although there are some exceptions (Potting et al., 1999), many examples of interspecific antagonism among unrelated species have not come from research specifically studying this phenomenon, but rather from mixing pheromone blends for other purposes (Lopez et al., 1990; Haynes et al., 2002) or from mating disruption tests (Eizaguirre et al., 2002). Despite using the same host plant and having some seasonal overlap, there is little apparent selection pressure for the evolution of behavioral antagonism between *O. nubilalis* and *S. nonagrioides*, as they belong to different families, differ in size, do not share pheromone compounds, and mate at different times of the night. Consequently, an adaptive explanation for the existence of this antagonism may be the result of other species with which they both interact. In the case of *S. nonagrioides*, there is a sympatric sister species, *S. cretica*, whose pheromone consists of Z9-14:Ac and Z9-14:OH, and the former is a behavioral antagonist for *S. nonagrioides* (G. Rotundo, personal communication). Additional research looking at a number of different species will have to be examined in order to rigorously test this hypothesis.

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Isolation, Identification, Synthesis, and Field Evaluation of the Sex Pheromone of the Brazilian Population of *Spodoptera frugiperda*

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Abstract Several studies have shown intraspecific geographical variation in the composition of sex pheromones. Pheromone lures from North America and Europe were not effective against the fall armyworm *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae) in Brazil, so we examined the composition of the sex pheromone produced by females from Brazilian populations. Virgin female gland extracts contained (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac), (*E*)-7-dodecenyl acetate (*E*7-12:Ac), dodecyl acetate, (*Z*)-9-dodecenyl acetate, (*Z*)-9-tetradecenyl acetate (*Z*9-14:Ac), (*Z*)-10-tetradecenyl acetate, tetradecyl acetate/(*Z*)-11-tetradecenyl acetate (*Z*11-16:Ac), and (*Z*)-11-hexadecenyl acetate. The relative proportions of each acetate were 0.8:1.2:0.6:traces:82.8:0.3:1.5:12.9, respectively. This is the first time that *E*7-12:Ac has been reported from the pheromone gland of *S. frugiperda*. Only three compounds, *Z*9-14:Ac, *Z*7-12:Ac, and *E*7-12:Ac, elicited antennal responses, and there were no differences in catch between traps baited with either *Z*7-12:Ac + *Z*9-14:Ac or *Z*7-12:Ac + *Z*9-14:Ac + *Z*11-16:Ac blends. However, the *Z*7-12:Ac + *Z*9-14:Ac + *E*7-12:Ac blend was significantly better than *Z*7-12:Ac + *Z*9-14:Ac, indicating that *E*7-12:Ac is an active component in the sex pheromone of the Brazilian populations of *S. frugiperda*.

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Introduction

The fall armyworm *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) is an important economic pest of maize and other grass crops in North America, Central America, and parts of South America (Andrews, 1988). In Brazil, this species causes substantial damage in various crops, such as sugarcane and sorghum, and is the most important pest in maize causing yield reduction up to 34% (Cruz et al., 1996). At present, despite the negative environmental impact, as well as negative human and animal health effects, pesticides are the preferred means of controlling this pest. However, success is limited because of the location of larvae when feeding on the host plant. More effective and ecologically acceptable means of control are required.

The sex pheromone of *S. frugiperda* has been studied extensively with (Z)-9-tetradecenyl acetate (Z9-14:Ac) being the first pheromone component reported (Sekul and Sparks, 1967), whereas later studies have reported additional components (Jones and Sparks, 1979; Mitchell et al., 1985; Tumlinson et al., 1986; Descoins et al., 1988). However, blends that proved successful in trapping fall armyworm males in North America and Europe performed poorly when tested in Brazil (Cruz et al., unpublished results), Costa Rica (Andrade et al., 2000), and Mexico (Malo et al., 2001).

Moth sex pheromones are species-specific communication systems for mate location and would be under intense stabilizing selection when selection pressures are similar for all populations (Hansson et al., 1990). However, significant intraspecific geographic variation in the pheromone composition of insects, especially moths, has been reported (Anglade et al., 1984; Löfstedt et al., 1986; Hansson et al., 1990; Löfstedt, 1990; Tóth et al., 1992; Miller et al., 1997; Andrade et al., 2000; El-Sayed et al., 2003). These are probably the result of different selective pressures acting on allopatric populations of the same species. Thus, the failure to effectively capture Brazilian fall armyworm males with European and North American lures may be caused by geographic variability in the sex pheromone. Furthermore, in the case of *S. frugiperda*, there are at least two morphologically indistinguishable host-plant-specific strains (Pashley et al., 1985, 1992; Pashley, 1986; Levy et al., 2002) that are also present in Brazil (Busato et al., 2002). Therefore, we undertook a study of the sex pheromone of Brazilian *S. frugiperda*, in both laboratory and field experiments, and developed a short and efficient synthetic route for the preparation of the pheromone components.

Methods and Materials

Insect Rearing

The colony of *S. frugiperda* was established at the Insect Bioassay Laboratory of the Universidade Federal de São Carlos, Brazil, using pupae collected in maize plantations at Piracicaba, in São Paulo State, Brazil. All specimens were from the second

generation that had been reared on a pinto bean-based artificial diet (Parra, 1986). The pupae were sexed, placed in individual plastic vials (6 × 6 cm diam), and then held in an incubation chamber under a reversed 12:12 hr light/dark photoperiodic cycle, at 25 ± 1°C, 70 ± 5% relative humidity, until the adults emerged.

Pheromone Extraction

During peak calling activity, about the 4th hour of the second scotophase (Tumlinson et al., 1986), the abdominal tips, with pheromone glands, were excised from virgin females after emergence and extracted for 24 hr in hexane. The supernatant was collected and stored in dark microvials with Teflon[®]-lined screw caps at –20°C until needed. Five extracts of 24, 30, 30, 31, and 36 pheromone glands were prepared.

Chromatographic Analysis

Gland extracts and synthetic solutions were analyzed on a Shimadzu 17-A chromatograph, equipped with a DB-1 column (30 m × 0.25 mm, i.d. 0.25 μm; J&W Scientific) coupled to Shimadzu QP 5000 mass spectrometer using helium as carrier gas. Split (synthetic standards) and splitless (gland extracts) injections of 2 μl of the sample and standard solution [(Z)-7-dodecenyl acetate (Z7-12:Ac), (E)-7-dodecenyl acetate (E7-12:Ac), dodecyl acetate (12:Ac), Z9-14:Ac, and (Z)-11-hexadecenyl acetate (Z11-16:Ac)] were performed at 250°C. The initial oven temperature was 50°C for 1 min, then increased to 230°C at a rate of 5°C/min, and finally to 280°C at a rate of 25°C/min. The final temperature was retained for 21 min. Electron impact mass spectra were monitored at 70 eV in the *m/z* range of 33–250.

The geometric isomers were separated on a Shimadzu 17-A chromatograph with a flame ionization detector (FID) equipped with a 30 × 0.25 mm, i.d. 0.25 μm DB-5 column (J&W Science) using H₂ as carrier gas. The oven temperature program was 50°C for 1 min up to 145°C at a rate of 3°C/min, then to 230°C at 5°C/min, and finally to 280°C at 30°C/min, where it was maintained for 21 min. The coinjection (isothermal analyses at 100°C) was carried out by injecting simultaneously 2 μl of the gland extract and 1 μl of a diluted standard solution containing E7-12:Ac and Z7-12:Ac.

Coupled Gas Chromatography–Electroantennographic Detection

Pheromone extracts were subjected to gas chromatography–electroantennographic detection (GC-EAD) analyses, using a Shimadzu 17-A gas chromatograph, equipped with a DB-5 column (30 m × 0.25 mm, i.d. 0.25 μm; J&W Scientific) and a Syntech electroantennography system (Hilversum, The Netherlands). The same chromatographic conditions as for the GC–mass spectrometry (MS) analyses were used for the chromatographic separation. The FID was kept at 280°C, whereas the temperature of the transfer capillary was maintained at 290°C to avoid condensation.

Electrophysiology

Electroantennogram (EAG) recordings of Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac alone and in various combinations were tested at 0.01, 0.10, 0.25, 0.50, 0.75, 1.00, 10.00, and 100.00 mg/ml, using antennae of 1- to 2-d-old *S. frugiperda* males. The

antenna was stimulated with 0.3-sec puffs of purified and humidified air (1.2 l/min) delivered through a Pasteur pipette, containing a filter paper strip (ca. 0.8 cm), impregnated with 5 μ l of the test solution. Puffs from a filter paper plus solvent were used as control stimulus. The test compounds (Z7-12:Ac, Z9-14:Ac, and Z11-16) alone were used at increasing concentrations, i.e., a range of doses starting from the lowest to the highest, and blends were applied randomly. All test compounds were applied at intervals of 30 sec. Hexane stimulation was made at the beginning and at the end of every series of EAG experiments. The Syntech EAG software calculated the normalized values automatically.

Synthesis of Pheromone Components

Unless otherwise noted, all commercially available reagents were purchased from Aldrich Chemical Co. and were purified, when necessary, according to the usual procedures described in the literature. The infrared (IR) spectra refer to films and were measured on a Bomem M102 spectrometer. ^1H and ^{13}C NMR spectra were recorded on both a Bruker ARX-200 (200 and 50 MHz, respectively) and a DRX-400 (400 and 100 MHz, respectively). Mass spectra were recorded on a Shimadzu GCMS-QP5000. Analytical thin-layer chromatography was performed on a 0.25- μm film of silica gel containing fluorescent indicator UV₂₅₄ (Sigma-Aldrich). Flash column chromatography was performed using silica gel (Kieselgel 60, 230–400 mesh, E. Merck). Gas chromatography was performed in a Shimadzu GC-17A with H_2 as carrier and using a DB-5 column.

Preparation of bromoalcohols 2: The diol (65 mmol), HBr 48% (8.2 ml, 78 mmol), and benzene (180 ml) were mixed and refluxed for 12 hr in a 250-ml flask with Dean-Stark and reflux condensers. Once the temperature reached rt, an ice bath was added to precipitate out the excess diol. The solid was filtered and washed with chilled benzene. The crude product was employed in the next step after concentration *in vacuo* without further purification.

1-Bromo-8-octanol (2a): 83% yield. IR (film): ν_{max} (cm^{-1}) = 3352, 2930, 2857, 1464, 1249, 1053. ^1H NMR (400 MHz, CDCl_3): δ (ppm) = 3.69 (t, 2H, J = 6.6 Hz), 3.41 (t, 2H, J = 6.8 Hz), 1.90 (quint, 2H, J = 8.0 Hz), 1.60 (quint, 2H, J = 6.6 Hz), 1.5–1.2 (m, 8H). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 25.51, 28.03, 28.70, 29.19, 32.76(2C), 34.02, 62.99. MS (70 eV): m/z (%) = 55 (100), 69, 83, 109, 111, 148, 150, 162, 164.

1-Bromo-10-decanol (2b): 78% yield. IR (film): ν_{max} (cm^{-1}) = 3367, 2927, 2856, 1460, 1366, 1245, 1041. ^1H NMR (200 MHz, CDCl_3): δ (ppm) = 3.63 (t, 2H, J = 6.4 Hz), 3.40 (t, 2H, J = 6.8 Hz), 1.85 (quint, 2H, J = 6.8 Hz), 1.50–1.70 (m, 2H), 1.20–1.50 (m, 12H). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 62.97, 34.00, 32.79 (2C), 29.47, 29.35 (2C), 28.71, 28.13, 25.70. MS (70 eV): m/z 55 (100%), 69, 83, 97, 135, 137, 148, 150, 162, 164.

1-Bromo-6-hexanol (2c): 75% yield after purification by column chromatography in silica gel and hexane: ethyl acetate 20:1 as eluent. IR (film): ν_{max} (cm^{-1}) = 3343, 2900, 1696, 1453, 1247, 1058. ^1H NMR (200 MHz, CDCl_3): δ (ppm) = 3.64 (t, 2H, J = 6.4 Hz), 3.42 (t, 2H, J = 6.6 Hz), 1.88 (quint, 2H, J = 6.6 Hz), 1.56 (quint, 2H, J = 6.7 Hz), 1.3–1.7 (m, 4H). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 62.17, 33.90, 32.65, 32.43, 27.86, 24.87. MS (70 eV): m/z 55 (100%), 67, 69, 83, 92, 94, 106, 108, 134, 136.

Alkylation of 1-hexyne with bromoalcohols **2**: At -20°C , 1-hexyne (1 mmol) was placed in a 25-ml flask containing THF (2 ml) under N_2 atmosphere, and then an *n*-BuLi solution in hexane (2.2 mmol) was added dropwise. The bath temperature was allowed to rise to 0°C over 30 min, then returned to -30°C , at which time a solution of the bromoalcohol **2** (1 mmol) in HMPA or DMI (2 ml) was added dropwise. Water was added, and the mixture was extracted with ethyl ether (3×15 ml). The organic layer was washed with an aqueous solution of HCl 10% (3×10 ml) and brine (3×10 ml), dried over NaSO_4 , and concentrated *in vacuo*. The crude product was purified by flash chromatography in silica gel with hexane/ethyl acetate 6:1 as an eluent (see Table 1).

9-Tetradecyn-1-ol (**3a**): IR (film): ν_{max} (cm^{-1}) = 3338, 2930, 2857, 1462, 1331, 1056. ^1H NMR (200 MHz, CDCl_3): δ (ppm) = 3.6 (t, 2H, $J = 6.7$ Hz), 2.52 (s, 1H), 2.13 (t, 4H, $J = 5.8$ Hz), 1.32–1.55 (m, 16H), 0.9 (t, 3H, $J = 7.1$ Hz). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 80.07 (2C), 62.68, 32.63, 31.19, 29.28, 29.06 (2C), 28.71, 25.66, 21.84, 18.65, 18.35, 13.53. MS (70 eV): m/z 54, 67, 81, 96 (100%), 110, 121, 135, 167.

11-Hexadecyn-1-ol (**3b**): IR (film): ν_{max} (cm^{-1}) = 3347, 2924, 2857, 1460, 1227, 1056, 745. ^1H NMR (200 MHz, CDCl_3): δ (ppm) = 3.6 (t, 2H, $J = 0.5$ Hz), 2.13 (t, 4H, $J = 6.0$ Hz), 1.29–1.55 (m, 20H), 0.90 (t, 3H, $J = 6.9$ Hz). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 80.15 (2C), 62.8, 32.68, 31.23, 29.35 (4C), 28.48, 25.90, 25.71, 21.88, 18.71, 18.40, 13.6. MS (70 eV): m/z 55 (100%), 69, 83, 102, 116, 123, 147, 152, 194.

7-Dodecyn-1-ol (**3c**): IR (film): ν_{max} (cm^{-1}) = 3376, 2932, 2863, 1455, 1364, 1225, 1053, 913, 733. ^1H NMR (200 MHz, CDCl_3): δ (ppm) = 3.53 (t, 2H, $J = 6.5$ Hz), 2.72 (s, 1H), 2.07 (t, 4H, $J = 6.7$ Hz), 1.28–1.52 (m, 12H), 0.9 (t, 3H, $J = 6.8$ Hz). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 80.15 (2C), 62.48, 32.50, 31.15, 29.00, 28.53, 25.23, 21.81, 18.57, 18.31, 13.49. MS (70 eV): m/z 54, 67(100%), 81, 93, 110, 121, 153.

Hydrogenation of alkynols **3**: Quinoline (0.6 ml) and Lindlar reagent (0.3 g) were added to a solution of alkynol **3** (14.3 mmol) in methanol (30 ml), and the resulting suspension was hydrogenated at rt under hydrogen atmosphere for 6 hr. The mixture was filtered, washed with ethyl ether (3×10 ml), and the filtrate was then washed with a 1 M aqueous solution of HCl (3×10 ml), saturated solution of CuSO_4 (3×10 ml), and brine (2×10 ml). The solvent was evaporated at reduced pressure to provide the desired product.

(*Z*)-9-Tetradecen-1-ol (**4a**): 85% yield. IR (film): ν_{max} (cm^{-1}) = 3338, 2926, 2857, 1459, 1046, 715. ^1H NMR (200 MHz, CDCl_3): δ (ppm) = 5.16–5.32 (m, 2H), 3.50 (t, 2H, $J = 6.5$ Hz), 2.85 (s, 1H), 1.8–2.0 (m, 4H), 1.45 (quint, 2H, $J = 6.3$ Hz), 1.1–1.3 (m, 14H), 0.89 (t, 3H, $J = 7.0$ Hz). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 129.7 (2C), 62.46, 31.84, 30.65, 29.63, 29.36 (2C), 29.13, 27.05, 26.77, 25.70, 22.20, 13.83. MS (70 eV): m/z 55(100%), 67, 82, 96, 109, 123, 138, 166, 194.

Table 1 Alkylation reaction of 1-hexyne with bromoalcohols **2** (Fig. 2) in different solvents^a

Entry	Bromoalcohol 2 (n)	Yield (%)	
		HMPA	DMI
1	5	90	87
2	7	90	91
3	9	83	88

^a Bromoalcohol **2** was added in dry HMPA or DMI and 1-hexyne in dry THF.

(*Z*)-11-Hexadecen-1-ol (**4b**) and (*Z*)-7-Dodecen-1-ol (**4c**) were obtained as described above and employed in the acetylation reaction without further purification or characterization.

(*E*)-7-Dodecen-1-ol: Small pieces of sodium metal (0.35 g, 15 mmol) were added to liquid ammonia (20 ml) held at -70°C . This was briskly stirred until all the sodium had dissolved to give a blue-colored solution, at which time a solution of alcohol **3c** (0.14 g, 0.75 mmol) in THF (0.5 ml) was added. The reaction was followed by GC analyses. The ammonia was allowed to evaporate, saturated ammonium chloride solution was cautiously added, and then the reaction mixture was extracted with ether (3×15 ml). The organic layer was washed with brine (30 ml), dried over MgSO_4 , filtered, and evaporated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate, 95:5), and 0.127 g of the (*E*)-7-dodecen-1-ol was obtained (90% yield). IR (film): ν_{max} (cm^{-1}) = 3320, 2920, 2860, 1465, 1040. ^1H NMR (200 MHz, CDCl_3): δ (ppm) = 5.28–5.47 (m, 2H), 3.64 (t, 2H, $J = 6.4$ Hz), 1.85–2.05 (m, 4H), 1.1–1.7 (m, 13H), 0.88 (t, 3H, $J = 6.6$ Hz). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 130.46, 130.13, 63.03, 32.75, 32.49, 32.25, 31.82, 29.57, 28.89, 25.59, 22.18, 13.94.

Acetylation of alcohols **4**: Alcohol **5** (7 mmol), acetic anhydride (2.0 ml, 21 mmol), pyridine (2.8 ml), and hexane (35 ml) were mixed in a 125-ml flask, stirred at rt for 8 hr, before adding ethyl ether (50 ml) and then washing the organic layer with a 1 M aqueous solution of HCl (3×50 ml) and 1 M NaOH (3×50 ml). The solvent was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel with hexane/ethyl acetate 9:1 as the eluent.

(*Z*)-9-Tetradecenyl acetate (**5a**): 90% yield, isomeric purity >99% by GC. IR (film): ν_{max} (cm^{-1}) = 3002, 2928, 2857, 1743, 1461, 1367, 1239, 1040, 721. ^1H NMR (200 MHz, CDCl_3): δ (ppm) = 5.26–5.42 (m, 2H), 4.05 (t, 2H, $J = 6.7$ Hz), 1.9–2.1 (m, 7H), 1.5–1.7 (m, 2H), 1.15–1.45 (m, 14H), 0.89 (t, 3H, $J = 7.0$ Hz). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 171.2, 129.8 (2C), 64.6, 31.9, 29.7, 29.4, 29.2 (2C), 28.6, 27.14, 26.9, 25.9, 22.32, 20.96, 13.96. MS (70 eV): m/z 55(100), 61, 67, 82, 96, 110, 124, 151, 194.

(*Z*)-11-Hexadecenyl acetate (**5b**): 91% yield in two steps, isomeric purity >99% by GC. IR (film): ν_{max} (cm^{-1}) = 3002, 2927, 2856, 1743, 1461, 1366, 1239, 1040, 721. ^1H NMR (200 MHz, CDCl_3): δ (ppm) = 5.25–5.50 (m, 2H), 4.04 (t, 2H, $J = 6.7$ Hz), 1.85–2.1 (m, 7H), 1.60 (quint, 2H, $J = 6.7$ Hz), 1.1–1.4 (m, 18H), 0.89 (t, 3H, $J = 6.9$ Hz). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 171.05, 129.77 (2C), 64.56, 31.93, 29.72, 29.48 (3C), 29.23 (2C), 28.58, 27.14, 26.87, 25.88, 22.30, 20.88, 13.93. MS (70 eV): m/z 55, 67, 82, 96 (100), 110, 124, 138, 152, 166, 180, 222.

(*Z*)-7-Dodecenyl acetate (**5c**): 85% yield in two steps, isomeric purity >99% by GC. IR (film): ν_{max} (cm^{-1}) = 3003, 2930, 2859, 1742, 1460, 1367, 1239, 1041, 725. ^1H NMR (200 MHz, CDCl_3): δ (ppm) = 5.23–5.4 (m, 2H), 4.05 (t, 2H, $J = 6.6$ Hz), 1.95–2.1 (m, 7H), 1.62 (quint, 2H, $J = 6.5$ Hz), 1.25–1.45 (m, 10H), 0.89 (t, 3H, $J = 6.8$ Hz). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 171.00, 129.92, 129.48, 64.48, 31.87, 29.52, 28.79, 28.52, 26.98, 26.83, 25.75, 22.25, 20.83, 13.87. MS (70 eV): m/z 55, 61, 67, 81 (100), 96, 123, 138, 152, 166.

(*E*)-7-Dodecenyl acetate: isomeric purity >99% by GC. IR (film): ν_{max} (cm^{-1}) = 3010, 2935, 2860, 1740, 1453, 1368, 1045, 730. ^1H NMR (200 MHz, CDCl_3): δ (ppm) = 5.15–5.40 (m, 2H), 4.05 (t, 2H, $J = 6.6$ Hz), 2.04 (s, 3H), 1.50–2.00 (m, 4H), 1.05–1.50 (m, 12H), 0.89 (t, 3H, $J = 6.8$ Hz). ^{13}C NMR (50 MHz, CDCl_3): δ (ppm) = 171.40,

130.52, 130.05, 64.61, 32.43, 32.23, 31.80, 29.45, 28.70, 28.57, 25.76, 22.16, 20.95, 13.89. MS (70 eV): m/z 55, 61, 67, 81(100), 96, 123, 138, 152, 166.

Field Experiments

All tests were conducted in Embrapa experimental maize plantations at Sete Lagoas, Minas Gerais State, Brazil. Pherocon 1C traps were suspended at the top of the plant canopy (100 cm above ground level) at 20-m intervals. In all trials, traps were emptied every second day. They were baited with red rubber septa (Aldrich Chemical Co.) impregnated with pheromone components in hexane. We ran the following trials to compare the efficacy of:

- (1) Z7-12:Ac + Z9-14 (0.01:1.00 mg) and Z7-12:Ac + Z9-14:Ac + Z11-16:Ac (0.01:1.00:0.10 mg). There were three replicates run over 26 d.
- (2) 10, 1, and 0.1 mg of Z7-12:Ac + Z9-14:Ac + Z11-16:Ac [at a 1:100:15 (m/m) ratio]. There were five replicates run over 24 d.
- (3) 10, 1, and 0.1 mg of Z7-12:Ac + Z9-14:Ac [at 1:100 (m/m) ratio]. There were five replicates run over 24 d.
- (4) Z7-12:Ac + Z9-14:Ac (0.01:1.00 mg), E7-12:Ac + Z9-14:Ac (0.01:1.00 mg), and Z7-12:Ac + E7-12:Ac + Z9-14:Ac (0.01:0.01:1.00 mg). There were 5 replicates run over 28 d.

In the last three trials, traps baited with either rubber septa impregnated with hexane or two virgin females, provided with 10% sucrose solution as food source, were used as controls. The treatments were replicated in a Latin square design (Perry et al., 1980).

Statistical Analysis

The mean values of the EAG responses calculated automatically by software EAG for Windows, as well as the data from the first field trial, were submitted to one-way analysis of variance (ANOVA) and compared using Tukey's test ($P < 0.05$). The last three field experiments were submitted to two-way ANOVA and compared using Tukey's test ($P < 0.05$).

Results

The virgin female gland extracts of Brazilian *S. frugiperda* showed seven peaks with spectral characteristics of long-chain acetates (Fig. 1). The two largest peaks, 4 and 7, were identified, based on mass spectral analysis, retention times compared with known synthetic standards, and index comparisons, as Z9-14:Ac and Z11-16:Ac, respectively. The mass spectrum of peak 5 indicated an acetate with one double bond based on the retention index data reported by Marques et al. (2000) and was identified as (Z)-10-tetradecenyl acetate (Z10-14:Ac). Mass spectrum of peak 6 showed characteristics of saturated and unsaturated long-chain acetates (abundance of the m/z 61, M-60), suggesting a coelution of (Z)-11-tetradecenyl acetate (Z11-14:Ac) and tetradecyl-1-ol acetate (14:Ac). This assumption was supported by the similarity of our results with the retention index data of these two compounds

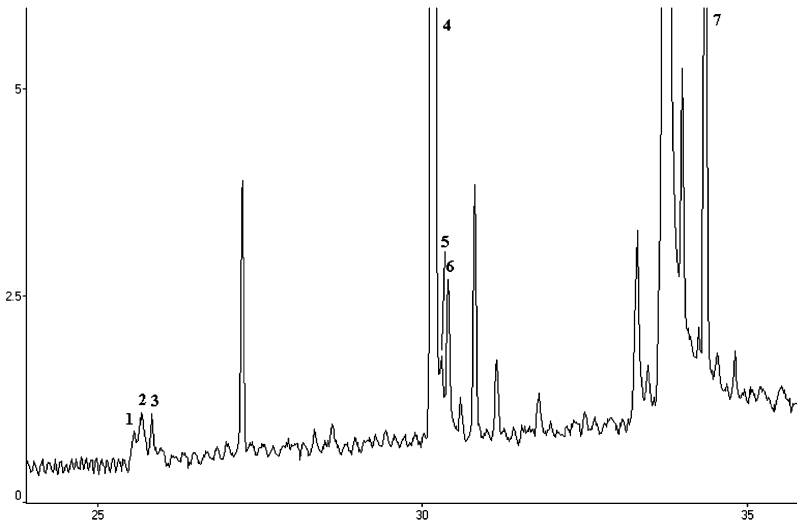


Fig. 1 Chromatogram of the gland extract of *Spodoptera frugiperda* virgin females, analyzed on a DB-1 column. Identification of peaks: (1) Z7-12:Ac, (2) E7-12:Ac, (3) 12:Ac, (4) Z9-14:Ac, (5) Z10-14:Ac, (6) 14:Ac/Z11-14:Ac, and (7) Z11-16:Ac. ($N = 30$)

reported by Marques et al. (2000). The last three acetates present in minor quantities were identified as Z7-12:Ac (peak 1), E7-12:Ac (peak 2), and 12:Ac (peak 3) using mass spectrum analysis and retention time comparisons and coinjection with the synthetic compounds (Fig. 2). While confirming the presence of these geometric isomers of the dodecanyl acetate by using a prolonged temperature program during the GC separation, we found another substance in trace quantities coeluting with Z9-12:Ac, but, given the small quantities, were unable to carry out any further analyses. The relative proportions of Z7-12:Ac, E7-12:Ac, 12:Ac, Z9-12:Ac, Z9-14:Ac, Z10-14:Ac, 14:Ac/Z11-14:Ac, and Z11-16:Ac in gland extracts of virgin females were 0.8:1.2:0.6:traces:82.8:0.3:1.5:12.9, respectively.

The straight chain (C_{10} – C_{16}) alcohols or acetates with a double bond that are found in many sex pheromones are typically prepared by a Wittig reaction or by alkylation of an acetylene with an alkyl halide followed by selective reduction. However, the Wittig reaction is not as stereoselective as the alkylation route,

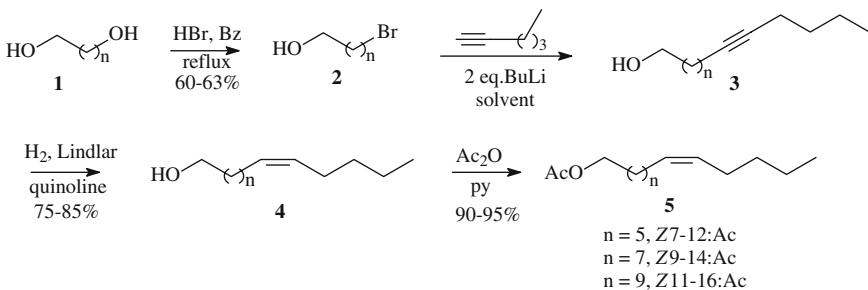


Fig. 2 Synthetic route for the preparation of the sex pheromone components of *S. frugiperda*

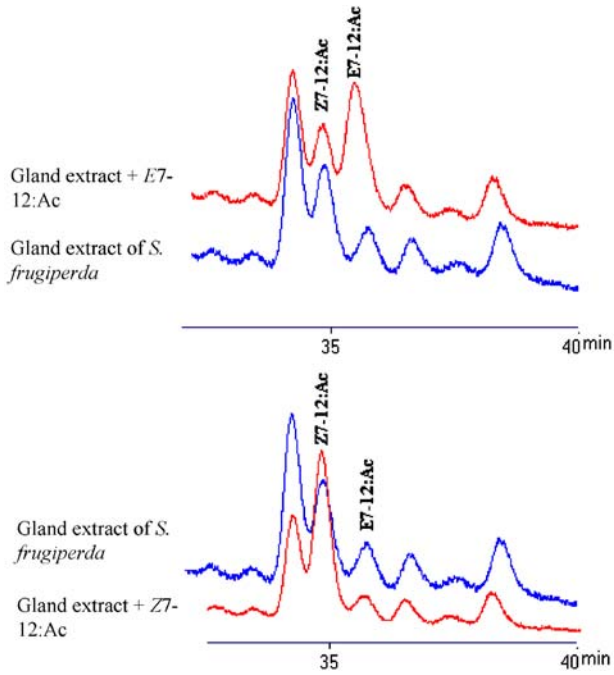


Fig. 3 Co-injection of gland extract of *S. frugiperda* virgin females and with standard solution containing *E7-12:Ac* and *Z7-12:Ac* analyzed on a DB-1 column (30 × 0.25 mm; 0.25 μm under isothermal conditions at 100°C)

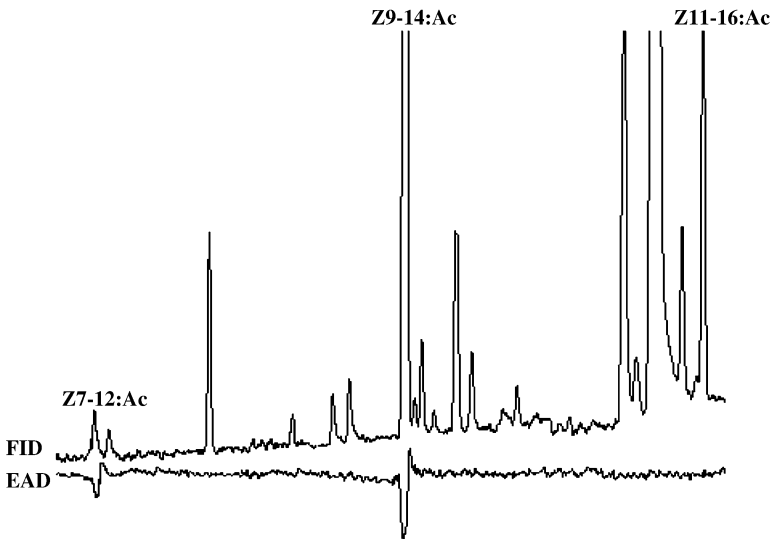


Fig. 4 Coupled gas chromatogram–electroantennogram (GC-EAD) of a male *S. frugiperda* antenna, stimulated by a gland extract of virgin female *S. frugiperda* ($N = 36$)

whereas the alkylation route usually includes a protection of the bromoalcohol as tetrahydropyranyl ether resulting in a decreased overall yield.

Mitra and Reddy (1989) synthesized Z9-14:Ac in ten steps starting from an alkylation of the dianion of 4-butyne-1-ol, prepared with two equivalents of n-BuLi in THF-HMPA, with 1-bromopentane that gave a 60% yield of 3-octyn-1ol. Lo and Chao (1990) substituted HMPA, which is quite toxic, for DMI as solvent in the alkylation step of ω -tetrahydropyranyloxy-1-alkynes with bromoalkanes in the synthesis of Z9-14:Ac and Z11-16:Ac.

We present an alternate four-step synthetic method for this type of compound employing alkylation of alkynes with bromoalcohols in HMPA at -30°C . We also investigated DMI as a solvent, and the alkylation occurred equally well (Fig. 3 and Table 1). Thus, using this methodology, Z9-14:Ac, Z7-12:Ac, and Z11-16:Ac, pheromone components of *S. frugiperda*, have been efficiently prepared.

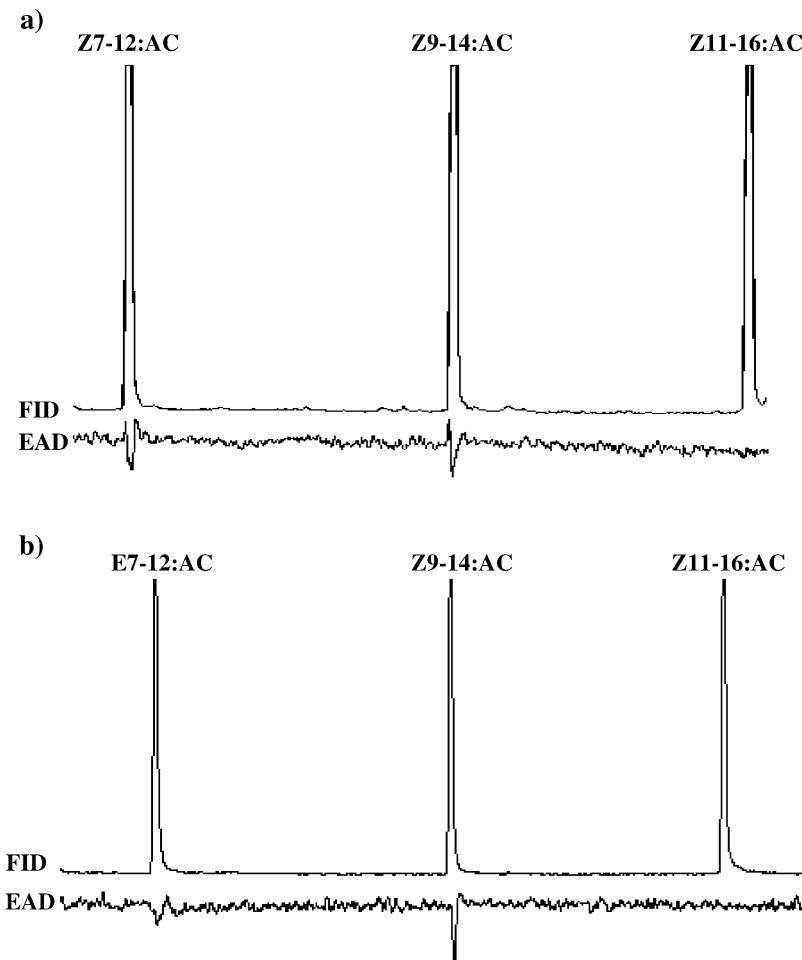


Fig. 5 Coupled GC-EAD of a male *S. frugiperda* antenna, stimulated by a mixture of synthetic pheromone blends: (a) Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac; (b) E7-12:Ac, Z9-14:Ac, and Z11-16:Ac

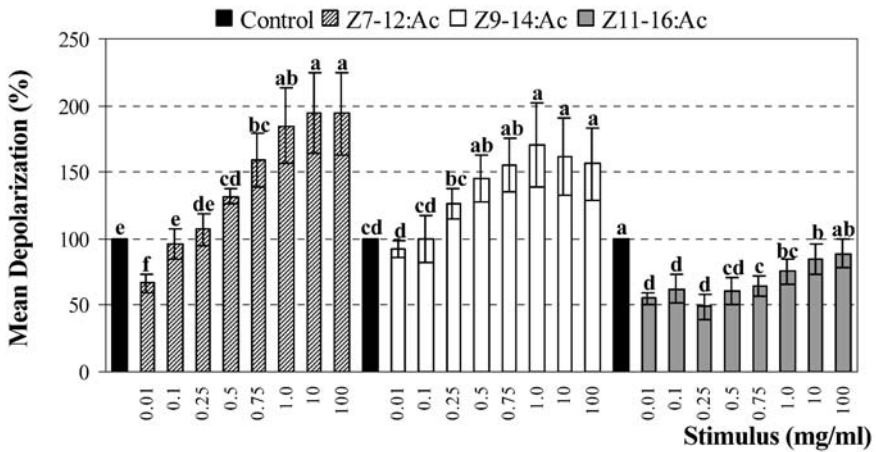


Fig. 6 Mean values (\pm SD) of EAG responses of *S. frugiperda* males to individual compounds Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac in different concentrations (0.01, 0.1, 0.25, 0.5, 0.75, 1.0, 10.0, and 100.0 mg/ml) and control (hexane). For any given compound, the mean values with the same letter are not significantly different at $P < 0.05$ based on Tukey’s test ($N = 10$)

The strongest EAD response from the pheromone gland extract was elicited by the major sex pheromone compound Z9-14:Ac (Fig. 4). We were unable to separate the geometric isomers Z7-12:Ac and E7-12:Ac, so the second peak (Fig. 4) could be the sum of the depolarizations from both compounds, as the two induced responses when antennae were stimulated by mixtures of synthetic Z7-12:Ac, Z9-14:Ac, and Z11-16:Ac (Fig. 5a) and E7-12:Ac, Z9-14, and Z11-16:Ac (Fig. 5b).

Over the range of concentrations from 0.01 to 100 mg/ml, Z11-16:Ac never showed greater electrophysiological activity than hexane, whereas Z7-12:Ac and Z9-14:Ac gave significantly higher responses than controls at concentrations of 0.5 mg/ml and above (Fig. 6).

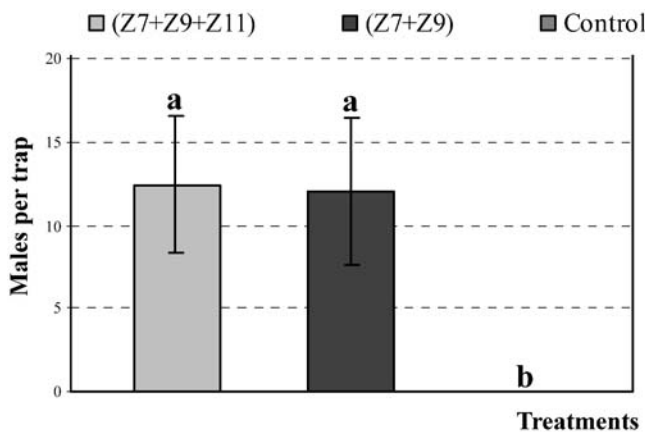


Fig. 7 Number ($X \pm$ SD) of *S. frugiperda* males captured in Pherocon 1C traps baited with Z7-12:Ac + Z9-14:Ac (0.01:1.00 mg), Z7-12:Ac + Z9-14:Ac + Z11-16:Ac (0.01:1.00:0.10 mg), or hexane solvent (three replicates and 13 collections). Mean values with the same letter are not significantly different (one-way ANOVA followed by Tukey’s test; $P < 0.05$)

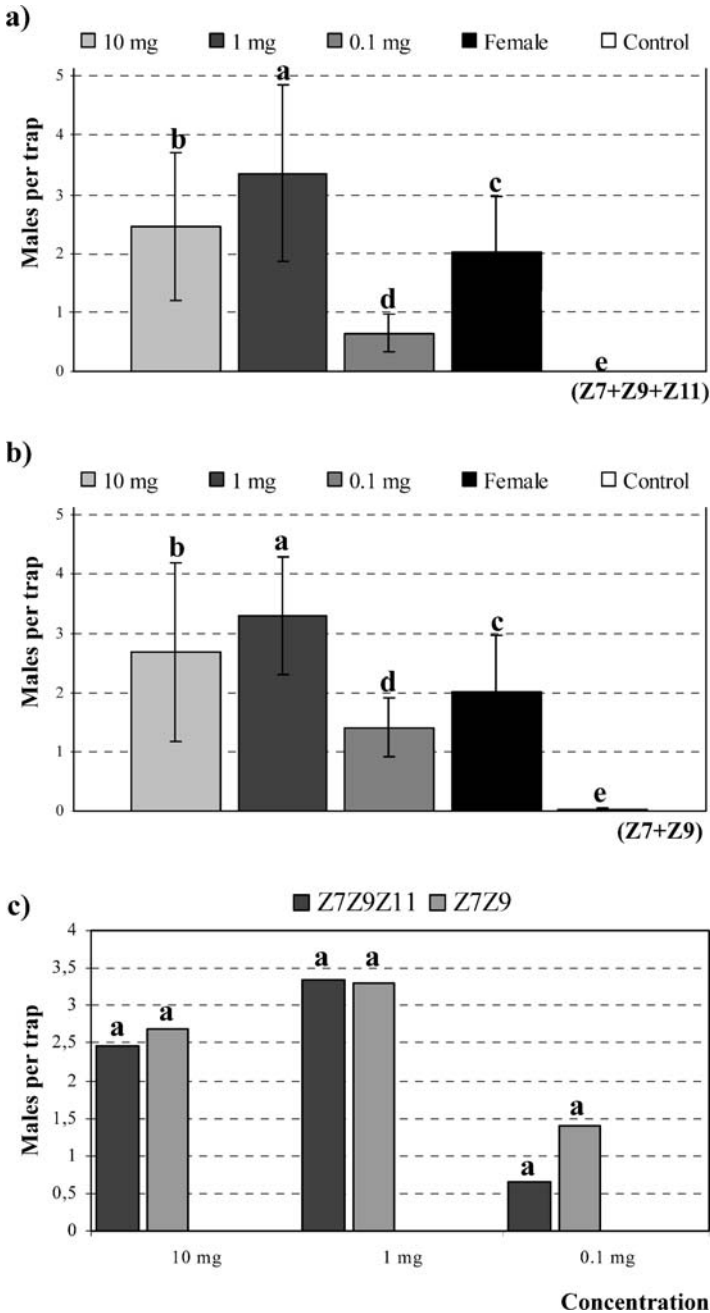


Fig. 8 Number ($X \pm SD$) of *S. frugiperda* males captured in Pherocon 1C traps baited with: (a) Z7-12:Ac + Z9-14:Ac + Z11-16:Ac (1:100:15 ratio) at doses of 10, 1, and 0.1 mg, hexane solvent, and two virgin females (five replicates and 12 collections); (b) Z7-12:Ac + Z9-14:Ac (1:100) at doses of 10, 1, and 0.1 mg, hexane solvent, and two virgin females (five replicates and 12 collections); (c) Z7-12:Ac + Z9-14:Ac + Z11-16:Ac (1:100:15 ratio) and Z7-12:Ac + Z9-14:Ac (1:100 ratio) at doses of 10 g, 1 g, and 0.1 mg, respectively (five replicates and 12 collections). Mean values with the same letter are not significantly different (two-way ANOVA followed by Tukey's test; $P < 0.05$)

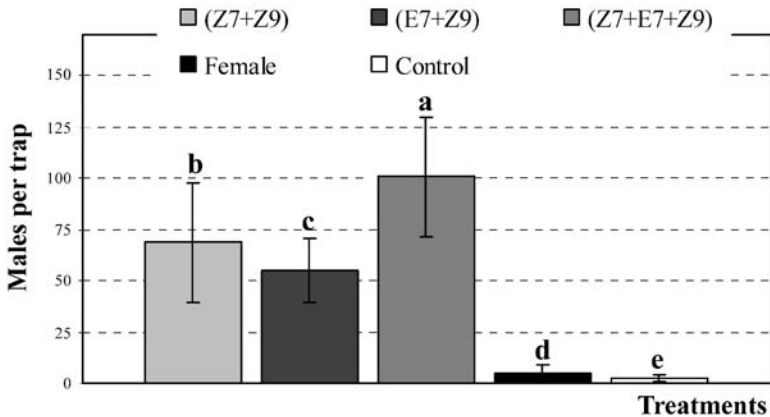


Fig. 9 Number ($X \pm SD$) of *S. frugiperda* males captured in Pherocon 1C traps baited with Z7-12:Ac + Z9-14:Ac at ratios of 0.01:1.00 mg, E7-12:Ac + Z9-14:Ac at ratios of 0.01:1.00 mg, Z7-12:Ac + E7-12:Ac + Z9-14:Ac at ratios of 0.01:0.01:1.00 mg, hexane solvent, and two virgin females (five replicates and 14 collections). Mean values with the same letter are not significantly different (two-way ANOVA followed by Tukey's test; $P < 0.05$)

Pherocon 1C traps, baited with either Z7-12:Ac + Z9-14:Ac (0.01:1.00 mg) or Z7-12:Ac + Z9-14:Ac + Z11-16:Ac (0.01:1.00:0.10 mg), captured similar numbers of males and, in both cases, were significantly higher than controls (Fig. 7). All concentrations of Z7-12:Ac + Z9-14:Ac + Z11-16:Ac (1:100:15 ratio) captured significantly more males than controls, and the two higher concentrations out-competed virgin females. The 1-mg lure was more effective than either the 0.1- or 10-mg ones (Fig. 8a), in the latter case probably because of receptor saturation. Similar patterns were observed with different concentrations of two- and three-component blends (Fig. 8), with all concentrations tested catching more than controls, and the higher doses performing better than virgin females (Fig. 8a,b). The addition of Z11-16:Ac to Z7-12:Ac and Z9-14:Ac did not increase trap catches (Fig. 8c), which is contrary to the results of Andrade et al. (2000) in Costa Rica, where Z11-16:Ac did result in a slight increase in trap efficacy. However, the addition of E7-12:Ac to the binary mixture resulted in a significant increase in the number of males captured (Fig. 9).

Discussion

The pheromone glands of *S. frugiperda* from North America were found to contain Z7-12:Ac, Z9-12:Ac, Z9-14:Ac, and Z11-16:Ac (Mitchell et al., 1985; Tumlinson et al., 1986; Descoins et al., 1988), whereas in Guadeloupe (Caribbean), the main components reported were Z9-12:Ac, Z9-14:Ac, and Z11-16:Ac (Andrade et al., 2000). In the present study, we also found Z9-14:Ac and Z11-16:Ac but for the first time report the presence of E7-12:Ac, present in higher quantities than Z7-12:Ac.

Malo et al. (2004) reported that Z9-14:Ac and Z9,E12-14:Ac evoked larger EAG responses than Z7-12:Ac in male antennae of *S. frugiperda*, from Mexico, whereas Z11-16:Ac and Z9,E11-14:Ac did not differ from the control hexane. The antennae of *S. frugiperda* males from Costa Rica respond to Z11-16:Ac (cited as unpublished

results of R. Gries in Andrade et al., 2000) but not in our studies, as significant EAG responses were only observed to Z9-14:Ac, Z7-12:Ac, and E7-12:Ac (Figs. 4 and 5). A comparison of our EAG data with those from North and Central America, together with the results of our field trials, supports the idea that different geographic pheromone races exist in *S. frugiperda*, as reported in other noctuids (Löfstedt et al., 1986; Tóth et al., 1992; Wu et al., 1999; Gemeno et al., 2000). These findings are important for the practical use of pheromones for monitoring of *S. frugiperda* populations in Brazil, and the Z7-12:Ac, E7-12:Ac, and Z9-14:Ac (0.01:0.01:1.00 mg, respectively) blend is currently being tested in integrated pest management of the fall armyworm in Brazilian maize crop.

Acknowledgments We thank Dr. K. Ogawa, Shin-Etsu Chemical Co., for furnishing pheromone samples, and G. C. R. Bernasconi for statistical analysis. This study was funded by FAPESP and CNPq/RHAE (Brazil) and IFS/OPCW (Sweden).

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Possible Mechanism of Inhibition of 6-Methoxy-Benzoxazolin-2(3H)-One on Germination of Cress (*Lepidium sativum* L.)

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Abstract 6-Methoxy-benzoxazolin-2(3H)-one (MBOA) inhibited the germination of cress (*Lepidium sativum* L.) seeds at concentrations greater than 0.03 mM. Inhibition was overcome by sucrose, suggesting that MBOA may inhibit sugar metabolism in cress seeds. Induction of α -amylase activity in seeds was also inhibited by MBOA at concentrations greater than 0.03 mM. Inhibition of both germination and induction of α -amylase activity increased with increasing concentrations of MBOA, and the extent of germination correlated positively with the activity of α -amylase in the seeds. MBOA added to a reaction mixture for α -amylase assay did not affect enzyme activity, indicating that MBOA does not inhibit *in vitro* α -amylase activity. Cress seeds germinated approximately 16 hr after incubation, and inhibition of α -amylase by MBOA occurred within 6 hr after incubation. These results suggest that MBOA may inhibit the germination of cress seeds by inhibiting the induction of α -amylase activity, because α -amylase plays a key role in the conversion of reserve carbohydrate into soluble sugars, a prerequisite for seed germination.

Keywords α -Amylase · Allelopathy · Germination inhibitor · *Lepidium sativum* · 6-Methoxy-benzoxazolin-2(3H)-one

Introduction

6-Methoxy-benzoxazolin-2(3H)-one (MBOA) was first found in *Croix lacryma-jobi* L. (Koyama, 1955) and later in several graminaceous plant species such as wheat, corn,

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and maize (Niemeyer, 1988). Benzoxazolinones, including MBOA and their precursor hydroxamic acids, have been recognized as resistance agents in plants because of their phytotoxic activity on insects (Dowd and Vega, 1996; Bravo and Copaja, 2002), fungi, and bacteria (Frey et al., 1997; Yue et al., 1998; Glenn et al., 2002).

MBOA and related compounds have also attracted attention because of their allelopathic effects (Barnes and Putnam, 1987; Inderjit and Duke, 2003; Belz and Hurlle, 2004). MBOA inhibits the germination and growth of several plant species (Pérez, 1990; Hayashi et al., 1994; Kato-Noguchi et al., 1998; Kato-Noguchi, 2000). At concentrations greater than 0.03 mM, MBOA inhibits the germination of *Digitaria sanguinalis* (L.) SCOP, *Lolium multiflorum* LAM, and *Pheleum pretense* L. (Kato-Noguchi, 2000). However, the physiological mechanism of MBOA on germination inhibition is not understood.

This study focused on the mechanism of inhibition of seed germination by MBOA. α -Amylase is considered to be essential for seed germination because this enzyme triggers starch degradation in the endosperm and enables seeds to germinate and grow (Perata et al., 1992, 1997; Vartapetian and Jackson, 1997). It is possible that MBOA inhibits germination because of its inhibition of α -amylase activity in seeds. Thus, the effects of MBOA on germination and α -amylase activity in cress seeds were investigated.

Methods and Materials

Plant Material

Seeds of cress (*Lepidium sativum* L.) were sterilized in a 2% (w/v) solution of sodium hypochlorite for 15 min and rinsed (4 \times 's) in sterile distilled water. All further manipulations were carried out under sterile conditions. MBOA (purchased from Sigma; 0.001, 0.03, 0.1, 0.3, 1, or 3 mM, final concentrations in Tween 20 solution described below) was dissolved in a small volume of methanol and added to two sheets of filter paper (No. 1, Merck) in a 9-cm Petri dish. The solvent was allowed to evaporate in a draft chamber for 1 hr. The filter paper in the Petri dish was then moistened with 4 ml of 0.05% (v/v) aqueous Tween 20. Sucrose (1 mM) was added into the medium in the Petri dish. Fifty seeds of cress were arranged on the filter paper and the Petri dishes were sealed. After incubation in the dark at 25°C for 6–48 hr, the lengths of emerged radicles of cress seeds were measured with a ruler. Seeds were considered to have germinated when their radicles emerged. Experiments were repeated three times, and the mean, standard error (SE), *t*-test value, and regression coefficient were calculated. For determination of α -amylase activity, cress seeds were harvested, frozen immediately in liquid N₂, and freeze-dried.

Pulse Treatment of MBOA

Cress seeds were incubated with 0 or 3 mM MBOA in 0.05% (v/v) aqueous Tween 20 solution in the dark at 25°C for 24 hr as described above. Then, the seeds were rinsed (5 \times 's) with sterile distilled water, transferred onto two sheets of fresh filter paper moistened with fresh Tween 20 solution containing 0 or 3 mM MBOA, and grown under the same conditions for 24 hr.

Extraction and Assay of α -Amylase

Freeze-dried cress seeds (10 seeds for each determination) were ground to a fine powder in a mortar by use of a pestle. The powder was homogenized with 1.5 ml of ice-cold 100 mM HEPES–KOH (pH 7.5) solution containing 1 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM $NaHSO_3$, and 50 mM bovine serum albumin. The homogenate was centrifuged at $30,000 \times g$ for 30 min; the supernatant was heated with 3 mM $CaCl_2$ at $75^\circ C$ for 15 min to inactivate β -amylase and α -glucosidase (Sun and Henson, 1991; Guglielminetti et al., 1995), then used for α -amylase assay.

α -Amylase was assayed by measuring the rate of generation of reducing sugars from soluble starch. Appropriate dilutions of enzyme preparations were made, and 0.2 ml of the diluted enzyme was added to 100 mM sodium acetate (0.5 ml; pH 6.0) containing 10 mM $CaCl_2$. The reaction was initiated with 2.5% (w/v) soluble starch (0.5 ml). After incubation at $37^\circ C$ for 15 min, the reaction was terminated by adding 40 mM dinitrosalicylic acid (0.5 ml) containing 400 mM NaOH and 1 M K–Na tartrate and then placing immediately into a boiling water bath for 5 min. After dilution with distilled water, the absorbance at 530 nm (A_{530}) of the reaction mixture was measured and reducing power evaluated using a standard curve obtained with glucose (Guglielminetti et al., 1995). Before MBOA was added to the assay, it was first dissolved in differing concentrations of dimethyl sulfoxide, then diluted to 0.001, 0.03, 0.1, 0.3, 1, or 3 mM with the reaction mixture. Experiments were repeated five times, with four assays for each determination.

Results and Discussion

Effects of MBOA on Germination

Radicles of control seeds emerged about 16 hr after sowing. MBOA inhibited germination, with germination completely inhibited by 3 mM MBOA (Fig. 1). When the length of cress radicles was plotted against the logarithm of MBOA concentration, there was a good logistic concentration–response curve (Fig. 2). At concentrations greater than 0.03 mM, MBOA inhibited the growth of cress radicles significantly. The concentration required for 50% inhibition of radicle growth was 0.18 mM (calculated from the regression equation of the concentration–response curve). These results suggest that MBOA inhibited the cress germination process and that the inhibition was increased with increasing MBOA concentrations (Figs. 1

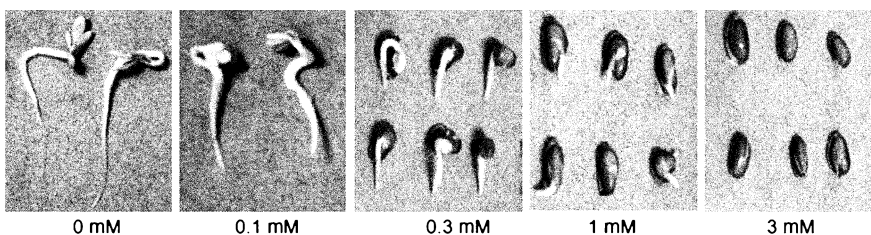


Fig. 1 Effects of MBOA on the germination of cress seeds. Cress seeds were incubated with MBOA in the dark at $25^\circ C$ for 48 hr

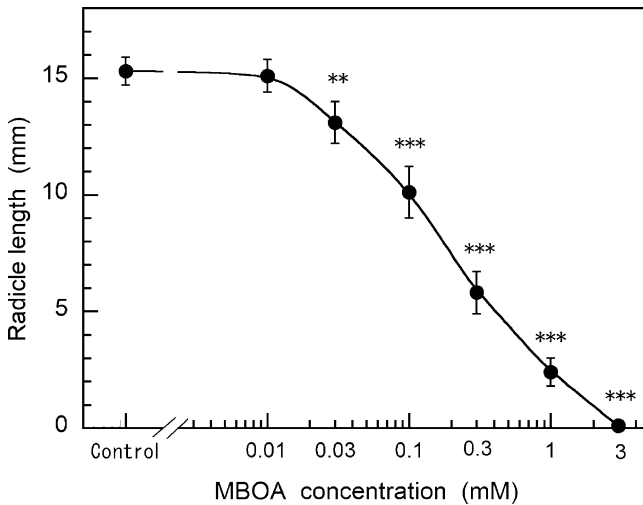


Fig. 2 Effects of MBOA on radicle length of cress seeds. Cress seeds were incubated with MBOA in the dark at 25°C for 48 hr. Means \pm SE from three independent experiments with 50 plants for each determination are shown. **Significant at $P < 0.01$, *** Significant at $P < 0.001$ level as compared with the control seedlings

and 2). MBOA also inhibits germination in several other plant species (Pérez, 1990; Kato-Noguchi, 2000), and uptake of MBOA by plant seeds is significantly faster than uptake of its precursor hydroxamic acid, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (Pérez, 1990).

Germination inhibition by MBOA was overcome by addition of sucrose to the medium (Fig. 3), which suggests that inhibition may not be due to a direct

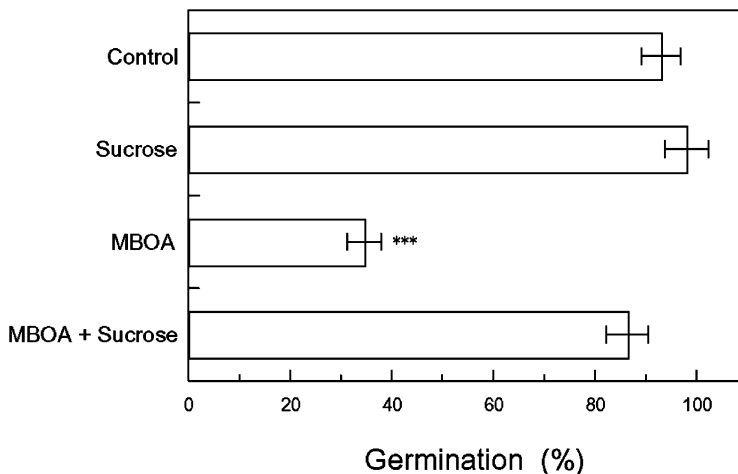


Fig. 3 Effect of sucrose on MBOA-induced inhibition of cress germination. Seedlings were incubated with 0.3 mM MBOA in the presence and absence of 1 mM sucrose in the dark at 25°C for 24 hr. Control seeds were incubated without MBOA and sucrose. Seeds were considered as germinated if their radicles emerged. Other details as for Fig. 2

influence of MBOA molecules themselves. Considering this observation, MBOA may inhibit sugar metabolism in the seeds because soluble sugars such as maltose and sucrose must be produced continuously from reserve starch to maintain the germination process (Ricard et al., 1998; Saglio et al., 1999; Guglielminetti et al., 2000).

Germination was inhibited when cress seeds were transferred into a solution containing 3 mM MBOA from one without MBOA (0 to 3 mM MBOA; Fig. 4). This suggests that MBOA is able to inhibit the germination process even after radicle emergence. In contrast, after the transfer of seeds into a solution without MBOA from one containing 3 mM MBOA, germination was initiated and radicles began to grow (3 to 0 mM MBOA; Fig. 4). This result indicates that 3 mM MBOA did not kill seeds, and that MBOA absorbed by seeds may be metabolized and detoxified in seeds (Inderjit and Duke, 2003; Glenn et al., 2003; Sicker et al., 2003). However, cress seeds did not germinate at concentrations greater than 300 mM, even after transfer into a solution without MBOA (data not shown).

Effects of MBOA on α -Amylase Activity

MBOA inhibited α -amylase activity in cress seeds at concentrations greater than 0.03 mM (Fig. 5). α -Amylase activity could be fitted to a logistic concentration-response curve when plotted against the logarithm of MBOA concentration. The concentration required for 50% inhibition of activity was 0.16 mM. This value was almost the same as the concentration required for 50% inhibition of radicle growth (Fig. 2).

MBOA was added into the α -amylase reaction mixture, but the enzyme activity was not affected by MBOA in the assay mixture (data not shown), indicating that MBOA does not directly inhibit *in vitro* α -amylase activity. Evaluating α -amylase

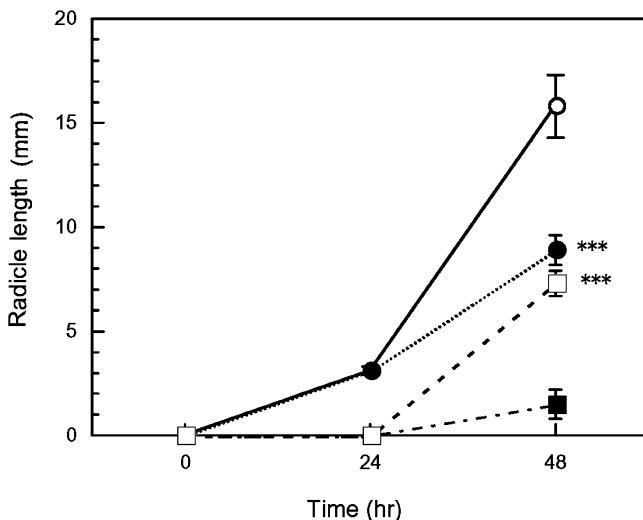


Fig. 4 Effects of pulse treatment of MBOA on cress germination. Cress seeds were incubated with 3 mM MBOA (or 0 mM) in the dark at 25°C for 24 hr, and then the seeds were transferred to medium of 0 mM MBOA (or 3 mM MBOA), and grown for 24 hr. ○, 0 to 0 mM MBOA; ●, 0 to 3 mM MBOA; □, 3 to 0 mM MBOA; ■, 3 to 3 mM MBOA. *** Significant at $P < 0.001$ level as compared between ● and ○, and □ and ■. Other details as for Fig. 2

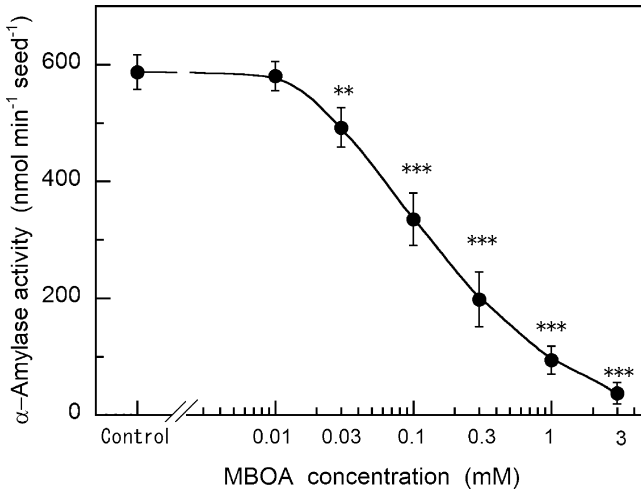


Fig. 5 Effects of MBOA on α -amylase activity in cress seeds. Cress seeds were incubated with MBOA in the dark at 25°C for 48 hr. Means \pm SE from five independent experiments with four assays for each determination are shown. Other details as for Fig. 2

induction at the protein level, as influenced by MBOA showed an increase in inhibition with increasing MBOA concentrations. In addition, both concentration–response curves (Figs. 2 and 5) indicate that length of cress radicles is positively correlated with α -amylase activity in seeds.

Figure 6 shows changes in α -amylase activity in cress seeds after sowing. The activity in control seeds (0 mM MBOA) was low at time 0 and increased as germination proceeded. Radicles emerged about 16 hr after sowing. MBOA

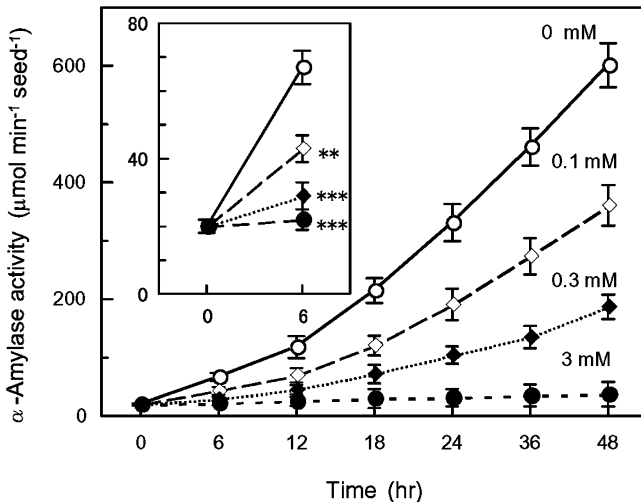


Fig. 6 Changes in α -amylase activity in cress seeds. Cress seeds were incubated with MBOA in the dark at 25°C. Means \pm SE from five independent experiments with four assays for each determination are shown. Other details as for Fig. 2

inhibited the induction of α -amylase activity within 6 hr after sowing, and the inhibition was greater with increasing MBOA concentrations. At 48 hr, the activities in seeds treated with 0.1 and 0.3 mM MBOA were 58 and 32% of that in control seeds, respectively, whereas the activity in seeds treated with 3 mM MBOA remained almost unchanged.

Although the α -amylase activity in control cress seeds increased continually (0 to 0 mM MBOA; Fig. 7), the rate of increase was reduced when cress seeds were transferred into 3 mM MBOA from a solution without MBOA (0 to 3 mM MBOA; Fig. 7), suggesting that MBOA may slow down the increase in α -amylase activity even after radicle emergence. In contrast, enzyme activity increased after transfer to 0 mM MBOA from a solution containing 3 mM MBOA (3 to 0 mM MBOA; Fig. 7) and radicles of these seedlings resumed a growth rate similar to that of untreated seedlings (Fig. 4). This suggests that α -amylase activity in cress seeds correlates well with the germination process (Figs. 4 and 7), and the induction of α -amylase may occur after MBOA is metabolized and detoxified, as suggested above.

Starch breakdown during seed germination is triggered by α -amylase that catalyzes the hydrolysis of α -1,4 glucan linkages to produce maltose and larger oligosaccharides (Beck and Ziegler, 1989; Vartapetian and Jackson, 1997). α -Amylase induction in many seeds is regulated by gibberellin at the transcriptional level (Ritchie and Gilroy, 1998). Inhibition of α -amylase activity by MBOA occurred within 6 hr after sowing (Fig. 6). Considering that radicles emerged around 16 hr after sowing, this inhibition may not be too late to inhibit the translation process of α -amylase.

Although it is possible that MBOA inhibits gibberellin biosynthesis, inhibitors of gibberellin biosynthesis do not inhibit the increase in α -amylase production during germination (Groselindemann et al., 1991). During germination, gibberellin precursors stored in seeds are mobilized, and the gibberellin produced triggers α -amylase induction (Groselindemann et al., 1991; Ritchie and Gilroy, 1998). Thus,

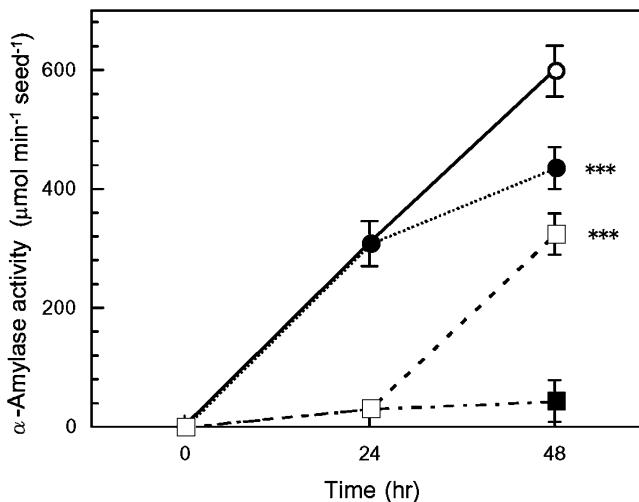


Fig. 7 Effects of pulse treatment of MBOA on α -amylase activity in cress seeds. Means \pm SE from five independent experiments with four assays for each determination are shown. Other details as for Fig. 4

MBOA may inhibit α -amylase induction in antagonism with gibberellin-induced events by affecting the α -amylase translation process rather than inhibition of gibberellin biosynthesis. Abscisic acid inhibits α -amylase induction in antagonism with gibberellin-induced events in this manner (Ritchie and Gilroy, 1998).

Germination Inhibition and α -Amylase Activity

Plant germination is a complex phenomenon involving many genes and enzymes. However, amyolytic breakdown of reserve starch in seeds is thought to be a prerequisite for seed germination and subsequent seedling growth. Starch breakdown during seed germination is principally triggered by α -amylase (Beck and Ziegler, 1989; Thomas, 1993; Conley et al., 1999).

During germination, respiration accelerates to produce metabolic energy and biosynthetic precursors for constructing cell structures (Perata et al., 1997). Therefore, soluble sugars that are readily used in respiration must be continuously supplied to maintain respiratory metabolism. However, the amount of utilizable soluble sugars in seeds is usually very limited (Ricard et al., 1998; Saglio et al., 1999; Guglielminetti et al., 2000). α -Amylase plays a major role in the conversion of reserve starch into soluble sugars during germination (Beck and Ziegler, 1989; Vartapetian and Jackson, 1997). Induction of α -amylase is essential to maintain active respiratory metabolism, which produces energy and carbon skeletons for the biosynthesis of new cellular components. Therefore, induction of α -amylase is a prerequisite not only for seed germination, but also subsequent seedling growth until photosynthesis is sufficient to support growth (Beck and Ziegler, 1989; Thomas, 1993; Conley et al., 1999).

MBOA inhibited seed germination and induction of α -amylase in cress seeds. Furthermore, α -amylase activity in the seeds reflected the extent to which they had completed the germination process. These results suggest that MBOA inhibits the induction of α -amylase, which is one possible mechanism of MBOA inhibition of the germination process. The observation that exogenously applied sucrose overcame the inhibitory effect of MBOA supports this hypothesis.

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Letter from the Editor-in-Chief

Since 2000, the *Journal of Chemical Ecology* has occasionally published special issues devoted to a single topic. Previous instances include: Allelopathy, September 2000; Aquatic Biology, October, 2002; and Molecular Chemical Ecology, December, 2004. These issues have been well received by the readership, and in many cases have both improved the quality of submitted papers, and moved research in the subdisciplines forward. This issue consists entirely of papers in Mammalian Chemical Ecology. Both invited and contributed papers are included.

The invited papers result from the International Mammalogical Congress held in Sapporo, Japan in August 2005 and focus on mammalian countermeasures against plant metabolites. Some contributed papers complement that theme, and others range from odor perception and mammalian pheromones, to improved methodology for chemical assays. It is a unique collection of work that is certain to provoke discussion, stimulate interest, and likely move the discipline in new directions.

The idea of the special issue was proposed by Editorial Board member William Foley of the Australian National University in Canberra. He has diligently worked with us since inception as a motivating force, a reviewer, and an editor to insure that the issue had broad appeal and appeared in a timely fashion. We acknowledge this major contribution with thanks and appreciation. We hope that *Journal of Chemical Ecology* readers find the issue educational, useful, and provoking.

John T. Romeo
Editor-in-Chief

Preface

This issue of *Journal of Chemical Ecology* focuses on aspects of the chemical ecology of mammals. The issue grew from a Symposium of the 9th International Mammalogical Congress held in Sapporo, Japan, in August 2005, entitled “Mammalian countermeasures against plant secondary metabolites.” This issue contains all but one of the invited papers in that Symposium, but we also are able to include several contributed papers directly related to the Symposium theme as well as a range of other papers, which illustrate the breadth of chemical ecology work on mammals.

Our purpose in initiating the Sapporo Symposium was to summarize current knowledge on the measures that mammals employ to counter plant defenses, in particular chemical defense mediated by plant secondary metabolites (PSMs). Thanks to a long history of studying the subject, we know that plants have evolved an enviable range of defenses. These include altering the foraging behavior of animals, depressing the efficiency of digestion, and ultimately impinging on growth, reproduction, and survival. In contrast to our knowledge of the chemical armory of plants and the variation it shows within species, we have a limited and scattered knowledge about the measures animals employ to counter PSMs. Here lies the key to understanding variation in reproductive success and, thus, mammalian evolution. For example, animals that can cope with particularly noxious diets can use feeding niches that remain beyond the reach of most other animals. There is no doubt that this field of chemical ecology is a fertile one for studying both theoretical and applied aspects of the interactions between animals and plants.

To further our understanding of the coevolution between plants and mammalian herbivores, we need to focus on the likely intraspecific variation in animals. For example, do some individuals or some populations tolerate the effects of PSMs better than others do? There are many examples of heritable variation in plant traits that affect herbivores and of insect traits that enable them to eat those plants. Regrettably, there are few examples where knowledge of variation among individuals has helped explain interactions between mammals and their food plants.

The fate of ingested PSMs provides a good example of the difficulties of exploring variation among individual animals. Ingested PSMs must be transformed to less toxic compounds and readied for excretion in the feces or urine. Toxicological studies in domestic species provide some guide to the types of metabolites we might expect a wild animal to produce. In most mammalian herbivores, however, significant microbial metabolism often precedes or follows enzymatic modification of the ingested compound by liver cytochrome P450 enzymes. Thus, as well as measuring the concentration of PSMs ingested, we also need to quantify the products of many excretory pathways.

Knowledge of the molecular targets and countermeasures that mammals use against PSMs provides the link to studying variation among individuals in enzyme activities and genes. The power of molecular approaches to understanding

variations in the susceptibility of mammals to PSMs is illustrated by the recent discoveries of bitter taste receptors and evidence of their variation among human populations.

We hope that the papers in this issue give readers an overview of the important types of mechanisms in the battles between plants and animals, but more importantly that they inspire them to quantify variation both between and within species. If so, we will improve our understanding of the coevolutionary relationships between mammals and plants.

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Behavioral Strategies of Mammal Herbivores Against Plant Secondary Metabolites: The Avoidance–Tolerance Continuum

Glenn R. Iason · Juan J. Villalba

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Abstract We review the evidence for behavioral avoidance of plant secondary metabolites (PSMs) and identify how, and the circumstances under which it occurs. Behavioral strategies of avoidance of PSM can only be fully understood in relation to the underlying physiological processes or constraints. There is considerable evidence that animals learn to avoid PSMs on the basis of negative postingestive effects. The extent to which this process determines foraging choices is limited by the ability of animals to experience the consequences of their behaviors and associate particular cues in foods with their specific effects in the body. The proposed learning mechanisms require that animals must at least “sample” plants that contain PSMs. They do not completely avoid PSMs, but there is evidence that they restrict their ingestion to within limits that they are physiologically able to tolerate, and that the amounts of PSM ingested result from a balance between toxicological considerations and the nutrient content of the plant material. These limits are influenced by the kinetics of PSM elimination, which underlies patterns of bite and patch selection from plant parts to landscapes. We suggest that altering spatial location of feeding (to alternative food patches or alternative foods within patches, including plant parts), and temporal distribution of feeding activity, by either cessation of feeding or by continuing to feed, but on alternative foods, can both lead to reduction of the intake and toxic effects of PSMs. We propose that the strategy of avoidance or reduction of intake of PSMs coevolved with the animal’s ability to physiologically tolerate their ingestion, and that avoidance and tolerance are inversely related (the avoidance–tolerance continuum). The animals’ propensity and ability to seek alternatives also vary with the dispersion of their food resources. Further work is required to test these proposals and integrate temporal and spatial aspects of foraging behavior and its nutritional consequences in relation to PSMs.

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Keywords Avoidance · Behavioral strategies · Food choice · Mammal herbivores · Plant secondary metabolites · PSM · Tolerance

Introduction

Mammalian herbivores are adapted to the ingestion of plants from which they derive the nutrients and energy for body maintenance, growth, and reproduction. A major problem to their nutrition is posed by the concomitant presence of plant secondary metabolites (PSMs), which are organic compounds usually considered to have no function in the primary metabolic processes of the plant, but which are known to mediate interactions with other organisms including plant defense against pathogens and herbivores (Rosenthal and Berenbaum, 1991). PSMs as a whole are highly diverse, with some representatives occurring in almost all higher plants, particularly dicotyledons (Bate-Smith and Metcalf, 1957; Jung et al., 1979). Their structural and chemical properties vary within and among the many different classes of compounds requiring them to be metabolized by numerous different routes (Scheline, 1991), and leading to a wide range of possible physiological effects on mammals (McArthur et al., 1991; Scheline, 1991). Most, but not all, of these effects are negative (Iason, 2005), including inhibition of digestion (Robbins et al., 1987), toxicity to many organs and tissues (Babich and Davis, 1981; Cheeke, 1988; Fenwick, 1989; Zhu et al., 1992; Vanleeuwen et al., 1995), acidosis (Foley et al., 1995), sodium imbalance (Pehrson, 1983; Reichardt et al., 1990), and diuresis (Dearing et al., 2001).

An early categorization of plant defenses considered them to be “quantitative” such as condensed tannins, thought to act in a dose-dependent manner, and toxic “qualitative” lower molecular weight compounds, whose actions were thought not to be dose-dependent (Feeny, 1976; Rhoades and Cates, 1976). This idea dominated ecological considerations for two decades. However, it is now accepted that all PSMs act in a dose-dependent fashion, and follow toxicological principles, although the forms of the functions, and thresholds and maximum tolerable concentrations differ widely (Timbrell, 1991). Although plant secondary compounds that are acutely toxic in small doses (Garland and Barr, 1998; Acamovic et al., 2004) should be avoided by herbivores, most observations suggest that mammals ingest at least some PSMs, but maintain their levels of intake of them within a range that is physiologically tolerable (Provenza, 1996; Foley et al., 1999). When an animal’s ability to excrete and detoxify PSMs is exceeded, it suffers toxicosis (Freeland and Janzen, 1974), the physiological effects of which are much greater than the costs of detoxification and excretion of compounds ingested at levels that lie within the normal range that can be readily detoxified and excreted (Iason and Murray, 1996). Therefore, in general, mammalian herbivores regulate their ingestion of PSMs, rather than completely avoid them. We propose that the ingestion or avoidance of PSMs should be considered as a behavioral strategy that is integrated with their physiological ability to detoxify and excrete them.

There are now numerous studies and reviews demonstrating that PSMs can underlie a reduction in intake of a single diet type (e.g., Bryant et al., 1983) or avoidance of a PSM-containing food source relative to other available foods either under experimental (e.g., Provenza et al., 1990) or field conditions (Palo and Robbins, 1991; Hjältén and Palo, 1992). To counteract the negative effects of PSMs,

which depend on the dose ingested, mammals must either partially or completely avoid ingestion of them and/or detoxify and eliminate them from their bodies. We review the behavioral means by which mammalian herbivores might limit their intake of PSMs, taking a process-based approach to interpret how these adaptations are underlain by physiological and pharmacological limitations and principles. We then propose a framework that integrates behavioral strategies for avoidance of PSMs with physiological avoidance and tolerance of their effects (the avoidance–tolerance continuum).

Limiting Ingestion of PSMs by Behavioral Means

Herbivorous animals need to feed on plants to obtain nutrients and energy for maintenance, growth, and reproduction. The overall determinants of choice and amounts of food items ingested are likely to be multifactorial. There are many correlative studies that have identified PSMs as determinants of diet choice by mammals, but a wealth of evidence suggests that the choices made by herbivores reflect both the concentration of PSM and nutrients in available food plants (Bergeron and Jodoin, 1987; Marquis and Batzli, 1989; McArthur et al., 1993; Barton and Whiten, 1994; Gauthier and Hughes, 1995; Rodgers and Sinclair, 1997). There are several ways in which PSMs might influence foraging behavior, the most significant of which are considered below.

Although the intake of food usually decreases with increasing concentrations or amounts of PSM (Lawler et al., 2000; Boyle and McLean, 2004), few studies have investigated the effects of PSMs on herbivore foraging decomposed into the specific parameters included in the process-based models of the intake-plant biomass (functional response) relationship (Spalinger and Hobbs, 1992; Gross et al., 1993). Experimental manipulations have shown the precise effects of plant spinescent defenses against mammals, as reducers of bite mass and bite rate (Gowda, 1996), and where secondary metabolites form a significant component of the mass of plant material, such as condensed tannins in woody plants, then they are likely to dilute the mass of nutrient-containing food ingested at each bite, thereby decreasing the foraging efficiency. To date, equivalent experiments with PSMs in whole plants have yet to be performed, but fine-scale temporal distribution of foraging behavior in relation to PSMs in artificial diets was recently quantified (Wiggins et al., 2003). Artificial diets enriched with PSMs can be a useful tool to demonstrate avoidance of PSMs (Tahvanainen et al., 1985) or to assess nutritional effects (Iason and Palo, 1991; Stapley et al., 2000), but they do not permit the study of normal foraging processes, including, for browsing herbivores, fine-scale selection among plant parts (Hofmann, 1989), habitats, and food patches (Bailey et al., 1996). Detailed behavioral responses to PSMs in natural forages are difficult to quantify, partly because of problems posed by acquisition of suitable plant material of known chemical composition. With increasing concentrations of sideroxylonol, a formylated phloroglucinol compound (FPC) of eucalypts, ringtail possums (*Pseudocheirus peregrinus*) decreased their total intake, rate of intake, and intake per feeding bout, and increased their cumulative time spent feeding (Lawler et al., 2000; Wiggins et al., 2005). Although PSM concentrations and composition vary among plant species and between tissues of the same species, they also covary with other parameters of relevance to animal foraging such as nutrient content, fibrousness, or relevant

physical parameters such as stem diameters (Reichardt et al., 1990; Shipley et al., 1999). Attribution of causality to PSMs is therefore difficult. Experimental manipulation of PSMs for quantification of their effects on fine-scale foraging processes will be most effective when plant material is available in which contrasting PSM concentrations or types are present, probably manipulated within a species by selection or genetic modification, independently of other characteristics.

A hierarchy of routes by which PSMs affect foraging behavior has been identified. These routes range from preingestive stimuli (Pass and Foley, 2000), e.g., taste and smell (Elliott and Loudon, 1987; Eppe et al., 1996) to postingestive mechanisms that involve feedback from reduced digestibility, or postabsorptive effects of PSM (Foley et al., 1999). The preingestive mechanisms are likely to act rapidly (e.g., via stimulation of the trigeminal nerve) to determine whether or not a food is eaten, whereas the postingestive mechanisms involve a lag before being sensed initially and take longer to affect foraging. The process of learning to forage can involve interactions between pre- and postingestive stimuli, for example, associating preingestive stimuli with postingestive consequences.

Effect of Postingestive Consequences on Subsequent Behavior

To survive in a world where concentrations of nutrients and toxins change in time and space requires that individuals experience the consequences of their behaviors and constantly modify those behaviors accordingly. Both *preferences* and *selection* (Hodgson, 1979) are probably the result of both positive influences of the nutrient and negative influences of the PSM content of the available foods (Foley et al., 1999; Villalba et al., 2002). A role for previous learning as a determinant of food choices is, however, particularly likely when PSMs are involved, since negative stimuli may produce stronger learning responses than positive ones (Ginane et al., 2005). Toxins ingested with food typically reduce the likelihood of future ingestion due to the negative effects of those chemicals on different metabolic and cellular processes in the consumer.

Physiological Basis for the Integration of the Senses and the Consequences of Feeding

The chemical senses interact with the body through neurophysiological feedback loops (Garcia, 1989; Scott, 1990; Provenza, 1995). The information generated in the gastrointestinal tract is carried to the CNS mainly by the vagus (parasympathetic) and splanchnic (sympathetic) afferent nerves. Afferent nerves for taste converge with visceral afferent nerves in the nucleus tractus solitarius (NTS) of the brain stem. These nerves then proceed from the NTS to the limbic system and from the limbic system to the cortex and back to the NTS (Ricardo and Koh, 1978; Barber and Burks, 1987). Thus, anatomical evidence supports the notion that information from the gastrointestinal tract and taste converge in the CNS, suggesting that how animals evaluate a particular taste is shaped by information on consequences, i.e., postingestive feedback. Taste and postingestive feedback are associated by an affective processing system (Garcia, 1989). This system causes changes in preference that depend on whether the effect on the animal is aversive or positive. The net result is a modified incentive to eat food. Affective changes in acceptability of food, through taste–feedback interactions occur automatically, in the absence of any cognitive

association or memory of the feedback event. Even when animals are anesthetized, postingestive feedback can still alter ingestive behavior. It has been shown that when sheep eat a nutritious food and then receive a toxin dose during deep anesthesia, they become averse to the food (Provenza et al., 1994). Thus, preference changes despite knowledge of the cause of the feedback event. On the other hand, *food selection* involves cognitive choices or decisions among alternatives, which involve cortical processes. The cognitive system integrates the odor and sight of food with its taste. Animals use the senses of smell and sight to differentiate among foods, and to select or avoid foods whose effect on the internal milieu is either positive or aversive (Provenza, 1995). The net result is behavior modification. Together, affective and cognitive processes enable animals to modify their diet as a function of their physiological state and the chemicals present in the environment.

Conditioned Food Aversion as a Mechanism to Prevent Toxicosis

Food intake and diet composition can be modified by herbivores learning to associate negative effects, such as nausea resulting from administration of emetic drugs, with particular food types (Provenza, 1995, 1996), and these conditioned food aversions have also been shown by using secondary metabolites (Provenza et al., 1990; Kyriazakis et al., 1997, 1998). Food aversion learning is the process by which after eating or drinking a specific food, a physiological event or physiochemical agent causes nausea (Garcia, 1989) that is then associated with the flavor of the food. Aversions are usually specific to a particular food, even though an animal may have eaten a meal of several foods (Burrirt and Provenza, 1991), and the aversive effect increases with the concentration of the aversive stimulus, for example, by ruminants in response to lithium chloride (Dutoit et al., 1991). Conditioned aversions in rats can occur within minutes of eating, or gradually over days and weeks (Garcia, 1989). They can be acquired in one single pairing of taste with malaise, and last for a long time even when malaise does not occur for hours after food is consumed (Garcia and Kimeldorf, 1957; Burrirt and Provenza, 1991). Nevertheless, it has been shown that when averted animals saw nonaverted animals eating the conditioned food, they began to sample this food, and eventually the aversion extinguished, as no subsequent adverse reaction followed the ingestive behavior (i.e., social facilitation; Ralphy, 1997). Extinction of a conditioned food aversion can occur rapidly when the target food is familiar instead of novel (Burrirt and Provenza, 1996; Kimball et al., 2002). Likewise, lack of alternative foods decreases the persistence of the aversion (Kimball et al., 2002).

Several mechanisms operate to allow animals to associate specific foods with the postingestive effects of PSMs even when several alternatives are available at the same time, as in diverse plant communities. Learning initially what to eat or not eat, can often be by way of experienced social models, like their mother distinguishing novel from familiar foods (Burrirt and Provenza, 1996). Novel foods are evaluated cautiously by the animal within a meal. Sheep have been shown to acquire an aversion to the novel food when toxicosis followed a meal of several familiar foods and a novel food (Burrirt and Provenza, 1989, 1991). Although the proximity in time between a behavior and its consequences is a key principle of animal learning (Yearsley et al., 2006), this can be overridden by the novelty of a food. Negative effects are associated with novel rather than familiar foods, even if they were not the most recently ingested (Provenza et al., 1993). For example, when toxicosis followed

a meal of five foods, one novel and one that had made them ill in the past, sheep subsequently completely avoided the novel food and markedly decreased their intake of the food that made them ill in the past; but they did not avoid the other three familiar foods (Burritt and Provenza, 1991). This also illustrates the effects of previous experience (Burritt and Provenza, 1996). Previously extinguished food aversion to a familiar food can be readily relearned even 3 years following the initial feeding event (Burritt and Provenza, 1991, 1996).

Flavor discrimination and generalization also allow animals to rapidly identify potentially toxic foods. For example, when lambs were offered cinnamon-flavored rice and then experienced toxicosis, they reduced their preference for cinnamon-flavored rice. When they were subsequently offered wheat, which they prefer, but with cinnamon flavor added, they refused to eat the cinnamon-flavored wheat. Thus, the lambs generalized an aversion from rice to wheat based on a common flavor—cinnamon (Launchbaugh and Provenza, 1993).

Limits of Learning Ability

If animals can learn conditioned food aversions, then why do they sometimes over ingest PSMs and even die from toxicosis? There are several possible reasons for imperfection in their system of learning. Free-ranging herbivores, especially generalists, often encounter and eat several foods during a meal (Zahorik and Houpt, 1981; Van Wieren, 1996), which has been shown to interfere with their ability to associate specific foods with their consequences (Duncan and Young, 2002). This is particularly true for ruminants and other foregut fermenters in which ingested food enters a relatively large fermentation organ that imposes transformations and delays prior to absorption.

To condition a food aversion, the agent ingested must affect the emetic system of the midbrain and brain stem (Garcia, 1989), so learning will not occur if the toxin does not stimulate the emetic system. When the ingested dose of PSM needed to produce the physical response is higher than the toxic dose, it similarly cannot produce a learned response (Molyneux and Ralphs, 1992), e.g., sesquiterpene lactones in bitter rubberweed (*Hymenoxys odorata*) and sneezeweed (*Helenium hoopesii*) have a cumulative effect, and stimulation of the emetic system frequently develops only after a lethal quantity of plant has been consumed.

Sheep in unfamiliar environments prefer familiar to novel foods, even if the familiar foods have previously caused toxicosis (Burritt and Provenza, 1997), e.g., animals familiar with PSM-containing plants in unfamiliar environments may consume amounts of PSMs beyond their capacity of detoxification. These processes would be expected to contribute to cases of acute toxicosis caused by PSMs. These typically involve cases where the plant or the herbivore, often a domestic species, has been introduced or translocated to a new area (Garland and Barr, 1998; Acamovic et al., 2004). They consequently lack the coevolutionary history known to be crucial in shaping the plant–herbivore interaction (Feeney, 1991).

Preingestive Processing of Forage

At least two examples are available of animals processing their forage prior to ingestion. These behaviors may be energetically costly but are hypothesized to manipulate forage quality in the animal's favor, including reduction of the effects of

PSMs. Meadow voles (*Microtus pennsylvanicus*) form piles of clipped twigs of coniferous trees, prior to ingesting them 2 or more days later, after which time their condensed tannins and total phenolic levels are reduced (Roy and Bergeron, 1990). Similar processes are thought to reduce the negative effects of forage phenolics in the hay-piles formed by pikas (*Ochotona princeps*) (Dearing, 1997). The processes of oxidation and complexation of PSMs occur naturally during decomposition, and it is hypothesized they may reduce the reactivity of PSMs rendering them less biologically active when ingested. However, the benefits of these strategies in relation to concomitant changes in nutrient value of the forage have not been rigorously tested in wild mammals. Removal of buds of arctic birch trees by snowshoe hares represents a prior manipulation of plants, hypothesized to reduce the intake of associated resinous material (Bryant and Kuropat, 1980), but it also has the disadvantage of removing the most nutrient-rich part of the twig (Palo et al., 1992).

Spatial Patterning of Foraging; Seeking to Limit PSM Ingestion

Seeking alternative foods is a common means by which animals either avoid or reduce ingestion of those food types containing high concentrations of PSMs. The degree to which animals must move their feeding location varies from a small shift of biting another part of the same plant containing lower concentrations of PSMs, or ingesting an alternative individual of the same or another species at the same location, to shifting to another feeding station, patch, or habitat (Bailey et al., 1996). There are numerous examples of contrasting PSM composition and concentration in different plant parts within species (Waterman et al., 1980) and associated demonstrations of their efficacy as determinants of avoidance of those parts (Bryant et al., 1983; Reichardt et al., 1984; Sinclair et al., 1988; Jogia et al., 1989). Patterns of selection and foraging processes at the larger patch scale derive to a large extent from smaller-scale bite processes, but also from multiple biotic and abiotic environmental considerations such as topography, shelter, sociality, and predator avoidance. PSMs have been implicated in patch-scale foraging processes most effectively when the patches are definable separate entities such as individual trees. Influences of PSMs on patch residence times have been demonstrated in red deer (*Cervus elaphus*) feeding on sitka spruce (*Picea sitchensis*) trees; although initial selection of the trees was based upon tree size, deer foraged for less time and removed less from those trees containing higher concentrations of monoterpenes, before moving to an alternative tree (Duncan et al., 1994). Moose also select trees on an individual basis rather than at the level of the stand (Danell et al., 1991). Use of individual trees leading to aggregated animal distributions was recently demonstrated in koalas (*Phascolarctos cinereus*). The size of the Eucalypt trees (*Eucalyptus* spp.) is the primary determinant of visitation frequency, which is also significantly positively related to foliar nitrogen concentration and negatively related to concentrations of formylated phloroglucinol compounds (FPCs) (Moore and Foley, 2005). This provides a further example that PSMs are merely one aspect of multifactorial determinants of the foraging process.

Temporal Patterns of Foraging Activity

It has been suggested that cattle can limit their ingestion of toxic alkaloids in larkspur pellets by alternating periods of a few days of high ingestion with a period

of low ingestion, when offered only a single food type (Pfister et al., 1997). Similarly, a cyclical, saw-toothed pattern of food intake between days has been observed in common brushtail possums (*Trichosurus vulpecula*) offered only a pelleted diet treated with the FPC jensenone (Stapley et al., 2000). These cyclical patterns are presumably caused by accumulation of toxins that leads to inappetence, during which the normal processes of detoxification and excretion reduce the toxic loads. Where absorption of a PSM is rapid, as in the case of the brushtail possum and the common eucalypt metabolite, 1-8-cineole, plasma concentrations of the compound and its metabolites increase rapidly, and subsequently decline rapidly due to metabolic clearance when foraging ceases, remaining low until the subsequent foraging bout (McLean and Duncan, 2006). When concentration of cineole increased in an artificial diet, the foraging parameters affected were the increased number of foraging bouts, and an overall slower rate of ingestion of food (Wiggins et al., 2003). Similarly, sheep ceased feeding when levels of 1,8-cineole in blood exceeded a critical threshold, and they resumed eating only after toxin concentrations in the body declined due to detoxification and elimination (Dziba et al., 2006). A model of the pharmacokinetic parameters associated with these processes shows the powerful effect of temporal phasing of foraging activity on the animal's ability to regulate its internal concentrations of PSMs below particular limits (Foley et al., 1999).

Integrating Temporal and Spatial Patterns of Foraging Activity

Despite our increasing knowledge on the role of PSMs as determinants of food quality, and an awareness of the spatial variation in PSMs (Lawler et al., 2000), relatively little consideration has been given to their importance in determining spatial ecology of herbivores (Scrivener et al., 2004). An understanding of the pharmacokinetics of PSM ingestion and excretion (Boyle and McLean, 2004) can be integrated with knowledge of rates of encounter and ingestion of PSMs in the environment (Fig. 1). This is likely to be a function of the herbivore's rates and directions of movement, and the characteristics of the PSM-containing patches such as their PSM concentrations and their size and shape, as well as the quality of the alternative forages. The longer the time interval an herbivore is exposed to a single food, then at least theoretically, the higher the likelihood of it correctly associating that food with its postingestive consequences (Yearsley et al., 2006). This leads to the hypothesis that an animal's spatial and temporal patterns of patch residency and toxin ingestion, and the pharmacokinetic characteristics of detoxification, interact to determine its decision making in relation to PSMs.

Dietary Mixing to Reduce the Effects of PSMs

An influential theory for diet diversity in mammalian herbivores is that by mixing food types, animals can avoid overingestion of a particular toxin and thereby limit the toxic effects suffered (Freeland and Janzen, 1974). This has been tested in several studies that show that animals are able to ingest a greater total amount of foods containing PSMs, when allowed to choose between them, than when offered the foods one at a time. It is implicit that once a particular pathway of PSM detoxification is saturated, then foraging on that food type and on others containing PSMs requiring the same pathway should cease, whereas foraging on alternatives containing PSMs

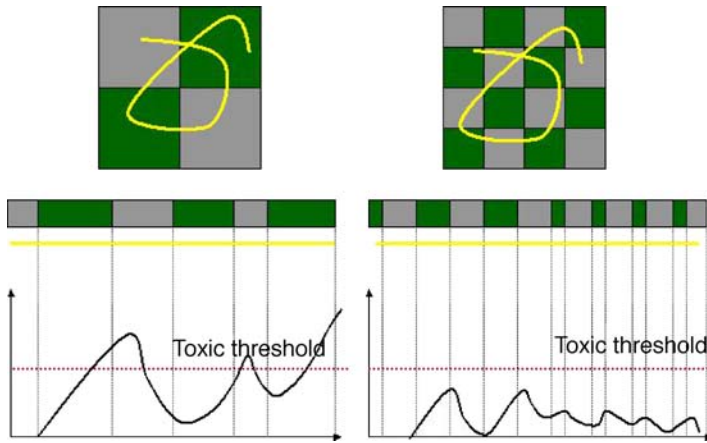


Fig. 1 Integration of temporal kinetics of processing PSM, with spatial patterns of foraging. Note the scale of the spatial patch sizes, determine the path length and rate of change of PSM-containing foraging bouts (darker patches) with bouts in non PSM-containing patches (paler patches). This in turn influences the temporal pattern of exposure of the animal to the PSM, a toxic threshold being more likely to be exceeded in the case of larger PSM patches, and longer foraging bouts within them (source: A.J. Duncan, unpublished data).

requiring other pathways would be permissible. Although choice experiments suggest this to be the case (Freeland and Winter, 1975; Dearing and Cork, 1999; Burritt and Provenza, 2000; Wiggins et al., 2003; Villalba et al., 2004), the critical test of the proposed underlying mechanism, by using PSMs whose metabolism by the animal is known, is yet to be undertaken. However, a crucial first step has been the recent demonstrations that brushtail possums are sensitive to their own detoxification capacity, and vary their food intake accordingly (Marsh et al., 2005).

A diverse diet leads to greater diversity of ingested phytochemicals that may also lead to mutual toxin neutralization or inactivation, which in turn could reduce susceptibility to PSMs. Rats eat more of a combination of foods containing tannins and saponins because tannins and saponins chelate in the intestinal tract, reducing the negative effects of both components (Freeland et al., 1985). If a mammal can evolutionarily or contemporarily learn to avoid certain plant parts because they lower its fitness due to PSM-related effects, it may also learn to seek out certain plant parts, or other substance in the environment because they raise its fitness (Janzen, 1978). The effects of condensed tannins, such as quebracho, in reducing digestibility by binding with dietary protein or digestive enzymes can be counteracted by concomitant ingestion of polyethylene glycol (PEG), which preferentially binds with tannin (Kumar, 1992; Waghorn and Shelton, 1995). In experimental tests, sheep increased their intake of PEG, as tannin concentrations in their diet increased (Provenza et al., 2000). They discriminated the “medicinal” effects of PEG from “nonmedicinal” substances by selectively increasing intake of PEG after eating a meal high in tannins (Villalba and Provenza, 2001). Sheep have also been shown to forage in locations where PEG is present, rather than where it is absent, when offered nutritious foods high in tannins in different locations (Villalba and

Provenza, 2002). Thus, interactions among PSM-containing foods could lead to complementary relationships such that eating a combination of foods may exceed the benefit of consuming any one food in isolation. However, evidence for food choices being driven by these interactions between naturally occurring PSMs is currently lacking. Similarly, it has been proposed that consumption of soil rich in kaolin by African elephants assists the animals in the digestion of browse, through its ability to detoxify the high concentration of PSMs found in tropical forest trees (Houston et al., 2001).

Evidence for Neutrality or Positive Selection for PSMs

Some studies have reported finding no effect of measured PSMs on foraging (Rangen et al., 1994), but examples of positive selection for PSMs are rare. However, the preference for natural forages by roe deer (*Capreolus capreolus*) is positively correlated with the presence of protein-binding phenolics (Tixier et al., 1997). Although roe deer exhibited lower rates of ingestion of artificial diets containing high concentrations of hydrolyzable tannins, compared with a control diet with no tannins, they ate relatively more of the diet to which moderate levels of hydrolyzable tannins had been added (Verheyden-Tixier and Duncan, 2000). We might expect that on occasions, no selection against PSMs will be found, due to the herbivore being effectively counteradapted to suffer no negative effects from their ingestion, for example, due to the roe deer's large salivary glands that secrete saliva containing a tannin-nullifying protein (Duncan et al., 1998). Positive selection, however, is—in this case—inexplicable, in that there is no demonstrated nutritional advantage in ingestion of hydrolyzable tannins, compared to an otherwise identical control diet containing none. However, it could be hypothesized that levels of PSMs that are low enough not to cause a detrimental effect, should occasionally be ingested as they may induce or maintain adaptation of the gut microflora or the animal's enzyme systems. This would protect against future ingestion of larger quantities that may be unavoidable, should foraging options be restricted. This bet-hedging strategy for ingestion of small amounts of PSM has yet to be tested, or distinguished from a general sampling strategy that provides updated information to the animal about the quality of available alternative foods.

It is known that low forage concentrations and low ingested doses of condensed tannins can be nutritionally advantageous by binding with and protecting ingested protein from rumen degradation, allowing it to dissociate from the tannin in the small intestine, thereby increasing its availability to the animal (Barry and Manley, 1984; Barry et al., 1986). Although the conditions under which such ingestion provides a nutritional advantage are quite narrow, this may explain the occasional reports of positive selection for condensed tannins, and ingestion of small amounts of tannin-rich foods (Iason, 2005). Condensed tannins are similarly thought to provide some protection against intestinal helminths, either by positively affecting immunity via nutritional benefits, or by directly inhibiting the parasites (Barry et al., 2002; Marley et al., 2003), in which case herbivores may be using the PSM-containing forages to self-medicate (Athanasiadou and Kyriazakis, 2004). Finally, many PSMs including phenolics have antioxidant properties as well as prooxidant detrimental effects (Appel, 1993), and may, therefore, account for the positive selection of PSMs. This has yet to be demonstrated.

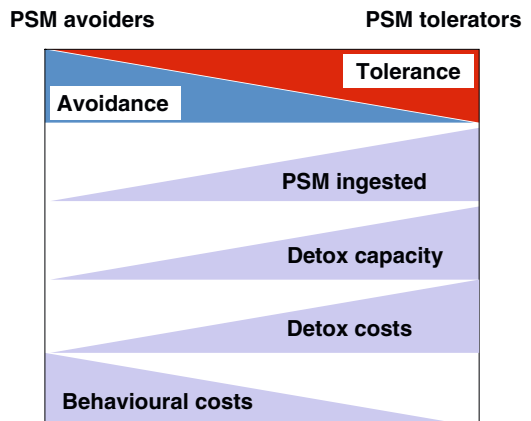
The Triangular Relationship

The examples above demonstrate that there is relatively little reason to suspect that at low levels in plants, PSMs should have a major, or indeed any, influence on mammal herbivore foraging. When PSM concentrations are low, other plant and animal characteristics can determine the overall quality of the food and the amounts of it that are eaten (Foley et al., 1999). The variation in food intake at low levels of dietary PSM is, therefore, high. At higher concentrations, however, the relative power of the effects of PSMs gains preeminence in determining foraging behavior, and they have a demonstrable negative influence on food selection and amounts ingested, leading to the so-called triangular or polygonal relationship with PSM concentration (Danell et al., 1990; Lawler et al., 2000; Scrivener et al., 2004). This is an important step in formulating our expectations of the effects of PSMs on foraging behavior, providing the appropriate perspective on how their effects integrate with other factors.

A Synthesis of Anti-PSM Strategies: The Avoidance–Tolerance Continuum

The way in which mammals trade off the behavioral and ecological costs of avoidance of PSMs with the alternative costs of detoxification, toxicosis, or other negative consequences of ingesting them, forms the basis of a framework that describes their strategy of circumventing PSMs: the avoidance–tolerance continuum. We hypothesize that the processes of detoxification and excretion, and behavioral avoidance are inversely related (Fig. 2). Both incur costs that are contrasting in nature; the former are metabolic and require provision of enzymes for detoxification such as the cytochrome P450 enzymes, and substrates for conjugation prior to excretion (Brattsten et al., 1977; Guengerich, 1997). The behavioral costs are hypothesized to consist mainly of reduced rates of intake arising from greater selectivity to avoid PSMs within food patches, by seeking alternative plant parts or alternative plant species, and/or by greater traveling or searching time for patches of

Fig. 2 Avoidance–tolerance continuum: illustrating the hypothesized negative relationship between the two extreme strategies



food or habitats containing higher nutrient levels or lower amounts of PSM (Hjältén and Palo, 1992; Hulbert et al., 1996; Iason et al., 1996; Wallis De Vries et al., 1998). This latter strategy is also likely to result in greater mortality risk.

At one extreme of the avoidance–tolerance spectrum is the strategy of complete avoidance of PSMs, while at the other extreme is that of no behavioral avoidance but strong physiological adaptation or tolerance to ingested PSMs. In reality, few if any, species are likely to be at the extremes of the continuum, ingesting no PSMs, or being completely without an upper physiological limit of tolerance requiring no behavioral avoidance; most likely they lie somewhere along the continuum between these extremes. Adaptation to PSMs, for example, by induction of enzymes for detoxification or salivary tannin-binding proteins on exposure to absorbed toxins (Pass et al., 2001) or condensed tannins (Mehansho et al., 1987), respectively, can shift an individual animal toward the tolerator end of the continuum. Such changes often take place seasonally with dietary change in response to changing composition of available and ingested food (Iason and Van Wieren, 1999). Conversely, the behavioral equivalent of this physiologically based counter adaptation is processes of associational learning to avoid PSM-containing foods and their negative consequences. This can effectively shift an individual toward the avoidance end of the continuum.

The avoidance–tolerance continuum maps approximately onto the grazer–browser continuum (Hofmann, 1989; McArthur et al., 1991), with avoiders including the grass and roughage feeders that relatively infrequently encounter or ingest PSM-rich food [e.g., sheep (*Ovis aries*), cattle (*Bos primigenius taurus*), topi (*Damaliscus lunatus*), oribi (*Ourebia oribi*)] compared to browser or concentrate selectors [e.g., roe deer (*C. capreolus*), bushbuck (*Tragelaphus scriptus*), greater kudu (*T. strepsiceros*)], which occupy habitats dominated by PSM-rich woody plants. When proposing the classification of ruminant herbivores according to their ecophysiological foraging and digestive strategy into bulk-roughage feeding “grazers” and selective browsers, Hofmann (1989) suggested that “grazers” avoid PSMs, whereas the more selective “browsers” are better able to deal with PSMs via physiologically based detoxification mechanisms. However, this provides a paradox since the browsers undoubtedly exploit more PSM-rich food sources (Iason and Van Wieren, 1999) despite their having finer mouthparts, more suitable for fine-scale selection and avoidance of PSMs (Perez-Barberia and Gordon, 2001). Similarly, the grazers’ avoidance of PSMs is—despite their having broader mouthparts—less conducive to selective foraging (Perez-Barberia and Gordon, 2001). Avoidance of PSMs can, therefore, take place either at the larger spatial scale by avoidance of the habitat, or at the finer scale by selective foraging within habitats, or both. Mammals can be generally avoiders of PSMs, but still highly selective foragers within habitats containing few PSMs [e.g., oribi, and most lagomorphs such as the European rabbit (*Oryctolagus cuniculus*), or European hares (*Lepus europaeus*)]. It is hypothesized that the selection between patches as a means of avoiding PSMs is likely to be more developed in animals close to the “avoiders” end of the continuum, such as cattle, rather than in the “tolerators.” However, recent evidence has shown that even koalas, a marsupial folivore strongly specializing in and adapted to PSM-rich foliages, discern between trees, being less likely to select those with lower concentrations of formylated phloroglucorinol compounds (Moore and Foley, 2005). This suggests that even those considered most specialist in being adapted to ingest PSMs probably have some upper limit of ingestion that influences their foraging behavior. Similarly, although selective avoidance of PSMs within patches

may occur in species lying anywhere in the continuum, it must be predominant among those that do not avoid PSMs at the habitat or patch scale. Any animal that selects positively for PSMs when balancing intake of PSM-rich food against nutrient intake (see above) must be able to tolerate those ingested levels, and is unlikely to be at the avoider end of the spectrum. As well as these foraging consequences of PSMs acting through seeking alternative forages elsewhere in space, animals that ingest PSMs may adjust the temporal distribution of their foraging activity in order to allow clearance of ingested and absorbed PSMs from the body prior to resumption of feeding activity (Foley et al., 1999).

We suggest that the animal's behavioral strategy against PSMs is intimately associated with its physiological strategy, and cannot be interpreted in isolation. It remains a challenge for nutritional ecologists to integrate and understand the relative magnitudes of the behavioral costs of avoidance with the physiological costs of ingestion of PSMs. We should remember that while we celebrate herbivores' ability to continue feeding and avoid toxicity of PSMs, the plants are simultaneously causing either considerable behavioral or physiological inconvenience, or both.

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Behavioral Responses of a Generalist Mammalian Folivore to the Physiological Constraints of a Chemically Defended Diet

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Abstract Mammalian herbivores, particularly browsers and folivores, encounter and consume a range of plant chemical defenses [plant secondary metabolites (PSMs)] on a regular basis. The physiological regulation of PSM ingestion and the resulting behavioral responses of mammalian herbivores directly affect their feeding decisions and the subsequent foraging strategies that they adopt. Generalist mammalian herbivores are hypothesized to consume a generalized diet because of physiological limitations of their detoxification systems. The consumption of a generalized diet is proposed to enable toxin (PSM) dilution through the use of multiple detoxification pathways. We tested the predictions of the detoxification–limitation hypothesis by offering two chemically different plant species, *Eucalyptus regnans* and *E. globulus*, to a generalist mammalian folivore, the common brushtail possum (*Trichosurus vulpecula*), as single- and mixed-species diets. By feeding more efficiently, brushtail possums benefited more, through increased intake, on the mixed-species diet than on either of the single-species diets. We argue that frequently switching between chemically diverse foliage reduces the physiological constraints imposed by a PSM-rich diet and enables more efficient feeding. The

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behavioral responses of brushtail possums were consistent with the proposed physiological constraints of a chemically defended diet, offering support for predictions of the detoxification–limitation hypothesis. We suggest that feeding behavior of herbivores may be a useful indicator of the physiological constraints imposed by a chemically defended diet.

Keywords Brushtail possum · Detoxification limitations · Eucalypt · Feeding behavior · Herbivore · Plant secondary metabolite

Introduction

By consuming plants, herbivores encounter a range of plant physical and chemical defenses (Feeny, 1970; Bryant and Kuropat, 1980). Plant chemical defenses, or plant secondary metabolites (PSMs), predominantly occur throughout woody plants as highly diverse compounds, both structurally and functionally. Ingestion of PSMs can incur physiological costs that include digestibility reduction (Robbins et al., 1987), toxicity (Pfister et al., 1997), and acidosis (Foley, 1992). To exploit woody plants, mammalian browsers (e.g., roe deer, *Capreolus capreolus*; Tixier and Duncan, 1996) and folivores (e.g., common brushtail possum, *Trichosurus vulpecula*; Kerle, 1984) must be able to process and deal with these chemical defenses. Mammalian browsers and folivores (hereafter referred to as herbivores) are indeed able to subsist on a chemically defended diet through a range of physiological and behavioral adaptations (Cork and Foley, 1991; McArthur et al., 1991).

Following ingestion and absorption, most PSMs must be detoxified to reduce the detrimental effects that they pose. Mammalian herbivores have evolved a series of detoxification pathways that convert absorbed PSMs into more hydrophilic and polar compounds through oxidation, reduction, or hydrolysis (phase I reactions), making them easier to excrete and/or preparing them for conjugation with an endogenous molecule (phase II reactions), again enhancing excretion (Sipes and Gandolfi, 1991). However, there are several limitations of these detoxification processes. Detoxification pathways rely on a series of rate-limited reactions, which, in turn, govern how much a herbivore can ingest per unit of time (Freeland and Janzen, 1974). Mammalian herbivores have been shown to regulate PSM ingestion at or below a proposed threshold level (Pfister et al., 1997; Lawler et al., 1998; Boyle and McLean, 2004). The inability to tightly regulate ingestion below a particular threshold may result in toxicosis, internal malaise, and, if severe enough, may even be fatal to the animal (Freeland and Janzen, 1974). Whereas most PSMs must be detoxified to aid in their excretion, the reactions involved can generate strong organic acids (Sipes and Gandolfi, 1991), which themselves may cause disturbances to a herbivore's acid–base status and, subsequently, disruptions to physiological and biochemical processes (Chilcott and Hume, 1984; Foley, 1992). All acid metabolites must, therefore, be buffered and excreted from the body (Foley et al., 1995). Detoxification of PSMs, through associated metabolic and excretory pathways, is an energetically expensive process (Cork and Foley, 1991; Freeland, 1991).

The capacity to which herbivores efficiently detoxify PSMs governs their subsequent feeding strategies. Only when a herbivore is able to minimize the energetic costs of detoxification (Sorensen et al., 2005), and efficiently metabolize and excrete ingested PSMs (Boyle et al., 1999), is it able to specialize on a relatively

homogenous diet. A generalized feeding strategy is by far the most common feeding approach adopted by mammalian browsers and folivores (Freeland, 1991). The consumption of a generalized diet is hypothesized to ensure adequate nutritional balance (Westoby, 1978). Acting over a much shorter timeframe, the detoxification–limitation hypothesis proposes that a generalized diet of chemically diverse plants enables toxin dilution, through the use of multiple detoxification pathways (Freeland and Janzen, 1974). Consistent with this hypothesis, research has shown that generalist folivores perform better on mixed than single forage diets through increased intake (Dearing and Cork, 1999; Wiggins et al., 2003). Explicit testing of the detoxification–limitation hypothesis is difficult because of limited knowledge of the mechanisms by which herbivores detoxify specific PSMs (Marsh et al., 2005).

Predictions of the detoxification–limitation hypothesis offer insight and understanding into the foraging decisions made by generalist mammalian herbivores. Consumption of a PSM-rich diet imposes direct physiological constraints to the herbivore. These constraints, in turn, should govern how generalists behaviorally respond to their diet, through the selection and mixing of different items. We have recently demonstrated that a folivore's rate of intake, and the frequency of switching between two chemically different plant species, was essential for maximizing intake (Wiggins et al., 2006). These feeding strategies were hypothesized to be directly related to the folivore's blood plasma PSM concentrations, a direct physiological constraint to the herbivore. A herbivore's feeding behavior is likely to be pivotal in how it responds to its dietary physiological constraints and could be used as an indicator of such constraints. Thus, the simultaneous investigation of three factors (intake, physiological responses, and behavioral responses) of herbivores to a mixed diet should enable us to evaluate predictions of the detoxification–limitation hypothesis. We know of no previous studies that have directly integrated these three components concurrently. Such integration is essential for the development of a more thorough understanding of the feeding decisions, and ultimately the foraging strategies, adopted by generalist mammalian herbivores.

We set out to test predictions of the detoxification–limitation hypothesis, in an attempt to explain why generalist mammalian herbivores are able to perform better on a chemically diverse diet than on a single species diet. We offered two chemically different plant species, *Eucalyptus regnans* and *E. globulus* (Wiggins et al., 2006), to a generalist mammalian folivore, the common brushtail possum (*T. vulpecula*), as single- and mixed-species diets. The four specific aims of this study were to (1) demonstrate differences in chemistry between the two eucalypt species; (2) determine whether intake was greater on the mixed- than single-species diets; (3) compare urinary characteristics between diet treatments as a crude indicator of the physiological status of the animals (e.g., pH, titratable ions, ammonia, urea); and (4) quantify differences in feeding behavior relative to maximizing intake.

Methods and Materials

Animals and Diet

Six adult brushtail possums (*T. vulpecula*), three males and three females [3.34 ± 0.59 kg (body weight mean \pm SD)], were collected from Hobart (Tasmania,

Australia), housed in a covered outdoor enclosure in individual mesh cages ($4.3 \times 1.7 \times 2.5$ m) at the School of Zoology, University of Tasmania, and provided with a nest box and logs for above-ground runways. Animals were maintained on a basal diet that was 18% dry matter (DM), consisting of [as % fresh matter (FM)] 46% apple, 35% silver beet, 10% carrot, 5% lucerne (ground to pass through a 1-mm sieve), and 4% raw sugar. Fruit and vegetable constituents were mixed in a food processor, then combined with dry ingredients. Food was provided at levels sufficient for maintenance (McArthur et al., 2000) and so was freshwater daily.

Feeding Trial

Possums were initially offered 50% of maintenance requirements of a basal diet and *E. regnans* and *E. globulus* sapling foliage *ad libitum* for 5 d, 1 wk before the trial. They received 120% basal diet two nights directly before the trial. Foliage was collected from Geeveston, Tasmania, and stored with stems in freshwater in a 5°C cool room. The feeding trial consisted of three treatment diets fed to possums in a balanced crossover design (Ratkowsky et al., 1993). The treatment diets were (1) *E. regnans* and (2) *E. globulus* single-species diets and (3) *E. regnans* and *E. globulus* mixed diet. Foliage was fed *ad libitum* in 300-g bunches, provided fresh each day. Each treatment was run for 4 consecutive nights, and immediately followed by 4 “rest” nights where possums were offered 120% basal diet to ensure they maintained body weight during the trial. Foliage intake was measured daily and expressed as grams dry matter per kilogram of body mass for each possum [$\text{g DM} (\text{kg BM})^{-1}$].

Foliage Properties

Two control bunches of foliage per species per night were pooled and subsampled for physical and chemical analyses to confirm that foliar secondary chemical properties differed. Foliage was assayed for DM, leaf toughness, nitrogen, fiber, oils, and phenolics.

Dry Matter

Subsamples of foliage were oven-dried at 55°C for 48 hr to determine percentage dry matter (% DM).

Leaf Toughness

Five leaves per species per night were sampled at three sections of the leaf (upper, middle, and lower) by using a dual tension gauge portable penetrometer (Chatillon, John Chatillon & Sons, Inc., NY, USA). Units are an index only, expressed as total grams of force required to puncture the thickness of foliage using a 1-g, 0.60-mm pin (Sands and Brancatini, 1991).

Nitrogen

Foliage was oven-dried at 55°C for 48 hr and ground to pass through a 1-mm sieve by using a cyclone grinder, then further oven-dried at 70°C for 24 hr. Following this,

a sulfuric acid and hydrogen peroxide digest was performed following methods of Lowther (1980). Digested samples were colorimetrically analyzed for nitrogen (QuikChem reference 10-107-06-2E, Lachat Instruments, WI, USA) on a continuous flow injector analyzer (QuikChem 800, Lachat Instruments). Results are expressed as % DM.

Fiber

Ground samples were analyzed for plant cell-wall components of neutral detergent fiber, acid detergent fiber, and lignin following methods in the ANKOM^{200/220} Technology Operator's Manual (1997). Results are expressed as % DM.

Total Oils

Fresh-frozen foliage was thawed prior to oil and phenolic assays. Oils were extracted following methods modified from Jones et al. (2002). Briefly, 1 g of foliage was cut into 1-cm² pieces and soaked in 10 ml dichloromethane for 1 hr. Extracts were analyzed by combined gas chromatography–mass spectrometry (GC-MS) as detailed in O'Reilly-Wapstra et al. (2004). Total ion currents were determined for the sum of all oil components (total oils) and the heptadecane internal standard. Results for total oils were standardized by dividing by the internal standard. Results are expressed as mg “cineole equivalents” per g DM.

Total Phenolics

One gram of foliage was cut into 1-cm² pieces, homogenized with a Polytron Homogenizer (POLYTRON[®] MR2100, Kinematica AG, Switzerland) in 20 ml acidified (pH 1) methanol (Close et al., 2001), and then boiled for 1.5 min. Samples were extracted overnight at 5°C in the dark, centrifuged at 10,000 rpm for 7 min, and analyzed by high-performance liquid chromatography, detailed in O'Reilly-Wapstra et al. (2004). Differences between major classes of phenolic compounds, the hydrolyzable tannins, and the formylated phloroglucinol compounds (FPCs) only were identified. Representative chromatograms of oils and phenolics were used as a visual reference to demonstrate differences in PSM composition between *E. regnans* and *E. globulus*.

Urine Collection

On night 4 of each treatment, four of the six possums (because of logistic constraints) were placed in individual metabolism cages (60 × 60 × 45 cm) for 24 hr for urine collection at the School of Pharmacy, University of Tasmania. Possums were fed with their allocated treatment diets of fresh foliage overnight. Urine was collected into containers held in liquid nitrogen and frozen immediately, then stored at –20°C until later analysis. Animals were acclimatized to the metabolism cages for a 24-hr period 1 week before commencement of the feeding trial. Urinary properties of pH, titratable ions, ammonia, and urea were measured for *N* = 3 possums because of low urinary output of one animal during the collection period. Ammonia and urea methods were modified methods of Faulkner and King (1970). The titratable ion variable was based on the concept of titratable acids (Foley, 1992).

pH

Urine samples were thawed and pH was measured with a TPS digital pH meter.

Titrateable Ions

Either 0.5 M HCl (for basic urine solutions) or 0.01 M NaOH (for acidic urine solutions) was used to titrate urine samples to pH 7.4 (neutral). Titrations were performed at 20°C, and results are expressed as mmol l⁻¹ to pH 7.4.

Ammonia

Tubes were set up with 0.1, 0.4, and 1.0 ml of 1:10 dilutions of urine and made up to 1.0 ml with deionized H₂O. Tubes containing a standard ammonium sulfate solution [800 nmol NH₄⁺ ml⁻¹; derived from 53 ml of 0.1% ammonium sulfate (Ajax Chemicals, Sydney, Australia) solution diluted in 1 l deionized H₂O] were also set up. To each tube, 5 ml of phenol–nitroprusside reagent [10.0 g phenol (May & Barker Ltd., Dagenham, England) and 0.05 g sodium nitroprusside (May & Baker Ltd.) dissolved in 1 l deionized H₂O] were added and mixed by vortex. Following this, 5 ml of NaOH–hypochlorate reagent [5.0 g NaOH (Sigma, St. Louis, MO, USA) and 44 ml of 4% sodium hypochlorite (Aldrich, Milwaukee, WI, USA) dissolved in 1 l deionized H₂O] were added to tubes and mixed by vortex. Tubes were incubated at 37°C for 30 min, and absorbance was read at 570 nm against the reagent blank. Results are expressed as μmol d⁻¹.

Urea

Tubes were set up with 0.1, 0.4, and 1.0 ml of 1:500 dilutions of urine and made up to 1.0 ml with deionized H₂O. Tubes containing a standard urea solution [250 nmol ml⁻¹; derived from 30.0 mg urea (AnalaR[®], Kilsyth, Australia) diluted in 2 l deionized H₂O] were also set up. To each tube, 0.1 ml of urease suspension [100.0 mg urease [Sigma] dissolved in 100 ml phosphate–EDTA buffer [made from 2.0 g Na₂EDTA (BDH Chemicals Ltd., Poole, England) and 5.7 g anhydrous Na₂HPO₄ (Ajax Chemicals, Auburn, Australia) dissolved in 100 ml deionized H₂O, adjusted to pH 6.5 with 1 M HCl (Ajax Chemicals), then made up to 200 ml with deionized H₂O]], stored on ice, were added and mixed by vortex, then incubated at 37°C for 15 min. Following this, 5 ml of phenol–nitroprusside reagent were added to tubes and mixed, followed by 5 ml of NaOH–hypochlorate reagent (see *Ammonia* in [Methods and Materials](#)), with tubes mixed by vortex. Tubes were incubated at 37°C for 30 min, and absorbance was read at 570 nm against the reagent blank. Results are expressed as μmol d⁻¹.

Feeding Behavior

Feeding behavior was measured for all six possums from nights 1–3. Animals were filmed nightly from 18:00 until 08:00 hr using individual B&W Bullet CCD Cameras (equipped with a Samsung[™] sensor, JayCar, Hobart, Tasmania), one camera per cage. Each camera was connected to a Panasonic[®] Video Cassette Recorder (Series NV-FJ630). An 80-W red flood lamp (Osram Par 38, manufactured in EC) was

positioned in each cage for enhanced visibility. Video footage was recorded directly onto BASF E-300 cassette tapes by using extended long-play recording speed. Data from video footage were summarized using The Observer[®] (v 4.1, Noldus Information Technology, The Netherlands, 2002). Behavioral variables were calculated using The Observer[®] and Microsoft[®] Excel (Microsoft Corporation, 1997). These variables were (1) time from first to last feeding bout (incorporating nonfeeding activity); (2) total feeding time; (3) rate of intake; (4) number of feeding bouts; (5) time per feeding bout; (6) intake per feeding bout; (7) total number of switches; and (8) the frequency of feeding bouts within which switching occurred. A single feeding bout was defined as the time from the possum's first bite to the end of its last chew, with at least 1 min of nonfeeding directly following this last chew (Wiggins et al., 2006). Total number of switches was a count of switches between plant species, which could occur both during and between feeding bouts. The frequency of feeding bouts within which switching occurred was calculated by dividing the total number of "switching bouts" per night by the total number of feeding bouts for that night. Switching behavior could only be measured on the mixed diet. Results for all intake and behavioral variables are expressed as mean values \pm standard errors (SE).

Statistical Analyses

The average of 3 d data for each treatment for all six possums was used in the analyses. Dependent variables of intake and aspects of feeding behavior were tested against the independent variables of possum, treatment, period, and carryover, using the general linear model procedure (PROC GLM) in SAS (SAS v6.12, SAS Institute Inc. 1989), following the methods of Ratkowsky et al. (1993). The Wilk-Shapiro statistic, normal probability plots, and standardized residual plots all indicated normality of the data. When an effect was significant, pairwise comparisons of the least-squares means were made by using the Tukey-Kramer adjustment. Data for three possums from d 4 were used for urine analysis. Dependent variables of intake, pH, titratable ions, ammonia, and urea were tested against the independent variables of possum, period, and treatment (PROC GLM, SAS Institute Inc. 1989). Differences between foliar physical and chemical properties of *E. regnans* and *E. globulus* were also tested (PROC GLM, SAS Institute Inc. 1989).

Results

Foliage Chemistry

Leaf percentage DM, leaf toughness, and nitrogen were similar between *E. regnans* and *E. globulus* foliage (Table 1). Of the primary constituents, only fiber differed between the two species (Table 1). Of the secondary metabolites, *E. regnans* contained higher levels of total oils than *E. globulus* (Table 1). Of the 15 major oil compounds identified, only one compound, bicyclogermacrene, was present in both plant species, with amounts similarly low in both species (Fig. 1). There were also major differences in phenolic profiles between plant species. The FPCs peaking between 9 and 11 min in *E. globulus* are not present in *E. regnans* (Fig. 2), whereas differences in the early major peaks indicate the presence of different hydrolyzable

Table 1 Physical and chemical constituents of sapling foliage of *Eucalyptus regnans* and *E. globulus*^a

Constituent	Units	<i>E. regnans</i>	<i>E. globulus</i>	<i>F</i> value	<i>P</i> value
Dry matter	DM	43.1 ± 1.5	39.7 ± 1.5	$F_{1,23} = 2.50$	0.128
Toughness	Grams force	165.9 ± 3.8	171.1 ± 3.8	$F_{1,23} = 0.93$	0.345
Nitrogen	% DM	1.3 ± 0.1	1.2 ± 0.1	$F_{1,23} = 0.83$	0.371
NDF	% DM	32.4 ± 0.9	29.8 ± 0.0	$F_{1,23} = 4.18$	0.053
ADF	% DM	27.4 ± 0.6	21.4 ± 0.6	$F_{1,23} = 45.68$	0.001
Lignin	% DM	15.4 ± 0.8	5.9 ± 0.8	$F_{1,23} = 63.15$	0.001
Total oils	Cineole equivalents (mg g DM ⁻¹)	36.1 ± 4.7	22.1 ± 4.7	$F_{1,23} = 4.36$	0.049

NDF = neutral detergent fiber, ADF = acid detergent fiber.

^a*N* = 12 for each species.

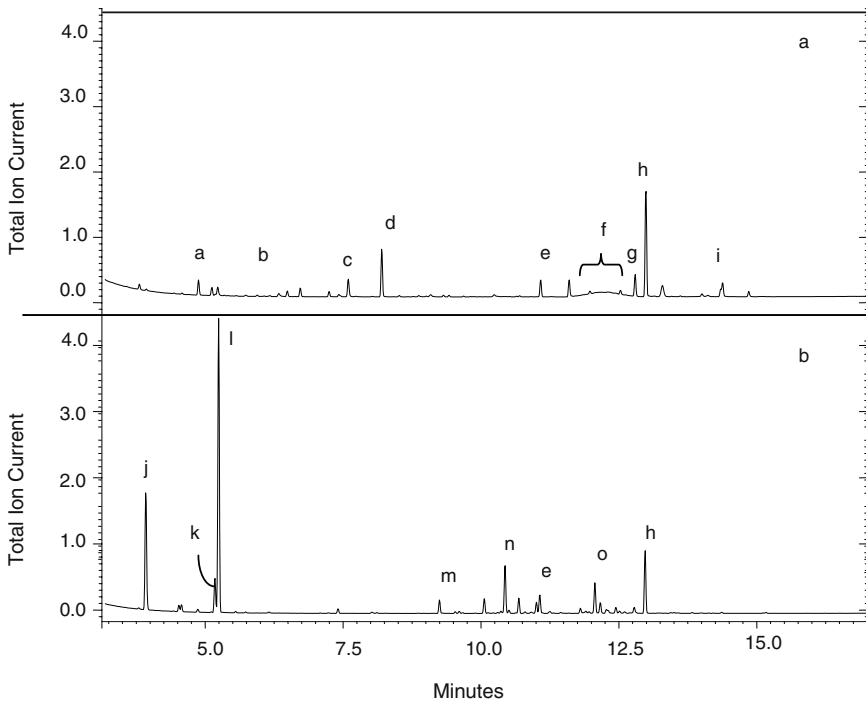


Fig. 1 Representative chromatograms of (a) *E. regnans* and (b) *E. globulus* oils derived from combined gas chromatography–mass spectrometry. Compounds are labeled with assigned letters, which stand for (a) α -phellandrene; (b) β -phellandrene; (c) piperitol; (d) piperitone; (e) bicyclogermacrene; (f) hedycaryol/elemol conversion; (g) β -eudesmol; (h) *n*-heptadecane (internal standard); (i) eudesmyl acetate; (j) α -pinene; (k) limonene; (l) 1,8-cineole; (m) terpinyl acetate; (n) aromadendrene; and (o) globulol

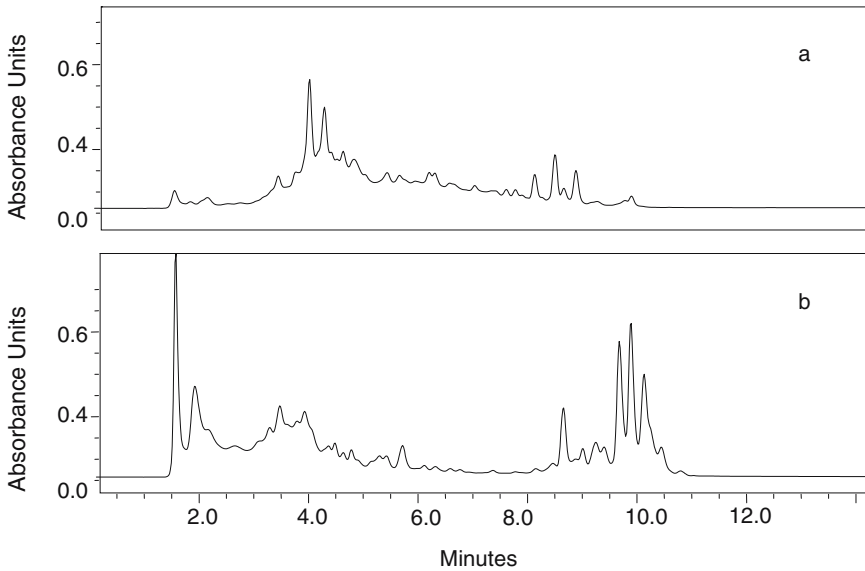


Fig. 2 Representative total phenolic profiles for (a) *E. regnans* and (b) *E. globulus* using high-performance liquid chromatography chromatograms at 280 nm

tannins in each species (peaks substantially differ from 1 to 4 min and from 4 to 6 min between the foliage species).

Intake

Possoms consumed substantially more foliage on the mixed- than on the single-species diets ($F_{2,17} = 14.99$, $P = 0.001$; Fig. 3).

Urinary Properties

Possoms produced a slightly acidic urine on *E. globulus* foliage, alkaline on *E. regnans* foliage, and urine at relatively neutral pH on the mixed diet ($F_{2,8} = 9.20$, $P = 0.032$; Fig. 4). Urine from the *E. regnans* single-species diet required the greatest titration to return it to neutral pH ($F_{2,8} = 7.68$, $P = 0.043$). Urinary properties of ammonia ($F_{2,8} = 0.95$, $P = 0.458$) and urea ($F_{2,8} = 1.42$, $P = 0.343$) were not significantly different between diets (Fig. 3).

Feeding Behavior

Possoms spent longer feeding on the mixed and *E. globulus* single-species diets than on the *E. regnans* single-species diet ($F_{2,17} = 20.15$, $P = 0.002$; Fig. 5a). They ate faster on the mixed diet than the respective single-species diets ($F_{2,17} = 4.53$, $P = 0.049$; Fig. 5b). Possoms did not alter the number of feeding bouts per night (mean 11.8 ± 1.3 , $P = 0.209$), but their time per feeding bout ($F_{2,17} = 7.55$, $P = 0.014$; Fig. 5c) and intake per feeding bout ($F_{2,17} = 8.09$, $P = 0.008$; Fig. 5d) were least on *E. regnans* and generally greater on the mixed diet. There was no difference between the time

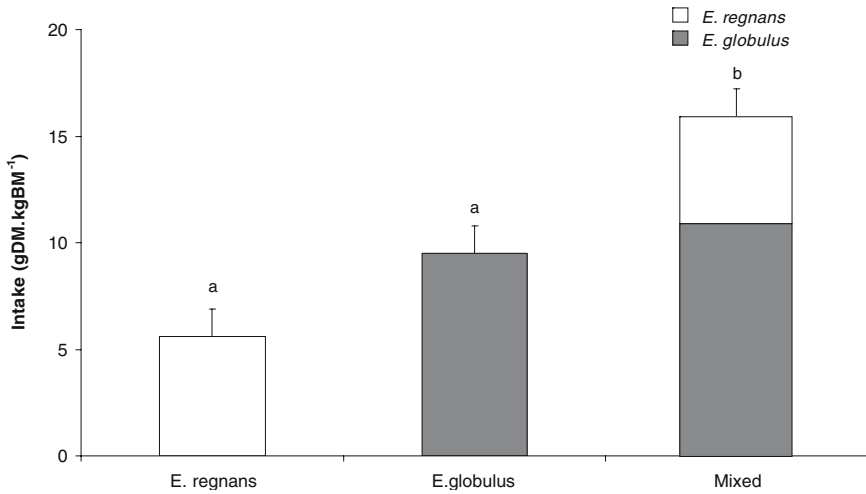


Fig. 3 Total intake [g DM (kg BM)^{-1}] of *E. regnans* and *E. globulus* foliage by brushtail possums across diet treatments. Values are least-squares means with SE bars for total intake. Letters that differ are significantly different ($\alpha = 0.05$ after Tukey-Kramer adjustment for multiple comparisons)

from the first to last feeding bout in response to treatment diets (mean 7.0 ± 0.2 hr, $P = 0.113$).

On the mixed diet, possums switched between foliage both within and between feeding bouts 10.6 ± 0.9 times per night. Of these, 46% of switches (4.4 ± 0.4) occurred within feeding bouts, whereas 54% (6.3 ± 0.4) occurred between feeding

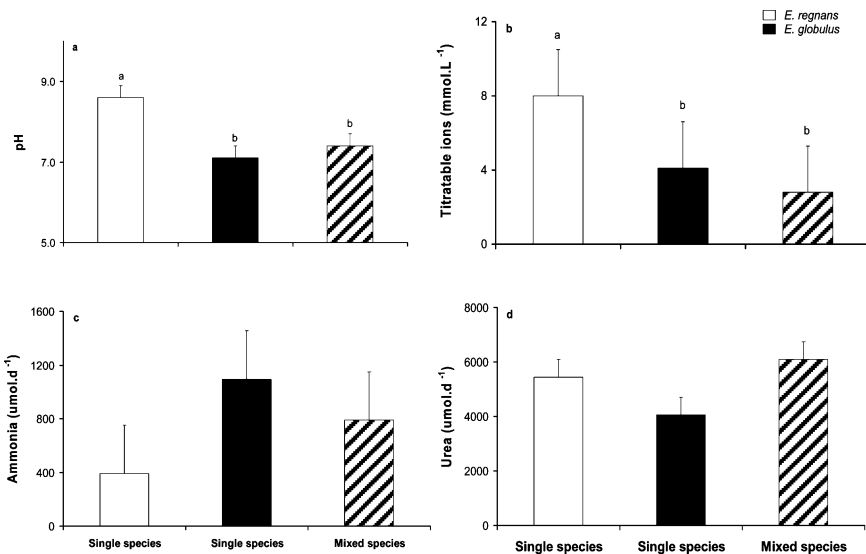


Fig. 4 Urinary properties for brushtail possums fed *E. regnans* and *E. globulus* foliage across treatment diets. (a) pH; (b) titratable ions (mmol l^{-1}); (c) ammonia ($\mu\text{mol d}^{-1}$); and (d) urea ($\mu\text{mol d}^{-1}$). Values are least-squares means for three possums with SE bars. Letters that differ are significantly different ($\alpha = 0.05$ after Tukey-Kramer adjustment for multiple comparisons)

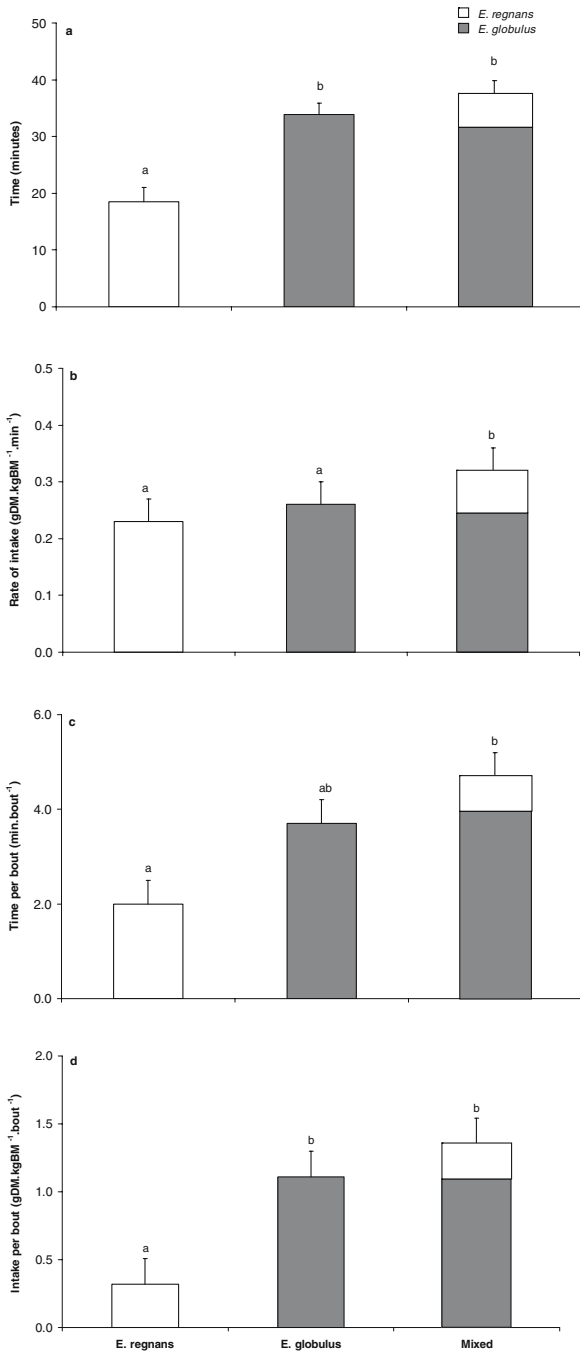


Fig. 5 (a) Total feeding time (minutes); (b) rate of intake [g DM (kg BM)⁻¹ min⁻¹]; (c) time per feeding bout (min bout⁻¹); and (d) intake per feeding bout [g DM (kg BM)⁻¹ bout⁻¹] of *E. regnans* and *E. globulus* foliage by brushtail possums across diet treatments. Values are least-squares means with SE bars for total intake. Letters that differ are significantly different ($\alpha = 0.05$ after Tukey-Kramer adjustment for multiple comparisons)

bouts. Of the within-bout switching figure, possums switched more often after 50% of their total feeding bouts had passed (3.0 ± 0.2 times) than before (1.5 ± 0.2 times; mean difference 1.2 ± 0.2 , $P = 0.001$).

Discussion

Foliar Chemical Properties

Foliar secondary chemical constituents of total oils and total phenolics differed substantially between *E. regnans* and *E. globulus*, as demonstrated by the minimal overlap in the major oils identified for either species and the absence of known herbivore feeding deterrents, the FPCs (O'Reilly-Wapstra et al., 2004), in *E. regnans*. There were also differences in the types of hydrolyzable tannins present in either species. This result confirms major differences between the foliar secondary chemistry in the two species, as previously demonstrated (Wiggins et al., 2006).

Herbivore Urinary Status

Consumption of each diet treatment generated different urinary characteristics in the common brushtail possum (*T. vulpecula*). Brushtail possums produced highly alkaline urine on the *E. regnans* single-species diet, slightly acidic urine on the *E. globulus* single-species diet, and relatively neutral urine on the mixed diet. Previous studies have shown that the consumption of *Eucalyptus* diets generally creates acidic urine to varying degrees, as a result of high buffering activity (as indicated by levels of ammonia excretion) because of organic acid production from the metabolism of dietary terpenes (Hamm and Simon, 1987; Foley, 1992; Foley et al., 1995). Interestingly, the consumption of *E. regnans* did not generate an acid urine. In fact, it was highly alkaline and showed similarities with urine of both brushtail possums and desert woodrats (*Neotoma stephensi* and *N. albigula*) consuming an artificial maintenance diet of fruit and vegetable matter (Dearing et al., 2000; Wiggins et al., unpublished data). The generation of alkaline urine may explain why the urinary properties of pH, ammonia, and urea indicated minimal disturbances to the acid–base status of possums feeding on *E. regnans*, as these measurements have only previously been used to demonstrate changes in the physiological properties of acid urine (e.g., Foley, 1992). Therefore, with the examination of acid and alkaline urine, titratable ions appear to be the most relevant indicator of herbivore physiological status. What the urinary results do suggest, more importantly, is that possums deal with dietary PSMs in *E. regnans* differently from *E. globulus* (confirming our premise for using these two species to test the effects of mixed diets).

Intake and Behavioral Responses

Brushtail possums ate more on a mixed diet of *E. regnans* and *E. globulus* foliage than on their respective single-species diets. This result is consistent with previous research (Dearing and Cork, 1999; Wiggins et al., 2003, 2006) and offers support for the predictions of the detoxification–limitation hypothesis (Freeland and Janzen, 1974). Marsh et al. (2006) recently demonstrated that brushtail possums were only able to eat more on a mixed PSM diet, compared with their respective single-PSM

diets, when individual PSMs were metabolized by using different (major) detoxification pathways. Brushtail possums were unable to eat more on a mixed diet when the PSMs were metabolized with competing detoxification pathways (Marsh et al., 2006). The fact that intake of the mixed diet in our trial was greater than either single-species diet indicates that the chemical properties of *E. regnans* and *E. globulus* foliage were sufficiently diverse to impose different physiological (detoxification) constraints to brushtail possums.

Brushtail possums ate more on the mixed-species than either of the single-species diets by eating faster, while tending to spend more time and eating more per feeding bout. They frequently switched between both species of foliage on the mixed diet, with the number of within-bout switches increasing in the latter half of their feeding bouts. By frequently switching between foliage on the mixed diet, possums may have been able to overcome the short-term intake constraints because of the toxic effects of each species. Wiggins et al. (2006) proposed that diet switching was a direct behavioral response to the detoxification limitations of a PSM-rich diet. That is, when intake on one species is constrained because of saturation of specific detoxification pathways, possums can continue feeding on the other species. They demonstrated that brushtail possums were only able to maximize their intake when able to frequently switch between dietary species during their nightly feeding activities: when foliage time and availability constraints were imposed on possums, they were unable to maintain intake (Wiggins et al., 2006). Results of this current study expand on this hypothesis through a more detailed investigation of the frequency and occurrence of dietary switching.

By switching between foliage more frequently during the latter half of their feeding bouts, we suggest that brushtail possums were responding to the accumulation of PSMs and/or detoxification metabolites over their nightly feeding timeframe. Brushtail possums have been shown to regulate dietary 1,8-cineole ingestion so as not to exceed a critical threshold level over a 3-hr feeding timeframe, but cineole metabolites accumulated throughout that sampling period (Boyle et al., 2005). Cessation of feeding on one plant species may have been a direct response to blood PSM levels that were nearing, or had indeed reached, a specific critical threshold level. By immediately switching to the next available plant species, possums may have been attempting to regulate PSM ingestion of *E. regnans* while continuing to feed on *E. globulus*. Increasing the frequency of this switching behavior during the latter stages of their feeding activities presumably reflects that detoxification limitations were at their most critical stages for both species of foliage. This result strengthens the argument that diet switching in a generalist mammalian folivore is a behavioral response to detoxification limitations of a PSM-rich diet (Wiggins et al., 2006).

The ability to frequently switch between foliage, which was not an option on the single-species diets, enabled possums to feed more efficiently, through a faster feeding rate and greater feeding bout size and length. These behavioral responses to a physiologically constraining diet represent an important facet that helps explain why, and how, generalist mammalian herbivores are able to benefit from a chemically diverse diet.

In summary, the common brushtail possum was able to benefit from the consumption of a chemically diverse diet, even over a short feeding timeframe. Possums fed more efficiently on a mixed- than on a single-species diet and achieved increased intake. We propose that diet switching is a behavioral strategy adopted by *T.*

vulpecula as a direct response to the physiological limitations of detoxification pathways, enabling them to efficiently consume a chemically diverse diet.

This integration of dietary chemical properties, animal physiological, and behavioral data has enabled us to test predictions of the detoxification–limitation hypothesis. Herbivore feeding behavior (e.g., diet switching) may be a useful indicator of the physiological constraints (e.g., detoxification limitations) imposed by a PSM-rich diet. Understanding the underlying processes that govern the intake of generalist herbivores is important: the way in which a herbivore is physiologically constrained by its diet, and the resulting behavioral responses, will directly impact on the herbivore's foraging decisions, and subsequently their foraging energetics.

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Salivary Proteins as a Defense Against Dietary Tannins

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Abstract Tannins, a diverse group of water-soluble phenolics with high affinity to proteins, are widely distributed in various parts of plants, and have negative effects in herbivores after ingestion. Some mammalian species are thought to counteract tannins by secreting tannin-binding salivary proteins (TBSPs). Several types of TBSPs are found in the saliva of laboratory animals, livestock, and wildlife. Among them, proline-rich proteins (PRPs) and histatins are effective precipitators of tannins. It is widely accepted that, at the least, PRPs act as a first line of defense against tannins. Many observations support this idea: *in vitro* affinity of PRPs to tannins is far higher than that of other proteins such as bovine serum albumin; complexes formed between PRPs and tannins are stable even under the conditions in the stomach and intestine; and PRP production is induced by ingesting tannins. It is believed that species that usually ingest tannins as part of their natural diets produce high levels of PRPs, whereas species not exposed to tannins produce little or no PRPs. This hypothesis is generally supported, although studies on TBSPs in wildlife are limited. This work stresses the importance of gathering basic information on such items as the characteristics of unidentified TBSPs, and seasonal and geographical variations in PRP production.

Keywords Affinity to tannins · Defense mechanisms against tannins · Feeding niche · Histatins · Nitrogen costs · Proline-rich proteins (PRPs)

Introduction: Tannins and Mammalian Countermeasures

Tannins, which are among the most widespread plant secondary metabolites (PSMs), are a diverse group of water-soluble phenolic compounds with high affinity for proteins. They are broadly distributed in various parts of plants (Blytt et al., 1988; Bernays et al., 1989; Waterman and Mole, 1994). On the basis of their chemical structures, tannins can be divided into two major groups: condensed and hydrolyzable (Zucker, 1983). Condensed tannins are oligomers and polymers of

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flavan-3-ols that are linked by C–C bonds and thereby difficult to hydrolyze. In contrast, hydrolyzable tannins are esters of polyols (usually glucose) with simple phenolic acids, such as gallic acid or hexahydroxydiphenic acid, and are readily hydrolyzed by acidic or basic conditions as well as by esterase enzymes.

Ingestion of tannins is known to cause various types of negative effects in mammalian herbivores, such as reduction in digestibility (Robbins et al., 1987; Chung-MacCoubrey et al., 1997; Shimada and Saitoh, 2003), damage to the gastrointestinal mucosa and epithelium (Blytt et al., 1988), kidney or liver failure (Fowler and Richards, 1965), and endogenous nitrogen loss (Blytt et al., 1988; Shimada and Saitoh, 2003). It has long been believed that tannins primarily act as protein digestion inhibitors by binding dietary proteins and digestive enzymes (see review by Zucker, 1983). However, recent studies have revealed that tannins do not bind to digestive enzymes in *in vivo* situations, because digestive enzymes are usually protected from tannins by occurring in a particulate membrane-bound form (Blytt et al., 1988; Bernays et al., 1989; Skopec et al., 2004). Instead, ingested tannins probably bind salivary proteins and the mucosa and epithelium of the gut. This may cause endogenous nitrogen loss, thereby reducing protein digestibility. Moreover, phenolic acid released by hydrolysis of tannins can be absorbed from the intestine and cause acute toxicity, such as necrosis and kidney and liver failure (Fowler and Richards, 1965; Niho et al., 2001). For these reasons, tannins should now be regarded not as digestion inhibitors that act relatively mildly, but as toxins that have acute negative effects on herbivores.

Animals can use both preingestive and postingestive countermeasures against dietary tannins. Avoiding highly tanniferous species or parts of plants is the simplest preingestive countermeasure. This behavior has been frequently reported in various taxa, such as ungulates (Cooper and Owen-Smith, 1985), rodents (Shimada and Saitoh, 2003), lagomorphs (Clausen et al., 1990; Dearing, 1997), and primates (Takemoto, 2003). Some herbivores have been shown to regulate their rates of intakes of tannins or other PSMs in order to circumvent overloading of one detoxification pathway (Dearing and Cork, 1999; Burritt and Provenza, 2000; Foley and Moore, 2005). Furthermore, reduction of tannin levels in plants through leaching during the hoarding period can be considered as a preingestive countermeasure (Dearing, 1997). Postingestive countermeasures, which mammalian herbivores are known to use, include the secretion of tannin-binding proteins in saliva, increased gastrointestinal mucus production, degradation of tannins by microorganisms in the gut, activation of detoxifying enzymes, and increased capacity of intestinal permeability glycoprotein (see reviews by McArthur et al., 1991; Dearing et al., 2005). These are thought to be key elements in overcoming, or at least mitigating, the negative effects caused by tannins.

This review concerns tannin-binding salivary proteins (TBSPs) as a defense against dietary tannins. TBSPs, which are defined as proteins secreted in saliva that have high affinity for tannins, can bind readily to dietary tannins in the oral cavity and are thought to prevent the tannins from interacting with other proteins (McArthur et al., 1995; Bennick, 2002). TBSPs interact with ingested tannins in the first stage of digestion. This feature stresses the importance of TBSPs in counteracting tannins, because the behavior of ingested tannins in the gastrointestinal tract may be inevitably regulated by these early encounters with TBSPs. In the last two decades, a considerable number of studies have been undertaken on TBSPs (Ann and Carlson, 1985; Asquith et al., 1985; Austin et al., 1989; Robbins et al., 1991;

Glendinning, 1992; Carlson, 1993; Hagerman and Robbins, 1993; Bacon and Rhodes, 2000; Gehrke, 2001; Isemura, 2003). However, most have been conducted on humans and laboratory animals. This is partly because TBSPs originally attracted attention in the field of dental science and nutrition. As a consequence, information on TBSPs in wildlife is still limited and scattered. In addition, studies on TBSPs have been relatively biased toward *in vitro* data, such as genetics, amino acid compositions, protein structures, and *in vitro* affinity to tannins. Thus, the straightforward question—to what extent TBSPs would be effective in overcoming the potential negative effects of tannins in herbivores—is still open, even in laboratory animals.

The aims of this review are to summarize the information on TBSPs (obtained mostly in laboratory animals) and to discuss the significance to wildlife of producing TBSPs.

General Characteristics of Tannin-Binding Salivary Proteins

Two families of salivary proteins, proline-rich proteins (PRPs) and histatins, are generally recognized as TBSPs. However, there is evidence for the presence of other, different types of proteins. These are characterized by their high affinity to tannins, but they differ greatly in terms of molecular size, amino acid composition, structure, and taxonomic distribution in mammals.

Proline-Rich Proteins

PRPs were first detected in human saliva (Mandel et al., 1965). They have also been found in the saliva or salivary glands of several laboratory animals and wildlife (Table 1). In these mammals, PRPs are thought to be the most prevalent group of proteins in the saliva; PRPs account for 70% of all salivary proteins in humans (Mehansho et al., 1987b) and 74% of those in the Japanese wood mouse *Apodemus speciosus* (T. Shimada, unpublished data).

The molecular weights of PRPs are estimated to range from a low of 5000 to over 25,000 (McArthur et al., 1995; Bennick, 2002). As their name suggests, the amino acid composition of PRPs is unique in that the content of proline is markedly high. Proline comprises at least 20% of total amino acid content of PRPs (Kauffman and Keller, 1979; Mole et al., 1990). Some species, such as humans, rats, and mice, produce PRPs containing about 40% proline (Mehansho et al., 1983, 1985; Mole et al., 1990). In light of the fact that most types of proteins contain, at most, 5% proline (Schulz and Schirmer, 1979), PRPs have a markedly characteristic amino acid composition. They are also rich in glycine, glutamine, and glutamic acid. Mole et al. (1990) compared the amino acid compositions of PRPs from several species and found that in all cases the sum of the quantities of these four amino acids accounted for 70–90% of the total protein. These four amino acids, especially proline, are the so-called alpha-helix breakers, which prevent proteins from forming secondary structures. As a consequence, PRPs do not have any recognizable conformational structures, thereby existing in solution as extended random coils (Hagerman and Butler, 1981; Murray and Williamson, 1994). This feature may enable PRPs to universally bind various types of tannins that have a variety of shapes and sizes.

PRPs are encoded by tissue-specific multigene families. The nucleotide sequences of PRP cDNAs from mouse (Clements et al., 1985), rat (Ziemer et al., 1984), human

Table 1 List of mammalian species, in which the presence of tannin-binding salivary proteins (TBSPs) has been examined, and the summary of the results

Order	Species		Feeding niches	Types of TBSPs	Induced secretion ^a		Reference
	Common name	Scientific name			By tannins	By β -agonist	
Rodentia	Mouse	<i>Mus musculus</i>	Omnivore	PRPs ^b	yes	yes	Mehansho et al., 1985; Glendinning, 1992
	Rat	<i>Rattus norvegicus</i>	Omnivore	PRPs	yes	yes	Mehansho et al., 1983; Mehansho and Carlson, 1983; Mole et al., 1990
	Japanese wood mouse	<i>Apodemus speciosus</i>	Omnivore	PRPs	yes	yes	Shimada et al., 2004
	Root vole	<i>Microtus oeconomus</i>	Intermediate	PRPs	yes	yes	Juntheikki et al., 1996
	Meadow vole	<i>Microtus pennsylvanicus</i>	Grazer	Not detected ^c		no	Dietz et al., 1994
Lagomorpha	Hamster	<i>Mesocricetus auratus</i>	Omnivore	PRPs	no	yes	Mehansho et al., 1987a
	Beaver	<i>Castor canadensis</i>	Bark eater	Unidentified ^d			Hagerman and Robbins, 1993
	Rabbit	<i>Oryctolagus cuniculus</i>	Intermediate	PRPs			Mole et al., 1990; Ferreira et al., 1992
	Mountain hare	<i>Lepus timidus</i>	Intermediate	PRPs			Mole et al., 1990
Artiodactyla	North American pika	<i>Ochotona princeps</i>	Intermediate	Unidentified			Dearing, 1997
	Sheep	<i>Ovis aries</i>	Grazer	Not detected	no		Austin et al., 1989; Mole et al., 1990
	Musk ox	<i>Ovibos moschatus</i>	Intermediate	PRPs			Vaithyanathan et al., 2001
	Mouflon	<i>Ovis orientalis</i>	Grazer	Unidentified			Gehrke, 2001
	Goat	<i>Capra hircus</i>	Intermediate	Unidentified			Vaithyanathan et al., 2001
	Cattle	<i>Bos taurus</i>	Grazer	Not detected	no		Austin et al., 1989; Mole et al., 1990
	Fallow deer	<i>Dama dama</i>	Intermediate	Other type ^e	no		Makkar and Becker, 1998; Gehrke, 2001
	Mule deer	<i>Odocoileus hemionus</i>	Intermediate	PRPs	no		Gehrke, 2001
							Austin et al., 1989; Hagerman and Robbins, 1993

	White-tailed deer	<i>Odocoileus virginianus</i>	Grazer	Other type	Mole et al., 1990
	Roe deer	<i>Capreolus capreolus</i>	Browser	PRPs	Fickel et al., 1998; Gehrke, 2001
	Moose	<i>Alces alces</i>	Intermediate	PRPs	Hagerman and Robbins, 1993; Juntheikki, 1996; Gehrke, 2001
	Reindeer	<i>Rangifer tarandus</i>	Intermediate	Unidentified	Gehrke, 2001
	Pig	<i>Sus scrofa</i>	Omnivore	Not detected	Mole et al., 1990
Perissodactyla	White rhino	<i>Ceratotherium simum</i>	Grazer	Unidentified	Clauss et al., 2005
	Indian rhino	<i>Rhinoceros unicornis</i>	Intermediate	Unidentified	Clauss et al., 2005
	Black rhino	<i>Diceros bicornis</i>	Browser	Unidentified	Clauss et al., 2005
Carnivora	Cat	<i>Felis catus</i>	Carnivore	Not detected	Mole et al., 1990
	Dog	<i>Canis familiaris</i>	Carnivore	Not detected	Mole et al., 1990
	American black bear	<i>Ursus americanus</i>	Omnivore	Unidentified	Hagerman and Robbins, 1993
Primates	Human	<i>Homo sapiens</i>	Omnivore	PRPs	Oppenheim, et al. 1971; Mole et al., 1990
	Crab-eating macaque	<i>Macaca fascicularis</i>	Omnivore	Histatins	Yan and Bennick, 1995
	Common brushtail possum	<i>Trichosurus vulpecula</i>	Browser	PRPs	Oppenheim et al., 1979
Marsupialia	Common ringtail possum	<i>Pseudocheirus peregrinus</i>	Browser	Histatins	Sabatini et al., 1989; Bennick, 2002
	Koala	<i>Phascolarctos cinereus</i>	Browser	Not detected	Mole et al., 1990
	Common ringtail possum	<i>Pseudocheirus peregrinus</i>	Browser	Not detected	Mole et al., 1990
	Koala	<i>Phascolarctos cinereus</i>	Browser	Not detected	Mole et al., 1990

^a These columns indicate the presence of inducibility of TBSP synthesis by intake of tannins or by injection of a β -agonist.

^b Proline-rich proteins (PRPs).

^c Any types of TBSPs have not been found.

^d The presence of TBSPs has been confirmed, but the detail characteristics have not been investigated.

^e The presence of TBSPs, which belong neither to PRPs nor to histatins, has been confirmed.

(Maeda et al., 1985), and crab-eating macaque (Oppenheim et al., 1987), and PRP genes from mouse (Ann and Carlson, 1985), hamster (Ann et al., 1987), and human (Kim and Maeda, 1986) have been reported. All proteins encoded by cDNAs and genes share a common structure consisting of a signal peptide, a transition region, a repeat region, and a carboxyl terminal region. The single peptides and repeated regions exhibit marked homologies among these species, whereas the transition regions and carboxyl terminal regions vary largely (Ann and Carlson, 1985; Clements et al., 1985; Ann et al., 1987). In mice, the PRP gene family is located on chromosome 8 (Azen et al., 1984). In humans, the PRP family is encoded by only six genes located on chromosome 12 (Maeda, 1985; Maeda et al., 1985); two encoding acidic PRPs, and four encoding basic and glycosylated PRPs (Kim and Maeda, 1986). Many of the proproteins encoded by these genes are subsequently cleaved before secretion, giving rise to a large number of secreted PRPs (Lyons et al., 1988). Consequently, more than 20 PRPs are present in human saliva (Bennick, 2002).

PRPs are generally classified as basic (BPRPs) or acidic (APRPs) (McArthur et al., 1995; Bennick, 2002). APRPs are composed of two major regions: the core C-terminal region, comprising 70–80% of the molecule, and the highly acidic N-terminal region. BPRPs are structurally equivalent to the C-terminal region of APRPs. The N-terminal region of APRPs contains little proline, and thereby the proline level of the whole molecule is higher in BPRPs than in APRPs. In humans, BPRPs are secreted only from the parotid glands, whereas APRPs are secreted from all salivary glands, including the parotid, submandibular, and sublingual glands (Kauffman and Keller, 1979). A similar observation has been made in rabbits (Ferreira et al., 1992). This phenomenon may, therefore, be common in mammals that produce salivary PRPs.

BPRPs, in general, have a much higher affinity to tannins than APRPs (Lu and Bennick, 1998), and the main function of BPRPs is regarded as counteracting dietary tannins (Bacon and Rhodes, 2000; Chan and Bennick, 2001). In contrast, APRPs act primarily in maintaining oral homeostasis. An extensive argument for the evolution of the different types of PRPs can be found in the review by McArthur et al. (1995); they hypothesized that the ancestral type is APRPs whose main function is maintaining oral homeostasis, and that BPRPs evolved later when mammals began to consume a diet with a high content of tannins. Interestingly, it has been proved that variation in the molecular structures within BPRPs and APRPs does not largely influence the affinity of PRPs to tannins (Lu and Bennick, 1998).

The affinity of PRPs to tannins differs among mammalian species. It is estimated as 5–80 times higher than that of bovine serum albumin (BSA) and 1000 times higher than that of lysozyme (Asquith et al., 1987; Austin et al., 1989; Mole et al., 1990). This high affinity enables PRPs to act as a first line of defense. Because tannins bind preferentially to PRPs, even in the presence of excessive amounts of other proteins of lower affinity (Asquith and Butler, 1985), PRPs can form stable complexes with tannins in the oral cavity. They thereby prevent tannins from interacting with other proteins, such as enzymes or those in the mucous membranes and epithelia, and then degrading into noxious phenolic compounds (Robbins et al., 1991; Hagerman and Robbins, 1993). The tannin–PRP complexes are also stable in the gastrointestinal tract, and most of the complexes formed are excreted in the feces (Mitaru et al., 1984; Skopec et al., 2004). These reactions of PRPs may present both quantitative and qualitative nitrogen savings to animals. The amount of protein

needed to bind all the tannins can be reduced. In addition, the four major amino acids of PRPs are all nonessential in mammals. This means that nonessential amino acids from PRPs are excreted instead of the dietary or endogenous essential amino acids (McArthur et al., 1995).

Interestingly, some species produce salivary proteins, which are rich in proline, but do not show the high affinity to tannins (Mole et al., 1990). For instance, salivary proteins in some marsupials, such as koalas and common ringtail possums, contain about 30% of proline, but their affinity to tannins is a tenth of that of BSA. Similar observations were also obtained in cattle and pigs (Mole et al., 1990). Some modifications in these proteins, such as extensive glycosylation, might reduce the affinity to tannins. If so, these nontannin-binding proline-rich proteins might include an important clue for studies on the evolutionary process of tannin-binding PRPs. These nontannin-binding proline-rich proteins cannot be regarded as TBSPs because of the lack of high affinity to tannins. Thus, in this review, this type of “proline-rich proteins” is not regarded as PRPs in the following discussion.

Another notable feature of PRPs is that their synthesis is induced by the presence of β -agonists and tannins, but this response varies among species (Table 1). PRPs are constitutively produced at high levels in humans and mule deer (Oppenheim et al., 1971; Austin et al., 1989). In contrast, in rats, mice, and hamsters, synthesis of PRPs is induced by injection of the β -agonist isoproterenol (Mehansho and Carlson, 1983; Mehansho et al., 1985, 1987a). This reaction is also found in the Japanese wood mouse (T. Shimada, unpublished data). PRP synthesis can also be induced by ingesting tannins. This response has so far been confirmed exclusively in rodent species, such as rats (Mehansho et al., 1983), mice (Mehansho et al., 1985), root voles (Juntheikki et al., 1996), and Japanese wood mice (Shimada et al., 2004). In black rhinos, the induction of TBSP synthesis by tannins has also been demonstrated (Clauss et al., 2005). Their TBSPs may also be PRPs, although this has not yet been examined. Interestingly, in hamsters, PRP synthesis is induced by β -agonist injection, but not by ingestion of tannins (Mehansho et al., 1987a). This induction mechanism supports the importance of PRPs as a defense against dietary tannins.

Histatins

Histatins are a group of relatively small proteins with high affinity to tannins; their molecular weight is, in general, less than 5000 (Yan and Bennick, 1995). Histatins are found only in the saliva of humans and some primates (Sabatini et al., 1989; Bennick, 2002). Histatins are characterized by a high content of histidine, which accounts for about 25% of all amino acids present (Yan and Bennick, 1995).

Unexpectedly, histatins in human saliva account for only 2.6% of all salivary proteins (Sugiyama and Ogata, 1993). They readily bind both hydrolyzable and condensed tannins (Yan and Bennick, 1995). The affinity of histatins to tannic acid is estimated to be twice that of gelatin, which is an effective binder of tannic acid. Furthermore, the complexes with tannins are stable in the gut. These findings indicate that histatins may act as defenses against dietary tannins, as do PRPs. However, PRPs are likely to be more important than histatins in defense against tannins if both of them are secreted coincidentally in an animal, because their levels of secretion in saliva are very different.

Histidine is regarded as an essential amino acid for most mammals, but its essentiality in adult humans is supposed to be relatively low (Schmidt-Nielsen,

1997), and it is regarded as semiessential. Assuming that other primates have similar requirements for histidines, this low essentiality of histidine might have enabled the evolution of histatin production in primates, although there is a possibility that histatins may be found in other taxonomic groups.

Other Types of TBSPs

PRPs and histatins are two major groups of TBSPs, but some researchers have found other types of salivary proteins with high affinity to tannins. First, Mole et al. (1990) investigated the presence of TBSPs in several mammals, including laboratory animals, livestock, and wildlife. In the saliva of white-tailed deer, they found TBSPs whose affinity to tannins was six times that of BSA. These salivary proteins contained only 7% proline, and the sum of the four amino acids (proline, glycine, glutamine, and glutamic acid) accounted for only 29% of the total. The molecular weight of these proteins was estimated to be over 10,000. These findings suggest that the salivary proteins from white-tailed deer are neither PRPs nor histatins.

In cattle saliva, Makkar and Becker (1998) found salivary proteins with high affinity to tannins: about six times that of BSA. Their proline content was only 6.5%, and the sum of the four amino acids accounted for about 31% of the total. Ingesting tannins did not alter the amino acid compositions or the affinity of these TBSPs to tannins. These authors speculated that the primary role of these proteins might not be to defend the animals against dietary tannins. Interestingly, Austin et al. (1989) and Mole et al. (1990) did not detect TBSPs in cattle saliva, even with the same isolation methods used by Makkar and Becker (1998).

Gehrke (2001) found TBSPs in the saliva of the fallow deer whose affinities to tannic acid and quebracho tannins (condensed tannins) were almost twice those of cattle saliva. These proteins contained 13% of proline, and the sum of the four amino acids accounted for 35.3% of the total. This proline content is lower than typical PRPs but relatively high as a usual protein. The author concluded that these TBSPs were not PRPs.

It is unknown whether such salivary proteins possess any evolutionary relationship to PRPs or histatins. However, the presence of several types of TBSPs suggests that parallel evolution may have occurred at least several times in herbivorous mammals, that some salivary proteins that originally had other functions may have incidentally acquired a high affinity to tannins and thus consequently have come to act as a defense mechanism against dietary tannins.

TBSPs in Relation to Feeding Niches

The possibility that TBSPs act as defense products against dietary tannins has promoted surveys of the presence of these proteins in various species of mammals. Some of these have tried to link the presence or affinity of TBSPs to the feeding niches of those animals. Table 1 shows the summary of previous studies that examined the presence of TBSPs in various mammalian species. Among 33 species examined, TBSPs are found in 26 species, including two controversial ones—cattle and sheep. The wide prevalence of TBSPs in various taxa implies a staple role in counteracting dietary tannins. Characterizations of TBSPs have been carried in 16

species, but include only 8 species of wild mammals. Thus, our knowledge of TBSPs is quite limited for wildlife.

It is hypothesized that animals with high tannin contents in their natural diets have developed high levels of TBSPs, but that those with low tannin contents produce little or no TBSPs (McArthur et al., 1995; Fickel et al., 1998; Clauss et al., 2005). In general, the leaves of monocots and forbs contain less tannin compared to fruits, seeds, and the leaves of shrubs and trees (Waterman and Mole, 1994). Thus, the above hypothesis implies that browsers, frugivores, and omnivores produce TBSPs at high levels, but grazers should have only small amounts.

Among rodents, rats, mice, and hamsters, which are all omnivores, are confirmed as secreting PRPs (Table 1). Furthermore, Shimada et al. (2004) found PRPs in the saliva of the Japanese wood mouse (an omnivore that is close to being frugivorous). Root voles, which are supposed to be intermediate between grazers and browsers, also produce PRPs in their saliva (Juntheikki et al., 1996). In contrast, meadow voles (grazers) have neither PRPs nor other types of TBSPs (Dietz et al., 1994).

Among ungulates, Austin et al. (1989) found TBSPs in the saliva of mule deer (intermediate), but not in cattle (grazers) or sheep (grazers). Mole et al. (1990) also reported similar results: white-tailed deer (intermediate) produce TBSPs, but sheep and cattle do not. Furthermore, Gehrke (2001) examined the affinity of TBSPs in six species of ungulates and found that a browser (roe deer) and intermediates (reindeer, fallow deer, and musk ox) produce TBSPs with higher affinity to tannins than grazers (cattle and mutton). Similarly, the recent study by Clauss et al. (2005) also supports this hypothesis by comparing the affinity of TBSPs in three species of rhinos with different feeding niches: Indian rhinos (intermediate) and black rhinos (browsers) produce TBSPs with higher affinity than do white rhinos (grazers).

Hagerman and Robbins (1993) examined the affinity between various types of tannins and crude saliva from the moose, beaver, mule deer, and American black bear by using an electrophoretic tannin-binding assay. Interestingly, the black bear (omnivore) produced the most effective TBSPs, which showed high affinity to all types of tannins: linear and branched condensed tannins and two types of hydrolyzable tannins (gallotannin and ellagitannin). TBSPs from the mule deer (intermediate) were more specific than those from the black bear; they could bind to all types of tannins other than ellagitannin. The moose (intermediate) produced TBSPs that bound only to linear condensed tannins. They also found that TBSPs from the beaver also bound only to linear condensed tannins, but their affinity was much lower than those from the mule deer. The main food of beavers is the bark and sapwood of the aspen, birch, willow, and alder, which are tannin-free or contain relatively low levels of condensed tannins.

In this regard, the above hypothesis is mostly supported. However, as already noted, some contradictory studies have found unclassified TBSPs in the saliva of cattle and sheep, which are typical grazers (cattle, Makkar and Becker, 1998; Gehrke, 2001; sheep, Vaithiyanathan et al., 2001).

We note, however, that there are some obstacles that may complicate such comparative studies. The first of such barriers are methodological differences found in various studies—methods for extracting salivary proteins, examining the presence of TBSPs, and quantifying the affinity. Each method likely has a different sensitivity and applicability. Thus, comparisons between different studies require care. Second, these previous comparative studies did not necessarily deal with the same types of TBSPs. In other words, the presence or affinity of TBSPs that may have originated

from different evolutionary processes may complicate the examination of the hypothesis. For instance, among the four species examined in the study of Hagerman and Robbins (1993), the characteristics of TBSPs were determined only in the mule deer and moose, which have been confirmed to have PRPs (mule deer, Austin et al., 1989; moose, Gehrke, 2001). It is possible that TBSPs from the other two species may not have been PRPs. To investigate the evolutionary relationships among feeding niches and salivary proteins as defenses against dietary tannins, we need to characterize the TBSPs in each species.

***In Vivo* Effectiveness of TBSPs**

There are sufficient data to show that TBSPs, and especially PRPs, bind effectively to both condensed and hydrolyzable tannins *in vitro* (Mehansho et al., 1987b; Bennick, 2002). *In vitro* evidence cannot always be applied *in vivo*, because some factors that differ between *in vitro* and *in vivo* situations—such as the state of tannins in the diet and the presence of other dietary chemicals—may interfere with the *in vivo* reaction between tannins and TBSPs. Furthermore, the production of TBSPs may inevitably involve costs *in vivo* that are not considered in *in vitro* conditions (McArthur et al., 1995; Clauss et al., 2003). Thus, to evaluate the significance of TBSPs in counteracting dietary tannins, we need to examine their *in vivo* effects. However, the evidence that TBSPs are effective *in vivo* is limited, partly because it is difficult to measure levels of TBSPs nondestructively. Hereafter, I focused on PRPs, because data on this topic are lacking in other TBSPs.

Glendinning (1992) first demonstrated the significance of PRPs in dealing with tannins *in vivo* by using mice injected with isoproterenol (a β -agonist) to enhance PRP secretion. The isoproterenol-injected mice exhibited a greater preference for a tannin-rich diet compared to controls. Mole et al. (1993) found that injection of a β -antagonist (propranolol), which prevents PRP synthesis, amplified the negative effects of tannins in rats. Recently, Skopec et al. (2004), using fecal proline level as an index of secreted PRPs, demonstrated clearly that induction of PRP synthesis in rats reduces the negative effects of dietary tannins by preventing tannins from breaking up into small phenolics and being absorbed. In a similar way, Jansman et al. (1994), also using rats and fecal proline level as an index, revealed that PRPs are effective as defenses against tannins, but that this defense is incomplete when dietary tannins are not readily extracted from the diet during chewing. In addition, Shimada and coworkers (2004, 2006) suggested the significance of PRPs in counteracting tannins in terms of interindividual differences in secretion levels of PRPs in the Japanese wood mouse: individuals with high levels of PRPs were less sensitive to tannins in acorns.

Until now, *in vivo* evidence noted above has been obtained only from rodents. It is noteworthy that all of these rodents can induce PRP synthesis by ingesting tannins. This inducibility is presumably an adaptive mechanism that reduces the costs arising from PRP production in animals whose natural diet varies in tannin content. In other words, the *in vivo* effectiveness of PRPs as defenses against tannins is mostly untested in animals that, unlike rats and mice, do not have this feedback mechanism to reduce the costs involved in PRP synthesis. This topic is further discussed in the [next section](#).

Cost and Inducibility of PRP Synthesis

In this section, strategies for producing PRPs are argued in the light of the costs and inducibility of PRP synthesis. Production of PRPs may involve some costs in terms of metabolism and nitrogen balance. PRPs are poorly digested, because many peptide bonds involving proline are difficult to cleave (Muenzer et al., 1979; McArthur et al., 1995). Thus, production of excess PRPs may have a nitrogen cost, even if they consist mostly of nonessential amino acids.

There is some evidence for costs arising from PRP production. In rats, Haghight et al. (1996) found that injection of a β -agonist induced PRP synthesis, but by 15 d after the end of injections the production of PRPs had ceased. My colleagues and I have obtained similar results in the Japanese wood mouse (Shimada et al., unpublished data). The rapid termination of PRP synthesis observed in these studies implies that it is costly to keep producing them. Furthermore, Skopec et al. (2004) have demonstrated nitrogen costs involved in producing PRPs: the apparent nitrogen digestibility of a diet containing 3% pentagalloyl glucose (a hydrolyzable tannin) in PRP synthesis-induced rats was over 20% lower than in control rats. This reduction in nitrogen digestibility may be mostly attributable to excretion of the PRP–tannin complexes.

The costs of producing PRPs may be overcome by the benefits of PRPs when animals consume diets containing tannins. However, if the diet is tannin-free or includes little tannin, animals may still incur the costs involved in PRP production. Induction of PRP synthesis in response to tannin injection may reduce the costs of production. Animals whose natural diets vary in tannin content may benefit from such a feedback mechanism (McArthur et al., 1995; Bennick, 2002). The inducibility of PRP synthesis by tannins seems to have been reported only in rats, mice, root voles, and Japanese wood mice. As expected, the natural diet of the two wild rodents (excluding rats and mice) varies seasonally (Juntheikki et al., 1996). For instance, the Japanese wood mouse varies its primary food items seasonally; in summer these are soft plant parts or small invertebrates, and in autumn and winter these are seeds or acorns, which generally contain considerable amounts of tannins (Tatsukawa and Murakami, 1976; Shimada, 2001).

In contrast, some mammalian species lack this feedback mechanism, thereby secreting PRPs constitutively at relatively high levels (Austin et al., 1989). It has been hypothesized that the feedback mechanism may not have evolved or may have become lost in those animals whose diets constantly contain high tannin contents (Bennick, 2002; Clauss et al., 2005). Unfortunately, only two species have been reported to use this latter strategy—humans and mule deer—and there is, therefore, much to examine in regard to the above hypothesis. The tannin content of the natural diet of mule deer is likely to vary among seasons, because their diet varies seasonally (Sowell et al., 1985). If mule deer cannot adjust their levels of secretion of PRPs in accordance with their intake levels of tannins, they may suffer the costs of unnecessary PRP synthesis. Nonetheless, do they keep producing PRPs at a high level in any season? The inducibility of PRP synthesis in mule deer has been examined in captive animals by the semiquantitative method (Austin et al., 1989). Thus, the above question is still open, and it may be too early to conclude that such a wasteful strategy is adaptive for animals.

To understand the strategies of PRP production in accordance with tannin intake, it should be helpful to examine the variations in PRP production among populations

as well as among seasons, because levels of tannins in the natural diets will vary among populations even within a species. However, only a few researchers have addressed this topic; Juntheikki (1996) found that the binding affinity of TBSPs to tannins of Scandinavian moose was higher than that of North American moose.

Conclusions

TBSPs, and especially PRPs, probably act as a first line of defense against dietary tannins in far more mammalian species than have already been reported. However, our knowledge of TBSPs in wild animals is limited and is also biased toward *in vitro* data. Detailed characterizations of TBSPs in each species and further comparative studies among species, populations, or seasons will reveal the relationships between feeding niches and production of TBSPs and shed insight on the process of evolution of different types of TBSPs.

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Role of Tannin-Binding Salivary Proteins and Tannase-Producing Bacteria in the Acclimation of the Japanese Wood Mouse to Acorn Tannins

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Abstract We studied the defense mechanisms against the negative effects of tannins in acorns by using the Japanese wood mouse (*Apodemus speciosus*) and acorns of a Japanese deciduous oak *Quercus crispula*, which contain 9.9% tannins on a dry weight basis. For the experiment, we allocated 26 wood mice into two groups: acclimated ($N = 12$) and nonacclimated ($N = 14$). Mice in the nonacclimated group were fed only acorns for 10 d after 4 wk of receiving a tannin-free diet. In contrast, mice in the acclimated group received ca. 3 g acorns daily in addition to the tannin-free diet for the first 4 wk, then they were fed only acorns for 10 d. Body weight, food intake, and digestibility were monitored. In addition, the amount of salivary proline-rich proteins (PRPs) and abundance of tannase-producing bacteria (TPB) in the feces of mice were measured. Of the 14 mice in the nonacclimated group, 8 died, whereas only 1 of the 12 in the acclimated group died. During the first 5 d of feeding acorns only, mice in the nonacclimated group lost, on average, 17.5% of their body mass, while those in the acclimated group lost only 2.5%. Food intake, dry matter digestibility, and nitrogen digestibility were higher in the acclimated group than in the nonacclimated group. The results indicate that wood mice can mitigate the negative effects of tannins by acclimation. Path analysis revealed that increased

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secretion of PRPs and abundance of *Lactobacillus* type of TPB might explain the acclimation to tannins.

Keywords Acclimation · Defense mechanisms against tannins · Path analysis · Plant secondary metabolites · Proline-rich proteins · Tannase-producing bacteria · Wood mouse

Introduction

Chemical defense mediated by plant secondary metabolites (PSMs) is one of the most studied resistance mechanisms against herbivory. In the last two decades, impressive progress has been made in describing the effects of specific PSMs on herbivores. These effects are usually adverse and may include altered foraging behaviors and depressed efficiency of digestion, growth, and reproduction (Palo and Robbins, 1991). Nevertheless, we still have limited and scattered information about the countermeasures that herbivores use to overcome the effects of specific PSMs. The elucidation of these countermeasures will contribute to better understanding of the evolution of consumers and their interaction with plants. For example, animals that can cope with particularly noxious diets can use feeding niches that are unavailable to other animals.

Acorns of some *Quercus* species contain considerable amounts of tannins (Shimada, 2001; Kirkpatrick and Pekins, 2002). Tannins, one of the most widespread PSMs, are a diverse group of water-soluble phenolics with high affinity for proteins. They have several detrimental effects on herbivores: reduction in digestibility (Robbins et al., 1987a; Chung-MacCoubrey et al., 1997), damage to the gastrointestinal mucosa and epithelia (Bernays et al., 1989), kidney or liver failure (Fowler and Richards, 1965), and loss of endogenous nitrogen (Blytt et al., 1988; Bernays et al., 1989).

Nonetheless, the idea persists that acorns are a nutritional resource for wildlife, especially for forest-dwelling rodents. There are many reports describing the positive relationships between the acorn crop and rodent population densities (Jensen, 1982; Elkinton et al., 1996; McShea, 2000; Crespin et al., 2002). We have noted, however, that several studies have failed to find such positive relationships (Miguchi, 1988; Schnurr et al., 2002; Hoshizaki and Miguchi, 2005).

Recent studies reveal that acorns of some oak species can cause intense negative effects on forest-dwelling rodents, which consume acorns as a regular part of their diet (Chung-MacCoubrey et al., 1997; Shimada and Saitoh, 2003). In their feeding experiment, Shimada and Saitoh (2003) found that most Japanese wood mice (*Apodemus speciosus*), that were fed solely *Quercus mongolica* var. *grosseserrata* acorns, which contain 8.6% tannins [tannic acid equivalent (TAE)] on a dry weight basis, showed a marked decrease in food intake, lost body weight, and eventually died. The authors concluded that the negative effects were attributable to tannins rather than to nutrient deficiency or an insufficient supply of dietary energy. Complicating the picture is the fact that the wood mouse is known to forage intensively for acorns in autumn (Wada, 1993; Miguchi, 1994). Studies of the stomach contents revealed that seeds or acorns (a starch-containing diet) were the dominant item from autumn to winter (Ohta et al., 1976; Tatsukawa and Murakami, 1976; Sone et al., 1986).

These inconsistent findings imply that under natural conditions, *A. speciosus* displays some behavioral or physiological countermeasures to mitigate the negative

effects of tannins. Shimada et al. (2004) suggested that in *A. speciosus*, acclimation to tannins reduces their negative effects. We believe that acclimation, which the animal might achieve through a gradual increase in tannin intake, is a probable hypothesis for explaining how *A. speciosus* could overcome harm by tannins. Acclimation might be generated through a variety of processes. Two likely mechanisms are the induction of tannin-binding salivary proteins and the growth of tannase-producing bacteria in the enterobacterial flora.

Various mammalian species produce salivary proteins that have high affinity for tannins (Hagerman and Robbins, 1993; McArthur et al., 1995; Shimada, 2006), including *A. speciosus* (Shimada et al., 2004). Although there are several types of tannin-binding salivary proteins, proline-rich proteins (PRPs) are prevalent and well studied in mammals (Bennick, 2002). One of their key roles is thought to be defense against tannins by preventing them from interacting with other proteins, such as enzymes or those in the mucous membrane and gastrointestinal epithelia (Mehansho et al., 1987; Robbins et al., 1987b, 1991; Hagerman and Robbins, 1993; Fickel et al., 1998). In this way, PRPs may play a role in acclimation to tannins. In rats, induction requires several days after the initial ingestion of tannins (Mehansho et al., 1983; Skopec et al., 2004). Acclimation to a high tannin diet may require a gradual increase in tannin intake.

Tannase-producing bacteria (TPB) can degrade the complex between proteins and hydrolyzable tannins. They are known to exist in the cecum of some mammalian species, including koalas and ringtail possums (Osawa and Sly, 1992; Osawa et al., 1993). Koalas digest leaves of eucalyptus, which have a high tannin content, without any apparent severe damage, partly due to the activity of these bacteria (Osawa et al., 1993). Again, acclimation is important, with tannin ingestion appearing to increase the proportion of these bacteria in the enterobacterial flora (Osawa, 1991). The presence of two types of bacteria in *A. speciosus* has been recently demonstrated (Sasaki et al., 2005)—one is a gram-positive cocci identified as *Streptococcus gallolyticus*, and the other is a newly discovered gram-positive *Lactobacillus* sp.

In an earlier study (Shimada et al., 2004), we suggested the effectiveness of acclimation in reducing the negative effects of tannins, but we did not address the underlying mechanism. We did this in the present study, by feeding acorns of *Q. crispula* (synonymous to *Q. mongolica* var. *grosseserrata*) to two groups of *A. speciosus*, one of which was preexposed to tannins, while the other was not. We also investigated the relationship between the level of PRPs or TPB and the weight changes or digestibility of wood mice fed acorns.

Methods and Materials

Wood Mice and Acorns

Wood mice were caught in October 2002 in Kyoto, central Japan (34°48'N, 135°46'E). They were weighed, housed separately in plastic cages with 400 ml paper bedding (Alpha-dri, Shepherd), and were allowed free access to distilled water under regulated environmental conditions (20°C; photoperiod, 12 L:12 D). Humidity was not controlled and ranged from 50 to 80% during the experiment.

Acorns of *Q. crispula* were collected in October 2002 in Akita prefecture, in northern Japan (39°49'N, 140°18'E), because *Q. crispula* near Kyoto did not produce sufficient acorns during that year. Sound acorns that were free of deterioration and

Table 1 Nutritional constituents and tannin contents of the acorns of *Quercus crispula* used in the experiment

Water	Crude protein	Crude fat	Crude ash	Crude fiber	NFE	Tannin astringency
49.6	6.1	1.5	2.4	1.7	88.4	9.9

NFE: nitrogen-free extract.

Water content is expressed as percent of wet weight; the other values are given as per dry weight. Tannin astringency measured by the radial diffusion method (Hagerman, 1987) is expressed as tannic acid equivalent (%).

insect damage were stored at 4°C before being fed to the mice. Nutrient composition of the acorns is given in Table 1 (analytical procedures are the same as those in Shimada, 2001). Crude proteins and ash were determined by using an NC analyzer (NC-800, Sumika Chemical Analysis Service, Ltd.) and a muffle furnace set at 550°C, respectively. Crude fat (the diethyl ether extraction method) and crude fiber (the insoluble fraction after dissolving in sulfuric acid and sodium hydroxide; Robbins, 1983) were measured by Japan Food Research Laboratories. Nitrogen-free extract (NFE) was determined as a residual fraction of the other nutrients. Tannin astringency (i.e., the protein-precipitating ability, expressed as TAE) was measured by the radial diffusion method (Hagerman, 1987). The acorns used in this experiment averaged 9.9% TAE of tannins. Although we did not identify the types of tannins in these acorns, it is likely that they are mainly hydrolyzable tannins (Chung-MacCoubrey et al., 1997).

Feeding Experiment

Wood mice were allocated into two groups: acclimated and nonacclimated. The acclimated group ($N = 12$; eight male and four female mice) received a tannin-free diet and ca. 3 g shelled acorns daily during the 4-wk preexposure period (20% of that eaten in an earlier experiment; Shimada and Saitoh, 2003), whereas the nonacclimated group ($N = 14$; nine male and five female mice) received only the tannin-free diet. The composition of the tannin-free diet closely matched that of *Q. crispula* acorns in all major constituents except tannins (details of this diet are in Shimada and Saitoh, 2003). The preexposure period began on the day of capture and lasted 28 d. Immediately after the preexposure period, we started the acorns-only feeding period (10 d), the first day of which we denoted day 0. During this time, both groups of wood mice were given nothing except as many shelled acorns as they were willing to eat. Body weight on day 0 did not differ significantly between the acclimated and nonacclimated groups ($X \pm SD$; acclimated group, 44.2 ± 6.6 g; nonacclimated group, 42.0 ± 6.6 g; Mann–Whitney U test, $z = 0.94$, $P = 0.35$).

All feces and uneaten diet were collected each morning, dried at 105°C, and later weighed to determine food intake and apparent dry matter digestibility. Dried feces were milled, and nitrogen measured by using an NC analyzer to evaluate apparent nitrogen digestibility. Preliminary tests indicated no difference in the nitrogen content of dried and fresh feces. Weight change during the acorn-feeding period was determined on days 0 and 5. Specific weight changes were calculated as

$$\text{specific weight change} = (BW_t - BW_0)/BW_0,$$

where BW_t denotes body weight on day t and BW_0 represents initial weight.

Dry matter intake (DMI), fecal nitrogen content, apparent dry matter digestibility (DMD), and apparent nitrogen digestibility (ND), were determined by using data from days 1 to 5; the data for day 0 were omitted because they could be subject to carryover effects of the prior diet. In the case of mice that died before day 5, we used data based on the diets supplied on days 1 until the day before the day of death.

Saliva Collection and Estimation of PRP Production

Saliva was individually collected from all mice 3 d before the beginning of the acorn-feeding period. Wood mice were anesthetized with xylazine hydrochloride combined with ketamine hydrochloride and injected intraperitoneally with carbachol ($100 \mu\text{g kg BW}^{-1}$) to enhance the secretion of saliva. An anesthetized mouse lying on its back was held on a plastic board that was slightly inclined toward the head. Saliva flowing down the palate was collected by using a micropipette until flow stopped. The saliva was immediately weighed, and the flow rate (ml min^{-1}) was calculated from the volume of saliva collected during the first 10 min after the beginning of saliva secretion. We assumed that the specific gravity of crude saliva was the same as that of water. Saliva was stored at -80°C .

PRPs were extracted from the saliva as trichloroacetic acid (TCA)-soluble proteins (Robbins et al., 1987b). We mixed 0.2 ml saliva from each sample with an equal volume of 10% TCA. After a 20-min incubation at 4°C , the TCA-saliva solution was centrifuged at $17,000 \text{ g}$ for 20 min, and the supernatant was collected. The residue was mixed with 0.2 ml 10% TCA, and the supernatant was collected again after centrifugation. The supernatant fraction was neutralized with sodium hydroxide and dialyzed against distilled water by using dialysis tubing (Spectrapore 4D; molecular weight cutoff of $12\text{--}14 \times 10^3 \text{ Da}$). The dialyzate was diluted to 5 ml with distilled water, and the protein content of this solution was measured by the Bradford method (Bradford, 1976) using BSA (fraction V, fatty acid free, Sigma) as the standard. To quantify the ability to generate PRPs, we defined the PRP-secreting capacity ($\text{mg min}^{-1} \text{ kg body weight}^{-1}$; Fickel et al., 1998; Shimada et al., 2004) as follows: PRP-secreting capacity = (concentration of PRPs in saliva \times saliva flow rate)/body mass.

Isolation of TPB

Fresh fecal pellets were individually collected from mice on the day of capture to assess the presence of TPB as previously described (Sasaki et al., 2005). The fecal sample (ca. 50 mg wet weight) from each mouse was emulsified with sterile physiological saline by using a homogenizer and a vortex test tube mixer. A series of 10-fold dilutions (10^1 to 10^5) of the emulsified sample was made in saline and from each dilution, 0.1 ml was spread onto tannic acid-treated brain-heart infusion agar (T-TBHIA) plates. Inoculated plates were incubated anaerobically in Anaero-Pack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37°C for 5 d. After incubation, we counted those colonies that had a clear zone extending beyond their edges, indicating apparent bacterial degradation of tannin-protein complexes. Colonies of two types of TPB were distinguished by their appearance: colonies of *S. gallolyticus* [a tannase-producing *Streptococcus* (TPS)] are 3–5 mm in diam with distinct clear zones, whereas colonies of *Lactobacillus* sp. [a tannase-producing *Lactobacillus* (TPL)] are 2–3 mm in diam with less-distinct clear zones (Sasaki et al., 2005). We monitored the number of TPB colonies once per week until the beginning of the

acorns-only feeding period. The number of colonies was expressed in logarithmic form per gram of fresh feces.

Data Analyses

Fisher's exact probability test was used to test differences in frequency of survival between the acclimated and nonacclimated groups. Differences in weight change, components of digestion, PRP-secreting capacity, and the number of TPB colonies between the two groups were tested by using Mann–Whitney U tests.

Causal relationships among variables in the feeding experiment were assessed by path analysis to clarify the mechanisms causing acclimation to tannins. We regarded weight change from days 0 to 5 as a measure of acclimation. In other words, we supposed that wood mice that successfully acclimated to tannins would gain weight or at least have less weight loss. We built the path diagram based on three hypotheses: (1) weight change in mice would be influenced by the intake and digestibility of acorns, (2) digestibility would be influenced by intake, and (3) intake and digestibility would in turn be influenced by PRP-secreting capacity and the abundance of TPS and TPL.

Although this experiment involved two variables (DMD and ND) as a measure of digestibility, we used DMD in our hypothetical model because DMD—including the digestibility of protein, fat, and carbohydrate—may better explain body weight changes than ND, which indicates only protein digestibility. Apart from this, there was a strong correlation between DMD and ND ($r = 0.892$).

We conducted path analysis by using the program AMOS version 5.0 (Arbuckle and Wothke, 1999; Arbuckle, 2003) to calculate and test the statistical significance of path coefficients and to assess the goodness-of-fit of the model.

Results

Survival

During the acorns-only feeding period (10 d), 1 of the 12 mice (8.3%) in the acclimated group and 8 of the 14 mice (57%) in the nonacclimated group died. In the

Table 2 Weight change, survival rate, intake, and digestion in *A. speciosus* that were fed acorns

	Acclimated group ($N = 12$)		Nonacclimated group ($N = 14$)		Statistics (z)
	X	SD	X	SD	
Weight changes from Day 0 to Day 5 (%)	-2.5	1.6	-17.9	1.5	4.1**
Number of surviving individuals	11	–	6	–	0.015†
Dry matter intake ($\text{g day}^{-1} \text{g body weight}^{-1}$)	0.14	0.03	0.08	0.03	3.4**
Fecal nitrogen content (%)	5.17	0.13	3.80	0.12	4.1**
Apparent dry matter digestibility (%)	89.7	1.0	80.4	0.9	4.1**
Apparent nitrogen digestibility (%)	46.8	4.8	25.0	4.4	3.6*

Statistical analyses were conducted using Mann–Whitney U test, except for the number of surviving individuals.

†Fisher's exact probability test, * $P < 0.01$, ** $P < 0.001$.

acclimated group, one mouse died on day 3. In the nonacclimated group, one died on day 2; two died on each of days 3, 4, and 5; and one died on day 7 after substantial loss in body weight. The difference in survival between the two groups was significant (Fisher’s exact probability test, $P = 0.015$; Table 2).

Body Weight

Figure 1 shows the daily changes in body weight of the wood mice. Body weight decreased in all individuals of the nonacclimated group; the change ranged from -26% to -15% . In contrast, in the acclimated group, 5 of the 12 mice gained weight, and 7 lost weight, with changes in body weight ranging from -16% to 12% . Average weight change from days 0 to 5 was -2.5% in the acclimated group and

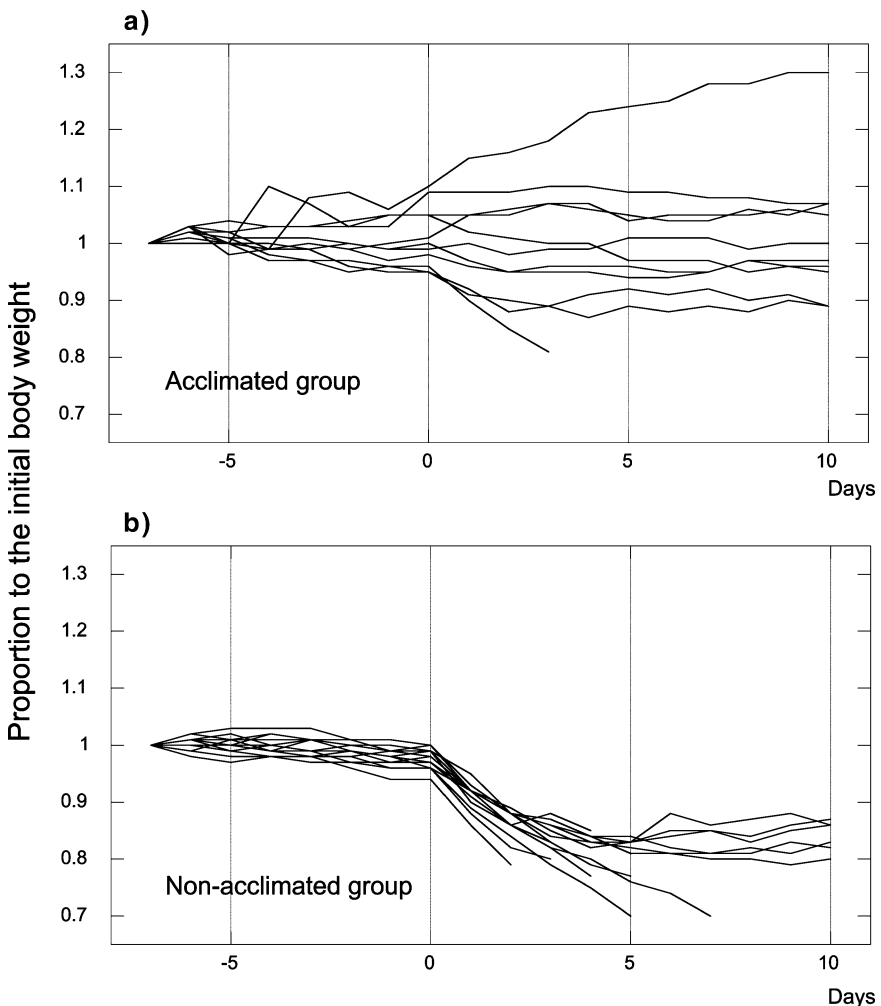


Fig. 1 Changes in body weight of *Apodemus speciosus*. Body weight is expressed as ratio to the body weight on day -7 . Mice in both groups ate only acorns from day 0. Each line shows the body weight of an individual wood mouse

–17.9% in the nonacclimated group (Table 2). This difference was statistically significant (Mann–Whitney U test, $z = 4.1$; $P < 0.001$).

Digestibility

Mice in the in the acclimated group ate 75% more than their counterparts in the nonacclimated group (Table 2) and produced feces with 36% more nitrogen. DMD and ND were 12% and 87% better in the acclimated group; these differences were statistically significant (Table 2).

Production of PRPs

Mice in the acclimated group secreted significantly more saliva and produced it 30% faster than those in the nonacclimated group (Table 3). The concentration of PRPs in saliva also appeared to be higher in the acclimated group than in the nonacclimated group; this difference, however, was not significant. Furthermore, PRP-secreting capacity in the acclimated group was 59% greater than in the nonacclimated group.

Isolation of TPB and Changes in the Number of Colonies

On the day of capture, TPS were isolated from the feces of 17 of the 26 wood mice (65%), whereas TPL occurred in the feces of all individuals. During the preexposure period, however, TPL disappeared from the feces of four mice (one in the acclimated group and three in the nonacclimated group). The animal in the acclimated group that lost TPL in its feces died during the acorns-only feeding period after losing 16% of its body weight.

Figure 2 shows the changes in the numbers of TPS and TPL colonies isolated from feces of the wood mice. The number of TPS colonies per gram of feces ($X \pm SD$, in logarithmic form) at the time of capture (day –28) was 5.05 ± 3.18 for the acclimated group and 3.55 ± 3.25 for the nonacclimated group, compared with 4.59 ± 2.85 for the acclimated group and 4.39 ± 3.99 for the nonacclimated group at the end of the preexposure period (day 0). The number of TPS colonies did not differ between the two groups at either the time of capture (Mann–Whitney U test, $z = 1.33$; $P > 0.1$) or the end of the preexposure period ($z = 0.91$, $P > 0.1$).

Table 3 Saliva flow rate and the secretion rate and concentration of proline-rich proteins (PRPs) in *A. speciosus* that were fed acorns

	Acclimated group ($N = 12$)		Nonacclimated group ($N = 14$)		Statistics (z)
	X	SD	X	SD	
Saliva flow rate (ml min ⁻¹)	0.400	0.116	0.308	0.065	2.2*
Concentration of PRPs in saliva (μg ml ⁻¹)	175.7	52.5	134.5	36.3	1.9†
PRP-secreting capacity (mg min ⁻¹ kg body weight ⁻¹)	0.157	0.060	0.099	0.033	3.2**

Statistical analyses were conducted using Mann–Whitney U test. * $P < 0.05$, ** $P < 0.01$, †: $0.05 < P < 0.1$.

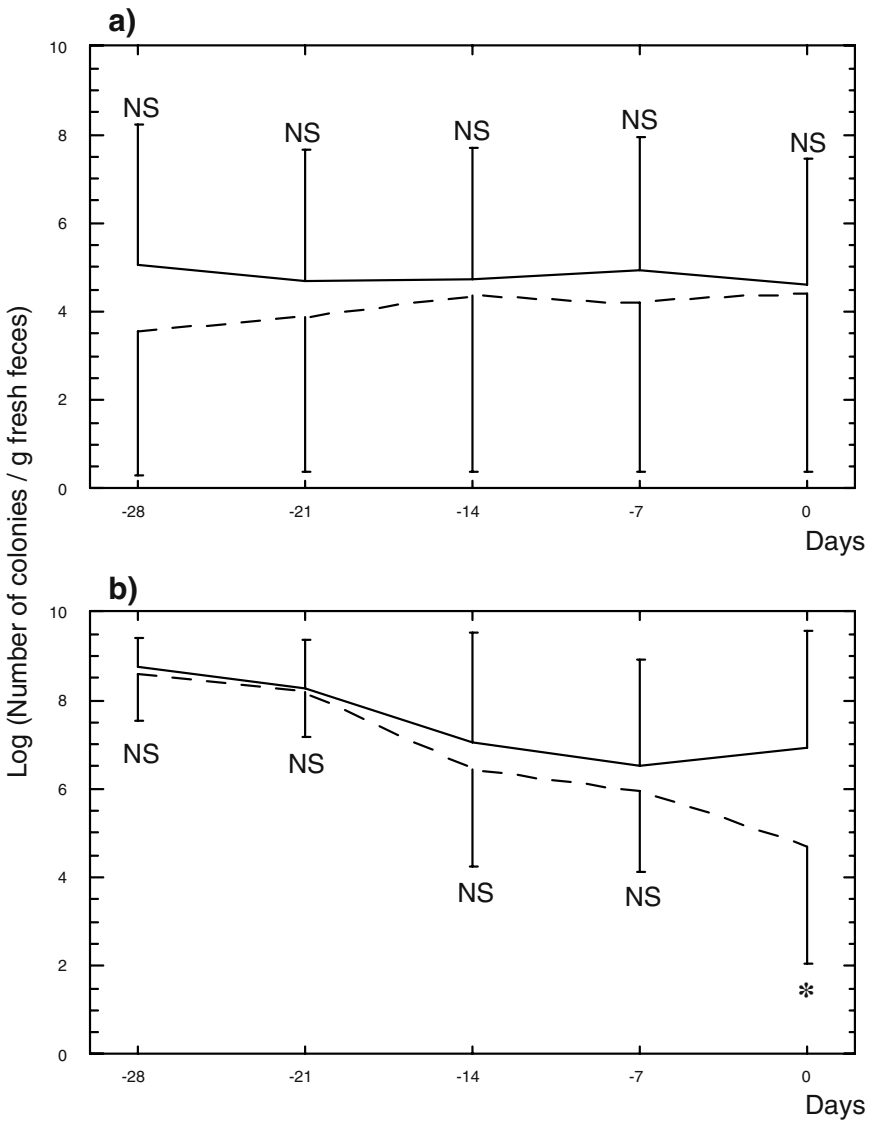


Fig. 2 Changes in the numbers of colonies of (a) TPS (tannase-producing *Streptococcus*) and (b) TPL (tannase-producing *Lactobacillus*) isolated from feces of the wood mouse, *A. speciosus*. Solid and dashed lines represent the acclimated and nonacclimated groups, respectively. Error bars represent SD. The results of Mann–Whitney *U* test between the two groups are shown by the letter beside the error bars (NS: not significant, **P* < 0.05). Mice were captured on day –28 and fed solely acorns from day 0

The two groups had a similar number of TPL colonies ($z = 0.48, P > 0.5$) at the time of capture (8.77 ± 0.63 for the acclimated group and 8.58 ± 1.06 for the nonacclimated group). By the end of the preexposure period, this had dropped to 6.93 ± 2.64 for the acclimated group and 4.71 ± 2.67 for the nonacclimated group, a significant difference between groups ($z = 2.35, P = 0.0185$) (Fig. 2).

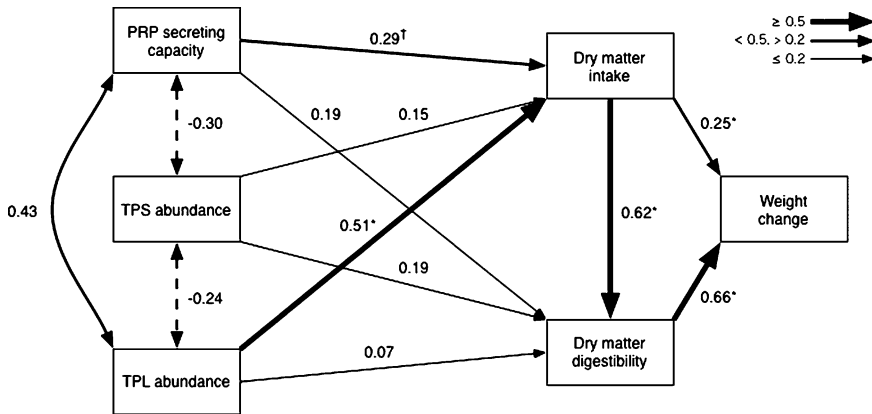


Fig. 3 Path diagram representing the influences on weight changes in the wood mouse, *A. speciosus*, that were fed acorns. Numbers above or beside the arrow refer to standardized path coefficients. Path coefficients significantly different from 0 are marked with symbols (*: $P < 0.01$, †: $0.05 < P < 0.1$). Continuous lines indicate positive effects and dashed lines negative effects, with the magnitude of the coefficient determining their width. A path with double-headed arrow denotes a correlation between the two connected variables

Path Analysis

Figure 3 shows the path diagram representing the hypothetical model of the causal relationship between variables in the acorn-feeding experiment. The goodness-of-fit test revealed that this model is statistically valid ($\chi^2 = 3.08$, $df = 3$, $P = 0.38$). The coefficient of determination for weight changes was 0.74, indicating that the model explains 74% of the variance in weight changes. The direct and indirect effects of DMI on weight change were significant, and thereby the total effect was highly significant (Table 4). DMD also influenced weight changes. In addition, PRP-secreting capacity had a positive effect on weight change (Table 4). Although PRP-secreting capacity did not have a significant direct path to both DMI and DMD, the total effect of PRP-secreting capacity on DMD was significant (Table 5). TPL abundance also positively influenced weight changes, but TPS abundance did not (Table 4). Likewise, TPS abundance did not significantly influence either DMI or DMD (Table 5). TPL abundance had a direct path to DMI but not to DMD, indicating that it could influence weight change primarily through DMI. The total

Table 4 Direct, indirect, and total effects, according to path analysis, on weight changes in the wood mouse *A. speciosus* that were fed acorns

Variables	Effects		
	Direct	Indirect	Total
PRP-secreting capacity	–	0.312*	0.312*
TPS abundance	–	0.220	0.220
TPL abundance	–	0.380*	0.380*
Dry matter intake	0.254*	0.403*	0.657**
Apparent dry matter digestibility	0.655*	–	0.655*

* $P < 0.05$, ** $P < 0.01$.

Table 5 Direct, indirect, and total effects, according to path analysis, on dry matter intake (DMI) and apparent dry matter digestibility (DMD) in *A. speciosus* that were fed acorns

Variables	Effects on DMI			Effects on DMD		
	Direct	Indirect	Total	Direct	Indirect	Total
PRP-secreting capacity	0.287†	–	0.287†	0.189	0.177†	0.366*
TPS abundance	0.146	–	0.146	0.189	0.090	0.279
TPL abundance	0.510*	–	0.510*	0.069	0.314*	0.383†

* $P < 0.05$, † $0.05 < P < 0.1$.

effects of TPL abundance on DMI and DMD were at least marginally significant, (Table 5). The relationships between weight changes and PRP-secreting ability or the abundance of TPLs are shown in Fig. 4.

Discussion

Effectiveness of Acclimation

The wood mice that were allowed to acclimate to tannins were better able to counteract the negative effects of tannins. This was evident in the mortality data, wherein 8 of the 14 nonacclimated mice died during the acorns-only feeding period, whereas only 1 of 12 mice in the acclimated group died (Table 2). All animals in the non-acclimated group lost much weight, while, except for one animal, those in the acclimated group lost little weight (Fig. 1). Furthermore, acclimated animals ate more and digested the dry matter and nitrogen better than nonacclimated ones (Table 2), even though they excreted feces with 36% more nitrogen than those excreted by mice in the nonacclimated group (Table 2). We speculate that fecal nitrogen content indicates the effectiveness of defense against tannins by PRPs. In rats, the increase in fecal nitrogen due to ingesting tannins comes mainly from PRPs, which form

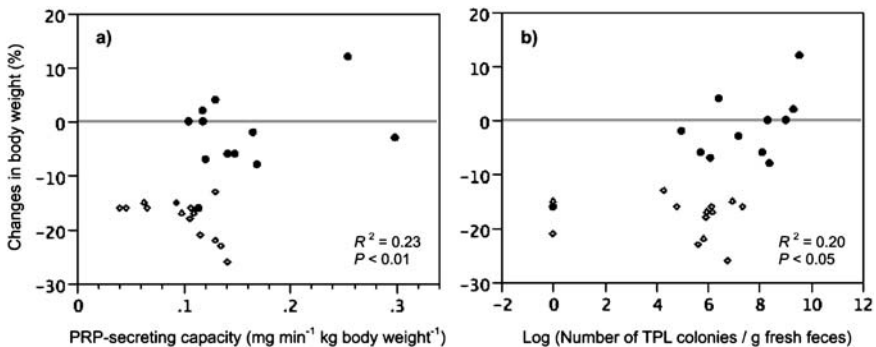


Fig. 4 Relationships between changes in body weight and (a) PRP-secreting capacity or (b) the abundance of TPL colonies in fresh feces in the wood mouse, *A. speciosus*, that were fed acorns. The data of TPL abundance were obtained on the first day of the acorns-only feeding period (day 0). Solid circles and open squares represent individuals in the acclimated and nonacclimated groups, respectively

complexes with tannins and are excreted in the feces (Eggum and Christensen, 1975). Thus, fecal nitrogen content appears to be a useful indicator of the success of PRPs in blocking tannins. Indeed, our preceding study (Shimada et al., 2004) found a significant positive correlation between PRP-secreting capacity and fecal nitrogen content in wood mice fed acorns.

These findings indicate that if *A. speciosus* have time to acclimate to acorn tannins, they can successfully negate their effects. In fact, all but one of the mice in the acclimated group appeared to be as healthy after the acorns-only feeding period as they were after eating the tannin-free diet supplemented with a few acorns. The exception was a mouse that lost TPL from its feces early during the preexposure period and died during the acorns-only feeding period after losing substantial weight. It may be that, unlike the other mice, the mouse might have somehow failed to accommodate enough exogenous TPL to cope with its dramatic dietary change. Consequently, this animal's response to tannins resembled those of mice in the non-acclimated group. As discussed below, acclimation to tannins may be linked to an increase in the abundance of TPL.

Mechanisms of the Acclimation

Path analysis yielded clues to the mechanisms underlying the acclimation. Weight change, which is regarded as an indicator of the extent of acclimation, was primarily affected by DMD and DMI (Fig. 3, Table 4). This result is understandable, because increased DMD and DMI lead to increased retention of dietary nutrients and energy (Robbins, 1983). Moreover, the PRP-secreting capacity and abundance of TPL were proved to positively influence weight changes in wood mice, whereas the abundance of TPS did not have a significant effect on weight change and, by extension, acclimation to tannins (Fig. 3, Table 4). These findings suggest that increases in both PRPs and TPLs are the underlying causes of acclimation to tannins in *A. speciosus*.

Acclimation by PRPs

PRPs are thought to defend animals against dietary tannins. They form stable complexes with tannins in the oral cavity, thereby preventing them from interacting with other proteins and degrading into noxious phenolic compounds (Robbins et al., 1991; Hagerman and Robbins, 1993; Fickel et al., 1998). In laboratory rats and mice, ingesting tannins is known to cause enlargement of the parotid glands and increased secretion of PRPs (Mehansho et al., 1985). Our *A. speciosus* probably responded in a similar way, as indicated by mice in the acclimated group secreting more PRPs than those in the nonacclimated group (Table 3).

This response probably takes several days to occur. For instance, Mehansho et al. (1983) used electrophoretic analysis to confirm that rats began to synthesize PRPs 3 d after they first ingested tannins. Likewise, Skopec et al. (2004) reported that rats started to produce PRPs in the first 8–10 d after encountering tannins. Thus, wood mice may require a gradual increase in tannin intake to tolerate the high concentrations in acorns.

Recent studies have been unveiling the *in vivo* roles of PRPs as a tannin defense. Shimada et al. (2004) found that *A. speciosus* exhibited acclimation to tannins during feeding on acorns and, in parallel, increased production of PRPs. They also

observed that wood mice producing more PRPs ate more and digested their diet better and consequently lost less weight. In rats fed radiolabeled pentagalloyl glucose (PGG), a hydrolyzable tannin, Skopec et al. (2004) demonstrated that PGG habituation increased the production of PRPs, which in turn increased the fecal excretion of PGG by forming stable PGG–PRP complexes. In this manner, PRPs may reduce the toxicity of PGG by minimizing the absorption of polyphenolic or its hydrolysis products. These observations support our hypothesis that PRPs may be one of the underlying causes of acclimation to tannins observed in the present study.

In our study, PRP-secreting capacity was used as an index of the level of PRP production instead of PRP concentration in saliva or salivary glands, which was often used in past studies (Robbins et al., 1987a; Mole et al., 1990). It has been well-documented, in laboratory rats and mice, that ingesting tannins causes the induction of PRP synthesis (Mehansho et al., 1985). Our results are the first to show that the ingestion of tannins can cause an increase in saliva flow rate as well (Table 3). To estimate the potency of PRPs as a tannin defense, it is presumably better to use PRP-secreting capacity, the product of PRP concentration and saliva flow rate, than PRP concentration alone. Another informative index to estimate the potency of PRPs is to measure the binding ability of PRPs or crude saliva to standard tannins (Austin et al., 1989; Clauss et al., 2005). The combination of PRP-secreting capacity with the salivary tannin-binding ability should be useful in future studies of the defensive role of salivary PRPs against tannins.

Acclimation by TPB

TPB can degrade complexes between proteins and hydrolyzable tannins and thereby transform these complexes into bioavailable forms for the host organism. Our results suggest that TPL (but not TPS) may be one of the underlying causes of acclimation to tannins in *A. speciosus*. This idea is supported by the following. First, TPL is far more prevalent than TPS in the feces of the wood mouse (Fig. 2). Second, the abundance of TPL in feces, but not of TPS, was related to the amount of tannins ingested by the mice. In addition, at capture, all wood mice had TPL, but 35% did not have TPS. Unlike TPS, TPL may be a key to enable mice to use acorns as a staple food source. The abundance of TPL decreased markedly while the wood mice were fed the tannin-free diet (Fig. 2). This finding shows that wood mice need to ingest tannins continuously to maintain a rich population of TPL. Interestingly, path analysis indicated that the abundance of TPL could positively influence body weight change in the wood mouse through DMI but not DMD. It is unlikely that TPL directly increases DMI in the wood mouse. It is likely that complicated physiological feedback mechanisms, which were not assumed in the path diagram (Fig. 3), are involved in the acclimation process.

TPL may complement the defense provided by PRPs. Ingested tannins are supposed to be blocked by PRPs in the oral cavity. Wood mice, however, would lose endogenous nitrogen if they could not metabolize the nitrogen-rich tannin–PRP complexes before their excretion (Shimada, 2006). In other words, while defense by PRPs may occur at the cost of endogenous nitrogen loss, we hypothesize that TPLs reduce this by degrading tannin–PRP complexes into forms suitable for recycling. Thus, PRPs and populations of TPL may both be essential to *A. speciosus* for protection against the deleterious effects of tannins.

Conclusion

The preceding discussion addresses how *A. speciosus* could overcome the negative effects of tannins in acorns through acclimation that allows them to increase their production of PRPs and establish viable populations of TPL. Acclimation, however, is not the only tannin countermeasure that *A. speciosus* might adopt. There are other hypotheses that explain how plant consumers could counteract tannins. One of the prevailing hypotheses is that consumers manipulate tannin concentrations by caching acorns, but some studies argue against this (Dixon et al., 1997; Koenig and Faeth, 1998; Shimada, 2001). Two other hypotheses, which are untested in *A. speciosus*, can be raised. One is that consumers regulate their intake of specific PSMs to circumvent overloading of any one detoxification pathway by feeding on a broad range of diets (Freeland and Janzen, 1974). The other is that, under natural conditions, consumers compensate for the decrease in nitrogen and energy digestibilities due to tannins by selective foraging on protein-rich diets, such as the larvae of the acorn weevil (Johnson et al., 1993). These two hypotheses should be evaluated in *A. speciosus*.

A. speciosus relies on acorns most extensively from late autumn to winter, when other resources, such as berries, the soft parts of plants, and invertebrates, are scarce (Tatsukawa and Murakami, 1976; Sone et al., 1986). If it did not feed on tannin-rich acorns, the mouse might find it difficult to survive winter in oak-dominated forests. Acquiring an effective defense against tannins may allow *A. speciosus* to survive and flourish in the temperate forest in Japan.

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Efflux Transporters as a Novel Herbivore Countermechanism to Plant Chemical Defenses

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Abstract The recent discovery of efflux transporters in the gut has revolutionized our understanding of the absorption and bioavailability of pharmaceuticals and other xenobiotics in humans. Despite the celebrity of efflux transporters in the areas of pharmacology and medicine, their significance is only beginning to be realized in the area of plant–herbivore interactions. This review integrates reports on the importance of gut efflux transporters to diet selection by herbivores. The diets of herbivores are laden with toxic plant secondary metabolites (PSMs) that until recently were thought to be processed almost exclusively by detoxification enzymes in the liver. We describe how efflux transporters in the gut may play a critical role in regulating the absorption of PSMs in herbivores and dictating diet selection. Recent studies suggest that the role of efflux transporters in mediating diet selection in herbivores may be as critical as detoxification enzymes. In addition to diet selection, gut efflux transporters have implications for other aspects of plant–animal interactions. They may be significant components of the evolutionary arms race that influences chemical diversity in plants. Furthermore, in agricultural systems, gut efflux transporters may play an important role in the effectiveness of pesticides. This synthesis paper introduces a new direction in plant–herbivore interactions by providing a complementary mechanism, regulated absorption, to detoxification that may define tolerance to PSMs by herbivores.

Keywords Herbivores · Plant secondary metabolites · Regulated absorption · P-glycoprotein · Mammals · Efflux transporters · Diet selection · Herbivore tolerance · Transporter proteins

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Introduction

Two central foci in plant–herbivore interactions have been identified to explain the diversity of plant chemical defenses as well as the variation in herbivore tolerance to defenses. Much progress has been made in identifying and quantifying plant secondary metabolites (PSMs), as well as understanding their influence on herbivore feeding (Freeland and Janzen, 1974; Palo and Robbins, 1991; Rosenthal and Berenbaum, 1992; Cheeke, 1998; Foley et al., 1999). Diversity in PSMs has been attributed to the need of plants to defend themselves against a wide range of herbivores and pathogens (Berenbaum et al., 1991; Fritz and Simms, 1992; Berenbaum, 1995, 1999). In contrast, relatively little progress has been made with respect to the other central goal, i.e., explaining the variation in tolerance to PSMs by herbivores. Variation in detoxification capabilities has long been suggested as the underlying mechanism responsible for differential tolerances to PSMs among herbivores (Freeland and Janzen, 1974; Foley et al., 1999; Pass et al., 2001; Karban and Agrawal, 2002; Li et al., 2004). However, individuals and species with the highest PSM tolerance do not always have the highest activity levels of detoxification enzymes (Neal, 1987) or the highest rates of PSM elimination. For example, *Neotoma stephensi*, a juniper specialist, can tolerate more juniper and PSMs in juniper than its generalist counterpart, *N. albigula* (Sorensen et al., 2005), but it does not have a faster rate of juniper PSM elimination from the blood (Sorensen and Dearing, 2003).

We propose that, in addition to enzymatic detoxification, variation in mechanisms that regulate absorption of PSMs in the intestine may further explain the variation in herbivore tolerance to PSMs. Although the importance of PSM absorption in herbivores is not a new concept, previously proposed mechanisms have focused on passive barriers in the gut such as peritrophic membranes (PMs) (Lehane, 1997; Barbehenn, 2001). In this work, we argue that in addition to passive barriers, herbivores can actively transport PSMs out of enterocytes and back into the lumen of the gut, limiting PSM absorption. We refer to this process and its predicted influence on the foraging behavior of herbivores as the “regulated absorption model.” We identify a plausible mechanism for regulated absorption and provide evidence that this mechanism can influence PSM tolerance in herbivores. In addition, we propose that regulated absorption of PSMs may further our understanding of chemical diversity in plants. Throughout, we provide several testable hypotheses regarding the role that regulated absorption plays in influencing the interactions between PSMs and herbivores. This synthesis paper introduces ecologists to a new direction in plant–herbivore interactions by providing a complementary mechanism, regulated absorption, to detoxification that may define tolerance to plant secondary metabolites by herbivores.

Introduction to Regulated Absorption

Ingestion of PSMs presents a physiological challenge for herbivores. PSMs that are ingested and absorbed exert a variety of deleterious effects on tissues and organs (Polya, 2003). Furthermore, the toxicity of PSMs is typically concentration-dependent and is therefore correlated with both the quantity of PSM ingested and the quantity of PSM that is absorbed and available throughout the body via the general circulation (Schumacher-Henrique et al., 2003; Orafidiya et al., 2004).

Because of the ubiquity of secondary metabolites in plants, herbivores cannot simply avoid them, but must employ mechanisms that minimize their levels in the circulation. Detoxification is currently regarded as the major physiological process that regulates the pharmacological distribution of PSMs in the body (Rosenthal and Berenbaum, 1992; Foley et al., 1999). In general, the process of detoxification biotransforms toxic PSMs into water-soluble metabolites that can be readily excreted. Although detoxification can lead to the formation of more toxic metabolites, the detoxification process typically results in a reduction in concentrations of toxic PSMs in the body. Moreover, several studies on insects have demonstrated that increased levels and activity of detoxification via enzymes are correlated with increased intake levels of certain PSMs in various herbivores. For example, the induction of cytochrome P-450 monooxygenases by PSMs, xanthotoxin and nicotine, is associated with high tolerance to these in the corn earworm (*Helicoverpa zea*) and tobacco hornworms (*Manduca sexta*), respectively (Snyder and Glendinning, 1996; Li et al., 2000; Sasabe et al., 2004). Although several studies have investigated detoxification enzymes in mammalian herbivores (Boyle et al., 1999, 2000a,b; Pass et al., 1999, 2001, 2002; Ngo et al., 2000; Lamb et al., 2001, 2004), few have directly linked higher PSM tolerance with higher detoxification enzyme activity in mammals (Miranda et al., 1991).

Although detoxification clearly plays a significant role in enhancing the excretion of PSMs and is predicted to facilitate PSM intake in herbivores, it is not the only mechanism that can regulate PSM concentrations in the body. For PSMs to gain access to tissues and organs, absorption across the gastrointestinal tract must first occur. Therefore, mechanisms in the gut that limit absorption provide the first line of defense against ingested PSMs. Detoxification in the intestine, whether via enzymes from microbes (Scheline, 1973; Jones and Megarrity, 1986; Hammond, 1995; Bhat et al., 1998) or the herbivore (Rosenthal and Berenbaum, 1992; Ilett et al., 1993; Watkins, 1997; Berenbaum, 1999; Von Richter et al., 2004), can regulate the absorption and subsequent distribution of ingested PSMs in herbivores. However, several studies demonstrate that many ingested PSMs that possess chemical properties conducive to absorption are neither detoxified nor absorbed in the gut (Ohmart and Larsson, 1989; Southwell et al., 1995; Schmidt et al., 2000; Cooper, 2001). For example, ingested terpenes, highly lipophilic PSMs, are excreted unchanged in the feces of two species of herbivorous mammals (*Neotoma* spp.; Sorensen and Dearing, 2003) and in frass of insects (Gómez et al., 1999; Müller and Hilker, 1999; Evans et al., 2000; Southwell et al., 2003). Physical barriers that limit the passive diffusion of PSMs across the gut, such as the peritrophic matrix and surfactants, have been the only mechanisms proposed to explain this phenomenon (Lehane, 1997; Barbehenn and Martin, 1998; Barbehenn, 2001). However, insights from biomedical and pharmaceutical research suggest that active transport of xenobiotics, including PSMs, out of enterocytes and into the gut lumen is also common and provides an efficient barrier against the absorption of lipophilic xenobiotics.

Mechanisms of Regulated Absorption

Despite the lack of attention from scientists who study plant–herbivore interactions, the biochemical mechanisms that actively regulate the absorption of ingested compounds are well known to medical scientists (Hunter and Hirst, 1997; Sharom,

1997; Watkins, 1997; Benet and Cummins, 2001; Doherty and Charman, 2002; Schinkel and Jonker, 2003). The molecules best known for their capacity to regulate the absorption of xenobiotics are collectively known as ATP-binding cassette (ABC) transporters. Permeability glycoprotein, or P-glycoprotein (P-gp), is encoded by the Multidrug Resistance (MDR) gene, and is one of over 200 ABC transporters that has been identified as playing a significant role in regulating bioavailability, or tissue distribution, of xenobiotics (Dietrich et al., 2003; Chan et al., 2004). Like other ABC transporters, P-gp is primarily expressed in the apical membrane of cells in barrier tissues, including the intestine, liver, kidney, testes, uterus, and blood-brain barrier. However, unlike other ABC transporters, P-gp is not substrate-specific and is capable of transporting a variety of structurally diverse compounds, including natural products (Sharom, 1997; Seelig and Landwojtowicz, 2000; Schinkel and Jonker, 2003). Although P-gp is a promiscuous transporter, most P-gp substrates are organic, relatively lipophilic, and contain polar or nonpolar domains (i.e., amphipathic, Sharom, 1997; Schinkel and Jonker, 2003).

P-gp is a major factor influencing the relationship between dose, blood concentration, and response of many orally administered drugs in humans (Van Zuylen et al., 2000; Ayrton and Morgan, 2001; Funakoshi et al., 2003). In general, individuals with higher quantities of P-gp exhibit lower blood concentrations and greater fecal excretion of certain orally administered drugs (Debenham et al., 1982; Van Zuylen et al., 2000). P-gp is also known to govern the bioavailability of many drugs resulting in lower pharmacological effect (Van Asperen et al., 1996; Jonker et al., 1999; Schinkel, 1999). P-gp reduces the bioavailability of ingested xenobiotics in three ways (Fig. 1). First, it can directly limit absorption by actively effluxing ingested xenobiotics out of cells against a concentration gradient via an ATP-dependent pump (Sharom, 1997; Dietrich et al., 2003). Second, it can indirectly

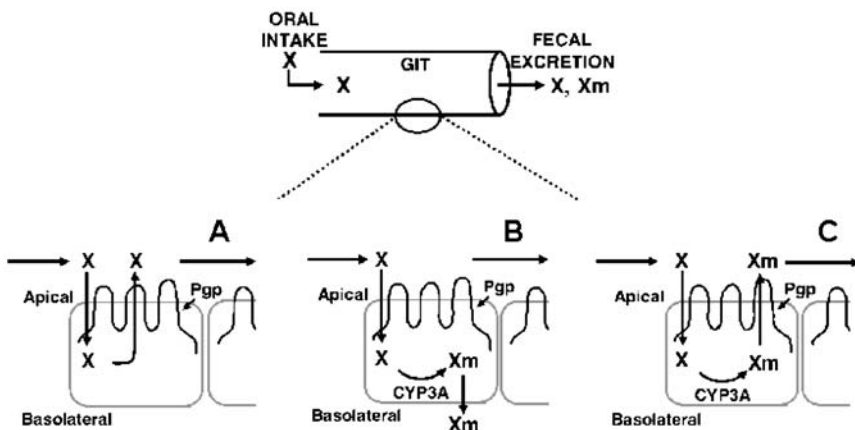


Fig. 1 Schematic representation of the functional role of P-glycoprotein (P-gp) and cytochrome P-450 3A (CYP3A) in regulating the absorption of ingested xenobiotics (X, i.e., drugs or plant secondary metabolites) in the gastrointestinal tract (GIT). P-gp and CYP3A can regulate the absorption of ingested xenobiotics (X) and detoxification metabolites (Xm) resulting in fecal excretion of these molecules via three scenarios. Ingested xenobiotics that are substrates of P-gp can be directly effluxed out of cells by P-gp (A). CYP3A metabolizes xenobiotics, which may result in absorption of the xenobiotic metabolite across the basolateral membrane (B). Finally, if the xenobiotic metabolite is a P-gp substrate, P-gp can efflux the metabolite out of cells (C)

reduce the bioavailability of ingested xenobiotics by regulating concentrations of intracellular xenobiotics to levels within the capacity of associated detoxification enzymes in cells, particularly cytochrome P-450 3A enzymes (Watkins, 1997; Ayrton and Morgan, 2001; Benet and Cummins, 2001). P-gp and CYP3A are colocalized in the villus tip of enterocytes, share many of the same substrates, and demonstrate broad overlap in substrate specificities (Kim et al., 1999; Katoh et al., 2001; Zhang and Benet, 2001). These attributes suggest that P-gp and CYP3A act in concert as a barrier to drug absorption. Finally, P-gp can efflux detoxification metabolites out of cells, thereby reducing the absorption of potentially toxic metabolites in addition to ingested parent xenobiotics (Katoh et al., 2001). In terms of drug therapy, mechanisms that limit absorption, enhance excretion, and minimize delivery of drugs to target tissues are medically problematic. For example, P-gp present in the transmembrane of cancer cells prevents the absorption of chemotherapeutic drugs (Lin and Yamazaki, 2003). Furthermore, because P-gp is a promiscuous transporter, it effluxes a wide range of anticancer drugs out of cells, resulting in the multidrug resistance phenomenon in cancer patients (Bellamy, 1996; Sparreboom et al., 2003). However, the same mechanisms that limit drug distribution in humans may also minimize the distribution of deleterious PSMs and prove beneficial to herbivores. In the following sections, we provide evidence that P-gp, or similar active transporters, may be employed by herbivores to enhance excretion and limit the bioavailability of ingested PSMs.

Evidence for Efflux Transporters in Herbivores

The first line of evidence that active transporters such as P-gp may be present in herbivores is the ubiquity and conservation of P-gp-like molecules among organisms. P-gp has been identified within mammals (humans, mice, rats, primates, dogs) and among distantly related taxa, such as bacteria, nematodes, insects, fish, birds, and plants (Dudler and Hertig, 1992; Lanning et al., 1996a; Davies et al., 1997; Barnes, 2001; Doi et al., 2001; Saier and Paulsen, 2001; Buss et al., 2002; Smith and Prichard, 2002; Mikkaichi et al., 2004). Furthermore, the sequence of P-gp is highly conserved across taxa (UCS Genome Bioinformatics website, <http://genome.ucsc.edu>). For example, the P-gp homologue in the plant, *Arabidopsis thaliana*, shares seven of nine introns with mammalian P-gp to within a few nucleotides (Dudler and Hertig, 1992). The shared sequence, organizational structure, and function of P-gp among distantly related organisms indicate that insect and mammalian herbivores will likely have P-gp homologues. Although empirical studies on P-gp in herbivores are minimal, the few species that have been investigated do possess P-gp or P-gp-like mechanisms. For example, P-gp-like transporters have been identified in the tobacco budworm, and appear to actively transport xenobiotics out of cells in these insects (Lanning et al., 1996a; Gaertner et al., 1998). Studies on herbivorous woodrats also demonstrate that several species of mammalian herbivores possess P-gp (Green et al., 2004).

A second piece of corroborating evidence is that a large number of PSMs that are potentially consumed by herbivores are substrates for P-gp (Patel et al., 2004; Zhou et al., 2004). Alkaloids, such as vinblastine from *Vinca rosea* (periwinkle, Burns, 1972; Sharom, 1997) and more recently, piperine from black (*Piper nigrum* Linn) and long pepper (*P. longum* Linn), are P-gp substrates (Bhardwaj et al., 2002).

Others include diterpene from *Euphorbia* (spurges, Hohmann et al., 2002; Appendino et al., 2003), phenolic glycosides from foxglove (*Digitalis lanata*, Kim et al., 1999; Katoh et al., 2001; Funakoshi et al., 2003), curcumin from *Curcumin longa* (Limtrakul et al., 2004), ginsenosides from ginseng (Choi et al., 2003; Kim et al., 2003), catechins from green tea (*Camellia sinensis*, Wang et al., 2002), bergamotol from grapefruits (Ohnishi et al., 2000; Wang et al., 2001), and hypericin from Saint John's wort (*Hypericum perforatum*, Wang et al., 2004).

The third line of evidence that active transporters such as P-gp are present in intestines of herbivores is the excretion of unmetabolized PSMs in the feces. A single study demonstrated that mammals (*Neotoma* spp.) excrete large quantities of ingested PSMs unmetabolized in the feces (Sorensen et al., 2004). Other examples of fecal excretion of unchanged compounds are found in herbivorous insects. For example, a large proportion of ingested nicotine is excreted unmetabolized in the feces of several insects specializing on tobacco (Self et al. 1964a,b; Snyder et al., 1994). Several other insect herbivores excrete terpenes in the feces that are identical in chemical structure and proportion to those found in the plants they consume (Gómez et al., 1999; Evans et al., 2000).

We propose that active transport of PSMs out of enterocytes is a plausible explanation for the fecal excretion of many unmetabolized PSMs in herbivores. Although passive barriers, such as peritrophic membranes (PMs), may be important in limiting the absorption of large complex PSMs, such as tannins (Barbehenn, 2001), these barriers would not be effective against small lipophilic compounds. Many of the PSMs excreted unmetabolized in the feces of herbivores, such as terpenes, are small molecules, highly lipophilic and, therefore, not likely to be passively filtered by PMs. Moreover, PMs cannot explain the excretion of terpenes unchanged in the feces of mammalian herbivores (Sorensen et al., 2004) because mammals do not possess PMs (Lehane, 1997). Instead, the excretion of many unmetabolized PSMs in the feces of herbivores is likely attributable to an active transporter such as P-gp. In support, a nicotine pump similar to P-gp has been identified in both malpighian tubules and in the blood–brain barrier of the tobacco hornworm (Murray et al., 1994; Gaertner et al., 1998). Finally, the strong correlation between the lipid solubility of a compound and its ability to interact with P-gp suggests that lipophilic PSMs are substrates for transporter proteins (Zamora et al., 1988; Schinkel and Jonker, 2003).

Although the excretion of unmetabolized lipophilic PSMs in the feces suggests that P-gp-like mechanisms are present in herbivores, the majority of studies fail to detect large quantities of unmetabolized PSMs in the feces of herbivores (Foley et al., 1987; Boyle et al., 1999, 2000a,b). We argue that these results do not necessarily indicate that most herbivores do not possess or utilize transporter proteins to minimize PSM bioavailability. Instead, the lack of unmetabolized PSMs in the feces may be attributable to the complex interactions between transporter proteins, detoxification enzymes, and microbes in the intestine prior to absorption. PSMs that are not absorbed may be detoxified or degraded by enzymes or microbes in the hindgut prior to excretion. For example, detoxification enzymes, particularly cytochrome P-450 3A, are locally associated and share substrate affinity with P-gp (Watkins, 1997; Kim et al., 1999; Zhang and Benet, 2001; Doherty and Charman, 2002). In addition, intestinal microbes from a variety of herbivores are known to metabolize and degrade PSMs (Carlson and Breeze, 1984; Jones and Megarrity, 1986; Hammond, 1995). Specifically, many microbes metabolize and degrade

lipophilic terpenes (Harder and Probian, 1995; Misra et al., 1996), which are likely P-gp substrates. Therefore, it is possible for P-gp to regulate the absorption of ingested PSMs in herbivores, but unmetabolized PSMs in the feces would not be present because of detoxification and modification of PSMs by enzymes and microbes prior to excretion. Alternatively, the rate of absorption of highly lipophilic compounds may exceed the rate of efflux by P-gp-like mechanisms resulting in absorption of PSMs that are actually P-gp substrates.

New Directions

It is essential to identify ways to isolate the contribution of active transport proteins in regulating PSM absorption in herbivores to better understand foraging constraints and disparate foraging strategies among herbivorous species. In this work, we suggest several areas of future research for chemical ecologists to better understand the interactions between PSMs and herbivores. Because of the preponderance of research on the function of P-gp, the following hypotheses will focus on assays and predictions for P-gp, but could be potentially applied to any efflux transporter protein.

The first needed area of research is to test the overall presence and level of transporter proteins in herbivores by using molecular techniques. The presence and quantity of particular proteins such as P-gp can be determined by using a combination of Western blots and quantitative polymerase chain reactions (qPCR). Western blots with monoclonal antibodies have been used to quantify P-gp in a variety of organisms, including herbivorous woodrats (Scheffer et al., 2000; Barnes, 2001; Doi et al., 2001; Green et al., 2004). Application of this method is accessible to a number of species because of the high conservation of monoclonal antibodies across species. Although this approach has been successfully applied to several species of animals, a potential limitation is the possible cross-reaction with proteins other than P-gp (Van Den Elsen et al., 1999) and the semiquantitative nature of the assay. Quantification of P-gp mRNA provides a more robust approach to identify and quantify P-gp in specific species of herbivores. Because sequences have not been determined for many wild species, species-specific sequences must be generated. In general, this process involves designing primary PCR primers from homologous sequences of nearest relatives, sequencing and verifying PCR products against known homologues to confirm identity, and then designing species-specific probes for qPCR. This approach was successfully used for plants (Dudler and Hertig, 1992; Yazaki et al., 2001; Sasaki et al., 2002), and will likely provide similar success in other species. An example of sequence alignment for a P-gp-like gene in plants and mammals is found in Dudler and Hertig (1992) and can provide an initial starting place for designing primers. In addition, homologous sequences can be designed based on P-gp (often referred to as MDR) gene alignments generated from the UCS Genome Bioinformatics website (<http://genome.ucsc.edu>). Quantitative PCR is beneficial in that it provides P-gp specificity and an opportunity to identify particular changes in P-gp sequence with respect to species, foraging strategies, and the type of PSM consumed. Quantification of P-gp-like transporters should not be restricted to segments of the gastrointestinal tract and should include studies in “barrier” tissues, such as the Malpighian tubules, liver, kidney, brain, testes, and uterus.

Given the likely applicability of these assays for P-gp quantification in herbivores, several hypotheses can be tested. First, these assays can be used to indirectly test the hypothesis that specific PSMs ingested by herbivores are substrates for P-gp. In general, ingestion of P-gp substrates yields an increase in P-gp concentrations (Benet and Cummins, 2001; Fromm, 2003, 2004; Lin and Yamazaki, 2003; Zhou et al., 2004). Determining whether a PSM is a P-gp substrate can be further verified with *in vitro* assays that use P-gp expressing cell lines (Varma et al., 2003; Moaddel et al., 2004; Wang et al., 2004) and/or *in vivo* studies that use P-gp knockout mice (Schinkel et al., 1997).

Second, assays can be employed to test the hypothesis that specialist herbivores rely on regulated absorption to a greater extent than generalist herbivores. Several studies that investigate the excretion of unchanged metabolites indirectly support this hypothesis. For example, the juniper specialist (*N. stephensi*) excreted a larger proportion (28%) of orally ingested alpha-pinene, the predominant PSM in juniper, in the feces than a closely related juniper generalists (*N. albigula*; 15%, (Sorensen et al., 2004). Similarly, specialist koalas excreted 15% of ingested eucalyptus oils, primarily composed of terpenes, in the feces (Eberhard et al., 1975) compared to 3% in a generalist counterpart, the brushtail possums (Foley et al., 1987). These results emphasize the need to quantify and compare P-gp activity, PSM intake, and fecal excretion of PSMs in additional specialist and generalist herbivores. Analytical techniques for quantification of unchanged PSMs include gas chromatography and high-pressure liquid chromatography; however, specific analysis is dependent on the chemistry of the particular PSM.

Quantitative assays can also be used to investigate the hypothesis that mechanisms regulating PSM absorption are positively correlated with expression of P-450 enzymes, specifically CYP3A, in the intestine. This prediction is based on the observation of large substrate overlap in CYP3A and P-gp within some species (Kim et al., 1999; Benet and Cummins, 2001; Katoh et al., 2001; Zhang and Benet, 2001). Furthermore, these molecules are typically located near one another and are thought to function in tandem. Chemical and physiological ecologists could, therefore, simultaneously compare P-gp and CYP3A levels along with the excretion of unmetabolized and metabolized PSMs in the feces when determining the role of P-gp in herbivory.

Finally, the ability to sequence, quantify, and test substrate affinity for P-gp makes it possible to investigate how generalists cope pharmacologically with a chemically diverse diet. Generalists are typically exposed to a wide and unpredictable array of PSMs because they consume taxonomically, and therefore, chemically diverse plant species, whereas specialists typically consume higher concentrations of a less diverse spectrum of PSMs because they consume plants within a narrow taxon range. It is theorized that generalists possess greater structural and functional flexibility in counterdefense mechanisms against PSMs (Gatehouse, 2002; Li et al., 2004). Greater polymorphisms in P-450 detoxification enzymes have been identified in a population of generalist insect herbivores (*H. zea*), and these are associated with wider substrate breadth compared to a population of specialists (*Papilio polyxenes*; Li et al., 2004). We predict that investigation on P-gp sequence and substrate affinity will reveal that generalist populations have greater P-gp polymorphisms that broaden substrate affinity compared to specialists. These tests will further describe the molecular and genetic disparity in counterdefenses between specialist and generalist herbivores.

Transporter proteins may not only be involved in the excretion of PSMs in feces, but may also explain the mechanisms for their sequestration in insects. Many herbivores “shuttle” ingested PSMs against a concentration gradient and sequester them unmetabolized in diverticular pouches, defensive glands, or hemolymph (Eisner et al., 1974; Müller and Hilker, 1999; Schmidt et al., 2000; Nishida, 2002; Pasteels and Hartmann, 2004). To date, no specific transporter has been implicated for the movement of these PSMs. However, there is recent evidence that selective transport systems are responsible for sequestration of certain metabolites in tissues of insects (Pasteels and Hartmann, 2004). In addition, transporter proteins have been proposed as a mechanism employed by plants to sequester PSMs in vacuoles, thereby protecting plant cells from the consequences of PSM exposure (Debeaujon et al., 2001; Sakai et al., 2002).

P-gp and Secondary Compound Diversity

P-gp transporters and the secondary compounds that interfere with them may be key factors that contribute in part to the bewildering diversity of chemical defenses in plants. The astounding diversity of secondary compounds in many species of plants is thought to be the result of a biochemical “arms race,” or reciprocal evolution between plants and herbivores (Berenbaum et al., 1991; Fritz and Simms, 1992; Berenbaum, 1995, 1999; Castellanos and Espinosa-Garcia, 1997). The presence and abundance of P-gp transporters in their guts could represent an evolutionary counterresponse on the part of herbivores that reduces the physiological effect of secondary compounds by decreasing the quantity of “potent” PSM absorbed into the tissues. As a consequence of P-gp transporters, plants may have evolved secondary compounds that directly interfere with P-gp transporters that thereby enhance toxicity through increased absorption of potent secondary compounds. We refer to the P-gp inhibitors as “potency enhancers” because they act to increase the absorption of compounds that are biologically potent, or bioactive. Examples of PSMs that reduce the effectiveness of P-gp and enhance the absorption of potent compounds include furanocoumarins from grapefruits (Ohnishi et al., 2000; Wang et al., 2001; Honda et al., 2004), quercetin in oaks and birches (Shapiro and Ling, 1997; Conseil et al., 1998), and diterpenoids in spurge (Hohmann et al., 2002; Appendino et al., 2003). Although no work exists on the synergism between “potency enhancers” that inhibit P-gp and secondary compound diversity, some plant species with P-gp inhibitors also produce potent lipophilic compounds (e.g., terpenes in grapefruit) that could be P-gp substrates. Obviously, more research is necessary to critically evaluate these speculations.

In contrast, secondary compounds have been described that activate P-gp transporters, thereby reducing absorption of other compounds (Dürr et al., 2000; Perloff et al., 2001). For example, in *in vitro* experiments with cell cultures, the administration of hypericin extracted from St. John’s wort elevates P-gp expression by 700% and significantly decreases the absorption of P-gp substrates (Perloff et al., 2001). The object of that study, as well as the others, was to identify compounds that interfere with the absorption of pharmaceuticals. Thus, the amount of activators applied may be far greater than the natural concentration of these compounds in plant tissues. Whether P-gp-activating compounds have synergistic effects on cooccurring secondary compounds at natural concentrations in plants when consumed by herbivores has not been examined. In theory, P-gp activators should

reduce the bioavailability of other coingested secondary compounds that are P-gp substrates. The adaptive significance to the plant of P-gp-activating compounds is not obvious. However, if P-gp activating compounds are produced at active concentrations in plants, the possibility exists that generalist herbivores take advantage of such compounds to reduce the absorption of secondary compounds from other plants in their diet. Thus, foraging behavior of herbivores may ultimately depend on the presence or absence of PSMs that are P-gp substrates, as well as on the presence of P-gp inhibitors and activators.

Broader Implications

Exploring how PSMs interact with transporter proteins and the relative role of these interactions in PSM tolerance in herbivores will not only contribute to understanding plant–herbivore interactions, but will likely have farther reaching applications. For example, the identification of PSMs that inhibit transporter proteins may contribute to a more efficient drug therapy. For example, a major complication in cancer therapy is due to multidrug resistance in cancer cells afforded by P-gp (Bellamy, 1996; Ambudkar et al., 2003). There is a pressing need to identify natural products and synthesize drugs that directly inhibit P-gp without concomitant toxicity to normal cells. Integration of knowledge from chemical ecologists who can predict which plants are most likely to possess P-gp inhibitors, and knowledge from pharmacologists who can predict which molecules are most likely to inhibit P-gp could facilitate the identification of compounds that enhance chemotherapy. Specifically, ecological knowledge of plant defenses and those acted on by active transporters may advance drug discovery. For example, assuming that P-gp is important in regulating the absorption of potent PSMs and that P-gp may be more important in specialist herbivores than generalists (Green et al., 2004; Sorensen et al., 2004), we can predict that leaves fed on by specialists contain compounds that are P-gp substrates, but are also likely to be potent drugs for humans. Moreover, we propose that individual plants or leaves within a host plant that are avoided by specialists may contain potency enhancers, compounds that inhibit the function of P-gp. We suggest that by applying the relationship between foraging strategies of herbivores and regulated absorption, a model can be used to efficiently target plants that contain potent compounds or potency enhancers. Further research into this area of plant–herbivore interactions could reveal the bioprospecting potential of herbivores.

Identifying active transporters in herbivores and PSMs that block the function of transporters could also be important in controlling both plant and animal pest species. Several pesticides, herbicides, anthelmintics, acaricides, and amebicides are P-gp substrates (Abu-Qare et al., 2003; Schinkel and Jonker, 2003). In addition, the absorption of many of these pesticides is limited by P-gp-like activity in insects (Lanning et al. 1996a,b; Buss et al., 2002; Srinivas et al., 2004) and plants (Windsor et al., 2003). The prevalence of P-gp may, therefore, help explain insect and plant resistance to pesticides and herbicides. Manipulation of transporters that regulate toxin absorption could be applied to development of more effective pest control agents. For example, pesticides that are P-gp substrates might be more effective if they were administered along with known P-gp inhibitors. This practice could result

in higher quantities of pesticides absorbed per unit exposure, thereby reducing the amount of pesticide used and/or increasing the susceptibility of insects to pesticides.

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Is alpha-Pinene a Substrate for Permeability-Glycoprotein in Wood Rats?

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Abstract alpha-Pinene is the dominant monoterpene in *Juniperus monosperma*. Wood rat species in the genus *Neotoma* that consume *J. monosperma* vary in their inclusion of it in their wild diet and in their tolerance of whole *J. monosperma* or alpha-pinene in laboratory feeding trials. A proposed mechanism for variable tolerance is a difference in absorption of alpha-pinene from the small intestine that is mediated by the intestinal transporter permeability glycoprotein (Pgp). To determine if alpha-pinene is a Pgp substrate, we tested whether it can competitively inhibit Pgp and thereby increase the accumulation of a known Pgp substrate (digoxin) in (1) everted sleeves of small intestine from *Neotoma stephensi*, a juniper specialist, *N. albigula*, a sympatric generalist that consumes juniper, *N. cinerea*, a more distantly related generalist, and Sprague-Dawley rats, and (2) in Caco-2 cells that over express Pgp. We also measured Pgp ATPase phosphate production in transfected insect membrane vesicles exposed to alpha-pinene. We found no significant increase in digoxin accumulation with competitive inhibition experiments, and no increase in phosphate production with transfected membranes, at any concentration of alpha-pinene up to 100 μ M. To test whether other compounds in juniper affect Pgp activity, we acclimated five *N. stephensi* to a juniper diet for 5 d, but found no significant effect

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compared to animals on control diet. Our data suggest that alpha-pinene is not a Pgp substrate.

Keywords P-glycoprotein · Wood rat · *Neotoma* · alpha-Pinene · Juniper · Small intestine · Everted sleeve · Monoterpenes · Rodents · Membrane bound transporter proteins · Biochemical defense mechanisms

Introduction

Mammalian herbivores differ in their tolerance of defensive chemicals found in plant diets (Atsatt and Ingram, 1983; Mead et al., 1985; Mangione et al., 2000). Within the *Neotoma* genus of wood rats (Edwards and Bradley, 2002) are both dietary generalists and specialists that consume plant species high in defensive compounds (Atsatt and Ingram, 1983; Dial, 1988; Edwards et al., 2001). *Neotoma albigula* (Hartley), a generalist, and *N. stephensi* (Goldman), a juniper specialist, are sympatric, and in the field *N. stephensi* includes more *Juniperus monosperma* leaves in its diet (Dial, 1988). In captivity, *N. stephensi* voluntarily consumes more *J. monosperma* and maintains body mass on diets of higher concentration of alpha-pinene, the dominant monoterpene in *J. monosperma*, than *N. albigula* (Dearing et al., 2000).

Sorensen and Dearing (2003), using a pharmacokinetic approach, found that *N. stephensi* exhibits a lower plasma concentration of alpha-pinene than *N. albigula* after oral gavage. Additionally, Sorensen et al. (2004) found that *N. stephensi* excreted more unchanged alpha-pinene in the feces than *N. albigula* when both species were either fed a juniper diet or gavaged with a single dose of alpha-pinene. These data implicate a barrier to intestinal absorption.

The hypothesized mechanism is a difference in the activity of permeability glycoprotein (Pgp). This member of the ATP-binding cassette (ABC) superfamily, is a 170-kDa membrane bound protein that actively pumps out numerous potentially toxic endogenous and exogenous compounds as soon as they diffuse into the cell membrane (Ambudkar et al., 1999, 2003). Pgp has been implicated as an important factor in the ability of cells exposed to a single drug/xenobiotic to develop resistance to a broad range of structurally and functionally unrelated drugs/xenobiotics. This has been termed multidrug resistance (MDR) or multixenobiotic resistance (MXR) (Kurelec, 1992; Hunter and Hirst, 1997). Initially discovered in cancer cells, Pgp has since been found on the secretory side of normal cells associated with secretion, absorption, or barrier function such as blood–brain barrier, blood–testis barrier, placenta, small intestine, pancreas, colon, adrenal cortex, kidney, and liver in humans and rodents (Hunter and Hirst, 1997; Ambudkar et al., 1999), and with gills and eggs in aquatic species (Bard, 2000).

In our previous work, we found the activity of Pgp summed over the small intestine to be higher in *N. stephensi* than in *N. albigula* (Green et al., 2004), which is consistent with their feeding behavior in both the field and laboratory. Thus, we hypothesized that alpha-pinene is recognized by Pgp and we tested this hypothesis in three ways. First, we determined if racemic alpha-pinene can competitively inhibit Pgp as indicated by increased accumulation of a known Pgp substrate, digoxin (Sababi et al., 2001; Stephens et al., 2001), in everted sleeves of small intestine. We made these measures in *N. albigula* and *N. stephensi*, and included a

congeneric generalist not known to consume *J. monosperma*, *N. cinerea* (Ord), to broaden our phylogenetic comparison, and Sprague-Dawley rat, a species we have used extensively in everted sleeve experiments. The everted sleeves of small intestine from wood rats and Sprague-Dawley rats were previously shown to exhibit the predicted increased accumulation of digoxin when exposed to a known Pgp inhibitor, cyclosporin A (CsA), and maintained both structural and functional integrity (Green et al., 2004, 2005). Second, we used the same indicator of competitive inhibition in a human colon carcinoma cell line (Caco-2) that overexpresses Pgp and is commonly used in the investigation of Pgp substrates (i.e., Chiou et al., 2001). Third, we eliminated unknown variables associated with tissue sections or whole cells by determining the effect of racemic alpha-pinene on phosphate production from Pgp ATPase activity in Pgp transfected membranes (Sarkadi et al., 1992; Ambudkar et al., 1999).

Pgp can be induced by ingestion of Pgp substrates (Salphati and Benet, 1998; Sandstrom and Lennernas, 1999), and may recognize alpha-pinene or some other compound(s) in *J. monosperma*. To investigate the possible induction of Pgp activity by juniper ingestion, we acclimated one group of *N. stephensi* to a diet high in juniper and measured the same endpoints as we did in animals on a control diet.

Methods and Materials

Chemicals

We purchased [^3H]digoxin and [^{14}C]polyethylene glycol (PEG; MW = 4000) from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA), [^{14}C]D-glucose and [^3H]L-glucose from Moravsek (Brea, CA, USA), cyclosporin A from Qbiogene, Inc. (Carlsbad, CA, USA), and phloridzin and a (1:1) racemic mixture of alpha-pinene from Sigma Aldrich (St. Louis, MO, USA).

Animals and Diets

We purchased Sprague-Dawley rats from Harlan Sprague-Dawley (Madison, WI, USA) and housed them in temperature-controlled facilities in the Department of Wildlife Ecology, University of Wisconsin–Madison with access to water and food *ad libitum*. Specialist (*N. stephensi*) (4 male, 6 female) and generalist (*N. albigula*) (4 male, 3 female) wood rats were trapped outside the south border of Wupatki National Park, 45 km NE of Flagstaff, AZ, USA (35°30'N, 111°27'W). The diets of these species were described in an extensive study by Dial (1988). We trapped *N. cinerea* (2 male, 3 female) at a private residence near Heber City, Summit Co., UT, USA. *N. cinerea* is a dietary generalist (Johnson and Hansen, 1979; Hauffer and Nagy, 1984; Frase and Sera, 1993), and the caches of *N. cinerea* used in this study suggested they were foraging on *Opuntia* clades, sagebrush (*Artemisia tridentata*) and various nonnative garden plants. Animals were transported to the University of Utah Animal Facility and were individually housed in shoe box cages (48 × 27 × 20 cm) with bedding and cotton batting at 20°C on a 12 L:12 D photoperiod. Animals were in captivity for 6–12 mos prior to the experiment. All animals on the control diet were fed Teklad ground rabbit chow (formula 2120) and water *ad libitum*. *N. stephensi* on the juniper diet were fed a rabbit chow based diet with

progressively increasing juniper (5, 25, 25, 70, and 70% juniper by dry mass, collected from Flagstaff, AZ, USA) over 5 d, a sufficient time to induce Pgp based on studies in cell lines (Fardel et al., 1996) and in rats (Salphati and Benet, 1998; Sandstrom and Lennernas, 1999), and which is a level of juniper on which *N. stephensi* are known to maintain body mass in the laboratory (M.D. Dearing, unpublished data). Based on weights prior to inclusion of juniper in the diet and immediately before the everted sleeve experiments, we found that *N. stephensi* maintained body mass over the 5-d dietary exposure (repeated-measures ANOVA, $F_{1,3} = 3.944$; $P = 0.141$).

All experiments on wood rats conformed to University of Utah IACUC protocol # 04-02012. All experiments on Sprague-Dawley rats conformed to University of Wisconsin–Madison IACUC protocol # A-07-6900-A00991-3-04-00.

Measures of Pgp Activity Using Tissue Accumulation of Digoxin

For tissue preparation and mounting, we closely followed the procedures described in Karasov and Diamond (1983) and Karasov et al. (1985) and reported by Green et al. (2005). Briefly, animals were euthanized with CO₂; then intestine from the stomach to the caecal attachment was quickly removed (4–6 min) and flushed with ice-cold Ringer solution (in mM: 50 mannitol, 100 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, and 20 NaHCO₃, gassed with 95% O₂ and 5% CO₂, 290 mOsm). After the intestine length was measured, the intestine was everted, and short sleeves (1.5 cm long) were cut and then mounted on stainless steel rods by tying the tissue down over grooves exactly 1 cm apart while kept in cold Ringer gassed with 95% O₂/5% CO₂. Limited intestinal length as well as concerns about experiment length precluded the use of all treatments in all animals. See Table 1 for allocation of samples among treatments.

Everted sleeves were first preincubated in 37°C Ringer solution bubbled with 95% O₂/5% CO₂ for 2 min (Pgp assays) or 10 min (D-glucose uptake) that contained either test compounds or vehicle control to maximize tissue exposure to our treatments. The tissue was then incubated in 8 ml gassed Ringer at 37°C in a flat-bottomed water-jacketed test tube with spinning stir bar to reduce and control the unstirred layer thickness (Karasov and Diamond, 1983). Incubation solution included radiolabeled probe ([³H]digoxin (9.1 kBq/ml) for measuring Pgp activity; Sababi et al., 2001; Stephens et al., 2001), or [¹⁴C]D-glucose (1.1 kBq/ml) for measuring D-glucose uptake activity) and marker solutes to correct for both non-absorbed probe in adherent mucosal fluid and diffusive flux ([¹⁴C]polyethylene glycol (PEG), MW = 4000; 1.1 kBq/ml) for Pgp measures, or [³H]L-glucose (31.4 kBq/ml), for D-glucose measures) (Karasov and Diamond, 1983). After 12 min (Pgp assay) or 4 min (D-glucose uptake), tissues were removed and then either blotted (Pgp assay) or rinsed in stirred cold Ringer and then blotted (D-glucose uptake). The mounted tissue was removed, weighed, incubated in 1 ml of tissue solubilizer (Soluene-350; Packard, Meriden, CT, USA), and counted in 10 ml of scintillation cocktail (Ecolume; Packard) with 0.5% by volume acetic acid on a Beckman LS 5801 (Beckman Coulter, Inc.) with channels set to minimize spill (counts of the alternate isotope appearing in the same counting channel) and programmed with a standard curve to correct for background quench. Calculation of digoxin accumulation followed (Karasov and Diamond, 1983), and for simplicity of expression, we assumed a digoxin concentration of 1 fmol/μl incubation solution (it was actually approxi-

Table 1 Allocation of everted sleeves to the different treatments in the proximal, mid, and distal sections of the small intestine of wood rats and Sprague-Dawley rats on control or juniper diets

Gut section	Proximal			Mid			Distal									
	Digoxin			Digoxin			Digoxin			D-Glucose						
	CsA	CsA	CsA	CsA	CsA	CsA	CsA	CsA	CsA	AP	AP	PHLOR				
Concentration (μM)	CON	10	CON	1	5	10	15	CON	10	100	CON	10	CON	15	100	1,000
<i>N. albigula</i>	7	7	7	5	4	7	6	7	7	7	7	7	7	6	5	5
<i>N. stephensi</i>	5	5	5	3	4	5	4	5	5	5	5	5	5	4	3	4
<i>N. stephensi</i> (juniper diet)	5	5	5	3	2	5	3	5	5	5	4	4	5	4	3	4
<i>N. cinerea</i>	5	5	5	3	4	5	4	5	4	5	4	4	5	4	3	4
Sprague-Dawley								4	4	4	4	4	4	4	4	4

mately 0.75 fmol/ μl), yielding units of fmol digoxin accumulated per milligram tissue. Total intestine mass was estimated as the product of the summed mass of the sixteen 1-cm tissues and the ratio of total intestine length: 16 cm.

We have previously determined that wood rat and Sprague-Dawley rat intestinal sleeves maintain structural integrity during the experimental procedure (Green et al., 2004, 2005). To verify the functional integrity and to check for the nonspecific effects of known and putative inhibitors, we measured the ATP-dependent mediated D-glucose uptake over the same combined preincubation and incubation time in adjacent tissues exposed to alpha-pinene (100 μM), vehicle control (0.15% ethanol), and phloridzin (1 mM), a known inhibitor of the intestinal glucose transporter SGLT-1, as previously described (Green et al., 2004, 2005).

Digoxin accumulation in the tissue is a balance between passive diffusion into and Pgp-mediated export out of the cells, so the desired measure of Pgp activity was the difference between digoxin accumulation in the presence of a maximal inhibitory concentration of a competitor of digoxin export, cyclosporin A (CsA) (Lan et al., 1996), and the accumulation in the absence of CsA (0.15% ethanol vehicle control), similar to Sababi et al. (2001). Pgp transport was maximally inhibited by 10 μM CsA in the rodent species on control diet (Green et al., 2004), and we verified this with *N. stephensi* on juniper diet by conducting a dose response with CsA [1, 5, 10 μM , and saturated ($\sim 15 \mu\text{M}$)] in the midintestine. To estimate intestinal Pgp capacity, which is activity summed over the entire small intestine, we used 10 μM CsA to determine Pgp activity in the proximal, mid, and distal intestinal sections. Because intestinal mass was not significantly different between the two dietary groups of *N. stephensi* (one-way ANOVA: $F_{1,7} = 1.771$, $P = 0.225$), any difference in Pgp capacity would be reflected in the Pgp activity along the intestinal tract.

Lacking radiolabeled alpha-pinene, we tested whether racemic alpha-pinene competitively inhibits the exclusion of our known Pgp substrate, [^3H]digoxin. We used racemic alpha-pinene because we lacked data on which enantiomer is found in *J. monosperma* and wished to account for both possibilities. We incubated intestinal sleeves of the midgut with alpha-pinene at concentrations that approximated 10 and 100 μM made by adding the appropriate amount of a 1000 \times solution in ethanol directly to the stirring incubation solution. These chosen concentrations bracket the concentration estimated to reach the intestine after gavage of 34.32 μg alpha-pinene/ml peanut oil used in a previous experiment, and corresponds to the amount in a full meal of *J. monosperma* (Sorensen and Dearing, 2003). Because of the volatility of alpha-pinene, we measured the loss from incubation solution over time by subsampling incubation solution every 2 min after an initial dose at the target concentration of alpha-pinene (i.e., 100 μM) and measured alpha-pinene concentration by using gas chromatography as described by Sorensen and Dearing (2003). We determined that adding half the initial amount every 2 min during both preincubation and incubation kept levels near to the desired dose (range approximately: 5–10 μM for the “10 μM ” dose, and 50–100 μM for the “100 μM ” dose). Vehicle control matched these additions with ethanol.

Caco-2 Cell Experiments

[^3H]Digoxin accumulation was measured in Caco-2 cell monolayers (passage 3–10; ATCC, Manassas, VA, USA) exposed to 1, 3, 10, 30, and 100 μM alpha-pinene made by diluting a 1000 \times solution in ethanol with cell media. First, cells were

washed twice with serum-free, phenol red-free Dulbecco's modified Eagles media (DMEM; Sigma Aldrich, St. Louis, MO, USA) and media replaced with phenol red-free DMEM containing 1% fetal bovine serum (FBS) and vehicle control (5% ethanol), or alpha-pinene (1, 3, 10, 30, 100 μM final concentration) for 30 min ($N = 4$ wells/treatment). Then, digoxin uptake was measured by adding [^3H]digoxin (37 kBq/ml) in base media with 1% FBS 45 min before the end of the trial. Subsamples (50 μl) of media were removed for specific activity, and the remainder of solution was aspirated and cells washed twice with 1 l ice-cold phosphate-buffered saline (PBS). Cells were solubilized in 0.5 ml 0.5 N NaOH and 200- μl aliquots were mixed with 200 μl 0.5 M HCl and counted on a Beckman LS 3800 (Beckman Coulter, Inc.) scintillation counter. Decays per minute were scaled to protein level in the wells, determined by spectrophotometrically comparing a 5- μl aliquot of solubilized cells from each well to bovine serum albumin standards.

Phosphate Release Assay with alpha-Pinene

Verapamil-stimulated, vanadate-sensitive ATPase activity associated with Pgp was assayed as described by the manufacturer (BD Biosciences, Woburn, MA, USA). Briefly, insect cell membranes containing human Pgp (BD Gentest Recombinant MDR1 microsomes; BD Biosciences, Woburn MA, USA) were used to assess Pgp ATPase activity in the presence and absence of vanadate and vehicle control (1% ethanol), verapamil (60 μM), a known Pgp substrate and positive control, or alpha-pinene at the same concentrations used in the everted sleeve experiments (10, 100 μM). Pgp membranes were added to reaction media (50 mM Tris-MES, 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol, 5 mM sodium azide, 5 mM MgATP) with and without 100 μM sodium orthovanadate and the test compound. The ATPase activity measured in the presence of orthovanadate represents non-Pgp ATPase activity and is subtracted from activity measured in the absence of orthovanadate. The reactants were incubated at 37°C for 20 min and terminated by the addition of 10% SDS and Antifoam A. Hydrolysis of ATP was measured on a spectrophotometric microplate reader (Dynatech MR-5000) as the release of inorganic phosphate (absorbance at 800 nm).

Statistical Analyses

When possible, we analyzed treatment effect in the everted sleeve experiments with repeated-measures ANOVA and examined interactions between treatment and species or diet. In experiments with Caco-2 cells, phosphate release assay, and everted sleeves when all treatments were not represented in every animal, we used one-way ANOVA. Effect of treatments on mediated D-glucose uptake was tested with a general linear model with treatment as a factor, and species and diet as interactions with treatment. In everted sleeves, the experimental unit was the individual rodent, while in the Caco-2 cell experiment and phosphate release assay the experimental unit was the well. We used Fisher's LSD for pairwise comparisons when global tests resulted in $P < 0.05$. A value of $P < 0.05$ was considered significant and $0.05 < P < 0.10$ a trend. All data are reported as mean \pm SE, N = sample size.

Plots of residuals were visually inspected to verify a random distribution. We used Systat 10 (SPSS Inc., Chicago, IL, USA) for analyses.

Results

Neither of our test compounds, CsA (15 μM) or alpha-pinene (100 μM), affected mediated [^{14}C]D-glucose uptake in any rodent species on control diet, or *N. stephensi* on the juniper diet, whereas uptake was significantly inhibited by phloridzin (1 mM) ($P = 0.005$), the specific inhibitor of the SGLT-1 Na/glucose cotransporter (general linear model, $F_{3,71} = 3.9$; $P = 0.013$, followed by pairwise comparisons, interactions not significant). One *N. cinerea* and one *N. stephensi* on juniper diet were removed from the study due to loss of functional integrity in intestinal sleeves as indicated by uniformly low [^{14}C]D-glucose uptake.

Consistent with our previous results in rodents on control diet (Green et al., 2004, 2005), net accumulation of [^3H]digoxin, corrected for adherent label, increased sublinearly with increasing concentration of CsA in the solution for intestinal sleeves of *N. stephensi* on the juniper diet, reaching an asymptote by 10 μM (Fig. 1; one-way ANOVA, $F_{4,11} = 11.4$; $P = 0.001$, followed by pairwise comparisons). There was no significant variation in Pgp activity along the intestinal tract for *N. stephensi* on either diet (Fig. 2; repeated-measures ANOVA, control, $F_{2,8} = 3.3$; $P = 0.091$; juniper, $F_{2,6} = 0.31$; $P = 0.748$), and there was no significant difference in Pgp activity/mg tissue between *N. stephensi* on different diets in proximal (one-way ANOVA,

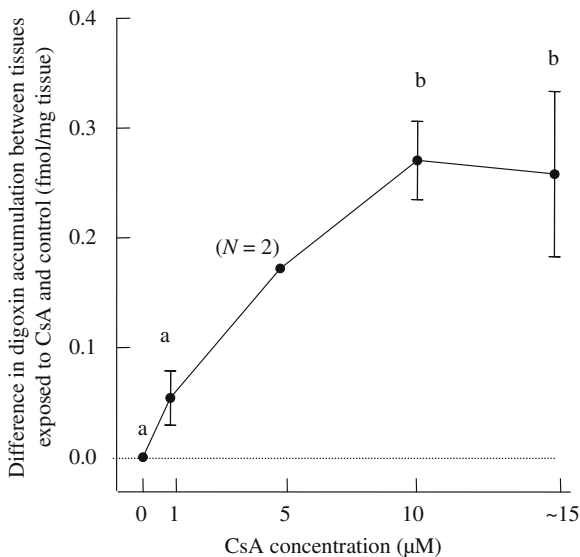


Fig. 1 Difference in digoxin accumulation (fmol/mg tissue) between midintestinal sleeves exposed to cyclosporin A (1, 5, 10, ~ 15 μM) and vehicle control (0.15% ethanol) during 2-min preincubations and 12-min incubations in *Neotoma stephensi* fed a diet containing *Juniperus monosperma* ($N = 2$ –5 sleeves/concentration). The difference shows a sublinear increase that reaches an asymptote by 10 μM (one-way ANOVA, $F_{3,8} = 5.736$; $P = 0.022$, followed by pairwise comparisons). Different letters (a, b) indicate significant differences between concentrations ($P < 0.01$). Points are mean \pm SE

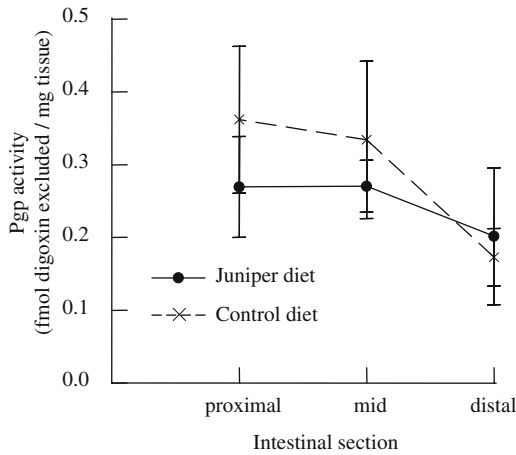


Fig. 2 Pgp activity (fmol digoxin excluded/mg tissue) along the intestinal tract in *N. stephensi* on control ($N = 5$) and juniper diet ($N = 4$) as determined by the difference in digoxin accumulation in intestinal sleeves exposed to ethanol vehicle control or 10 μM CsA during 2-min preincubations and 12-min incubations. There was no significant difference along the tract in either diet group (repeated-measures ANOVA, intestinal section: $F_{2,14} = 2.486$; $P = 0.119$, intestinal * section diet: $F_{2,14} = 0.500$; $P = 0.617$) and no difference in Pgp activity between diet groups in the proximal (one-way ANOVA, $F_{1,7} = 0.678$; $P = 0.437$), mid ($F_{1,7} = 0.332$; $P = 0.582$), or distal ($F_{1,7} = 0.130$; $P = 0.729$) sections. Points are mean \pm SE

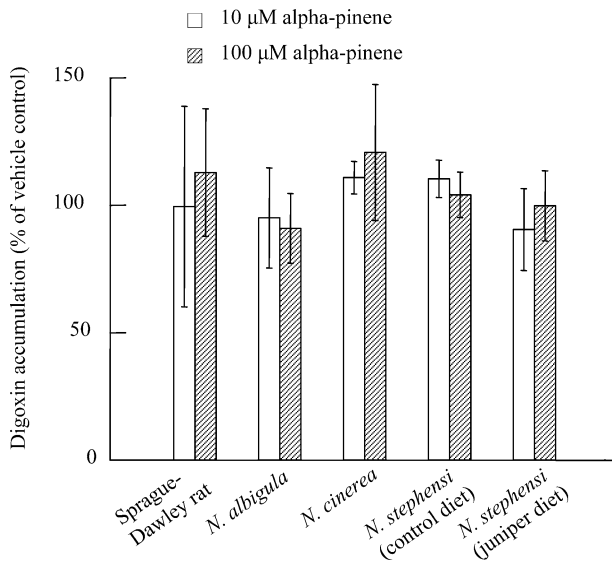


Fig. 3 Digoxin accumulation as percent of ethanol vehicle control in midintestinal sleeves exposed to alpha-pinene (10, 100 μM) during 2-min preincubations and 12-min incubations in Sprague-Dawley rats ($N = 4$), *Neotoma albigula* ($N = 7$), *N. cinerea* ($N = 3$), and *N. stephensi* on control ($N = 5$) and juniper diet ($N = 4$). There was no significant difference in digoxin accumulation in any group (repeated-measures ANOVA, $F_{2,36} = 0.150$; $P = 0.861$; interactions were not significant). Values are mean \pm SE

$F_{1,7} = 0.68$; $P = 0.437$), mid ($F_{1,7} = 0.33$; $P = 0.582$), or distal ($F_{1,7} = 0.13$; $P = 0.729$) sections (Fig. 2). Several tissue sections from one *N. stephensi* on juniper diet were damaged during the procedure eliminating this animal from the comparison with control diet and some samples from the dose–response curve. With no difference in intestinal mass (one-way ANOVA, $F_{1,7} = 1.77$; $P = 0.225$), we conclude that acclimation to a juniper diet over 5 d does not induce intestinal Pgp activity. The mean Pgp activity in proximal and midintestine of *N. stephensi* on juniper diet was lower than *N. stephensi* on the control diet, opposite of what would be expected if induction occurred, suggesting that a larger sample size would not affect this conclusion.

Accumulation of digoxin in mid-intestinal sleeves was not affected by alpha-pinene at 10 or 100 μM in any of the rodent species on control diet, or in *N. stephensi* on the juniper diet (Fig. 3; repeated-measures ANOVA, $F_{2,36} = 0.15$; $P = 0.861$; interactions not significant). This indicates that alpha-pinene does not competitively inhibit exclusion of digoxin by Pgp in wood rats or Sprague-Dawley rats over the range of concentrations tested.

We also measured digoxin accumulation in Caco-2 cells with and without alpha-pinene (1, 3, 10, 30, 100 μM) to test the ability of alpha-pinene to competitively inhibit Pgp in a commonly used and sensitive human cell line. alpha-Pinene, at 1 μM , yielded one extreme outlier, which was removed from analysis. We ob-

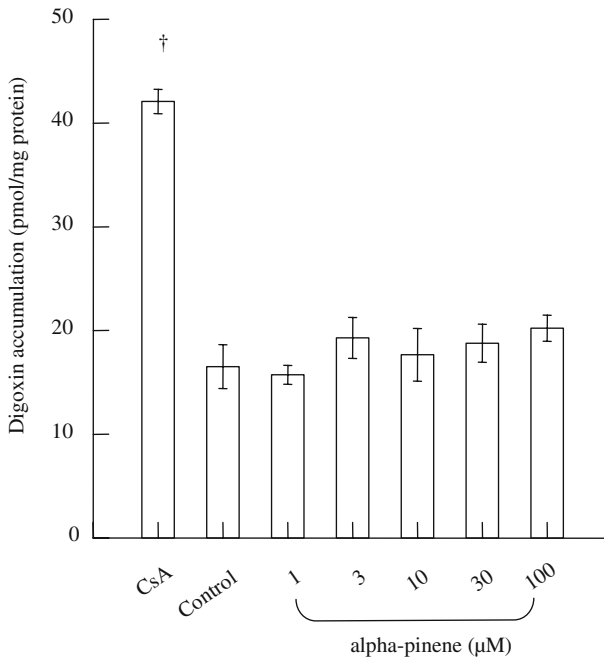


Fig. 4 Digoxin accumulation in Caco-2 cells exposed to cyclosporin A (5 μM), vehicle control (5% ethanol), or racemic alpha-pinene (1, 3, 10, 30, and 100 μM) for 75 min with [^3H]digoxin added 45 min before the end of the trial. Values are means \pm SE of 4 wells/treatment, except at 1 μM alpha-pinene where $N = 3$ wells. Decays per minute in cells were scaled to total protein in the wells. Significant differences from control in pairwise comparisons after one-way ANOVA ($F_{6,20} = 37.1$; $P < 0.001$) are indicated by ($\dagger P < 0.001$)

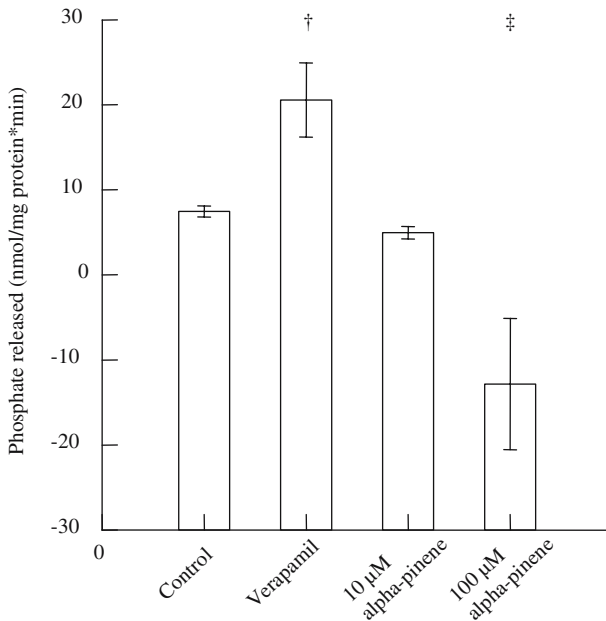


Fig. 5 Phosphate production from Pgp ATPase activity in Pgp transfected insect membranes exposed to verapamil (60 μM), racemic alpha-pinene (10, 100 μM), and vehicle control (1% ethanol). Values are means \pm SE of three replicates per treatment. Significant differences in pairwise comparisons after one-way ANOVA ($F_{3,8} = 16.607$; $P = 0.001$) are indicated by ($\dagger P < 0.05$) and ($\ddagger P < 0.005$)

served the expected increased accumulation of digoxin with the known Pgp substrate, cyclosporin A (5 μM), but no difference in digoxin accumulation between control and alpha-pinene at any concentration (Fig. 4; one-way ANOVA, $F_{6,20} = 37.1$; $P < 0.001$, followed by pairwise comparisons).

Phosphate Release Assay with alpha-Pinene

We observed the expected increase in phosphate release with our positive control, verapamil (60 μM), a known Pgp substrate, while alpha-pinene had no effect at 10 μM , and actually resulted in a decrease in phosphate release at 100 μM (Fig. 5; one-way ANOVA, $F_{3,8} = 16.61$; $P = 0.001$, followed by pairwise comparisons). These data, along with the Caco-2 cell-line results, indicate that alpha-pinene is not a substrate for human Pgp. The decrease in phosphate production by 100 μM alpha-pinene implies inhibition of ATPase activity.

Discussion

Although our previous results indicated a correlation between intestinal Pgp capacity and the tolerance of a *J. monopserma*-dominated diet (Green et al., 2004), we failed to establish a link between Pgp and alpha-pinene, the dominant

monoterpene in *J. monosperma*. Digoxin accumulation in wood rat intestine, Sprague-Dawley rat intestine, and Caco-2 cells was not affected by exposure to alpha-pinene. Phosphate released from ATPase activity did not show an increase in the presence of alpha-pinene at 10 μM , and 100 μM alpha-pinene actually decreased phosphate production, indicating an inhibition of ATPase activity. The possibility remains that wood rat Pgp may recognize alpha-pinene, but that digoxin has higher affinity for Pgp preventing inhibition by alpha-pinene in the everted sleeve and Caco-2 cell-line experiments. This would also require that the Pgp assay with human Pgp is not a valid surrogate for investigating wood rat Pgp. The best way to address this possibility would be to use radiolabeled alpha-pinene as the probe and CsA as the inhibitor, or to develop another method that measures alpha pinene concentration in tissue (i.e., gas chromatography). Another potential method would be to measure the accumulation of a probe of lower affinity than digoxin (i.e., propranolol; Lan et al., 1996) in everted wood rat intestinal sleeves with alpha-pinene as the potential inhibitor. A negative result, however, would still leave the question of relative affinities.

The highly lipophilic alpha-pinene molecule ($\text{C}_{10}\text{H}_{16}$, $\log K_{\text{ow}} = 4.83$; Li et al., 1998) is not ideal for recognition by Pgp. Although Pgp does recognize a number of compounds characterized as lipophilic, the majority appears to be amphipathic with polar oxygen or nitrogen groups attached (Ford and Hait, 1990; Hunter and Hirst, 1997; Ambudkar et al., 1999). Some diterpenes have been found to interact with Pgp (Hohmann et al., 2002; Appendino et al., 2003), but they have polar groups attached to the ring structure. It may be that one or more of the phase I metabolites of alpha-pinene are recognized by Pgp and are pumped back into the intestinal lumen. We would expect, however, that this would still result in competitive inhibition of Pgp and an increased accumulation of digoxin in both the everted sleeve and Caco-2 cell experiments. Furthermore, microbial conversion of a metabolite back to the parent compound is unlikely and, therefore, would not explain the difference in parent compound found in the feces of the two wood rat species (Sorensen et al., 2004).

The larger question of tolerance to a diet high in *J. monosperma* may involve compounds other than alpha-pinene. *N. albigula* decreased food intake and exhibited decreased urine pH after acclimating over 9 d to a diet containing alpha-pinene at a level corresponding to a 100% juniper diet (Dearing et al., 2000). Despite a decreased food intake, *N. albigula* did not lose significant body mass. In contrast, *N. albigula* and, to a lesser extent, *N. stephensi* lost significant body mass on a diet consisting of artificial diet (15% of maintenance levels) and *J. monosperma* leaves *ad libitum*. Both artificial diets with and without alpha-pinene matched nutrient levels of juniper.

In another plant herbivore system, Lawler et al. (1999) found that the feeding deterrence of common ringtail possums (*Pseudocheirus peregrinus*) and common brushtail possums (*Trichosurus vulpecula*) by the Eucalyptus terpene, 1,8-cineole, is a conditioned flavor aversion due to its association with the postingestive effects caused by diformylphloroglucinol compounds, such as jensenone. Could alpha-pinene serve a similar purpose in juniper as cineole does in Eucalyptus?

Because there may be other compounds in juniper that interact with Pgp, we also measured Pgp activity in *N. stephensi* acclimated to a juniper diet. We did not observe induction in Pgp activity, which suggests at least three possible explanations. (1) Although 5 d are sufficient to induce Pgp in laboratory rat studies using known Pgp substrates (Salphati and Benet, 1998; Sandstrom and Lennernas, 1999),

possibly the level of Pgp substrate(s) in juniper are not sufficient to induce Pgp on the same time scale. (2) *N. stephensi* maintains a high constitutive level of Pgp that is not inducible. Our previous results indicate that *N. stephensi* showed higher Pgp capacity than *N. albigula* when on a control diet (Green et al., 2004) in support of a high constitutive level of Pgp. Of interest would be whether the generalist, *N. albigula*, exhibits induced levels of Pgp when acclimated to a juniper diet. We lacked sufficient individuals to test this possibility. (3) Possibly Pgp does not recognize any component of *J. monosperma*, and our previous results on intestinal Pgp capacity are purely correlational. We consider this unlikely due to the long list of Pgp substrates and modulators, but a test would be to use extracts of *J. monosperma* in everted sleeve, Caco-2 cell line, and/or phosphate release experiments.

In addition to the possibility that other compounds besides alpha-pinene may be involved in juniper tolerance is the potential that other mechanisms besides Pgp may be involved. The difference in intestinal Pgp capacity between *N. stephensi* and *N. albigula* is only one difference in the arsenal of biochemical defense mechanisms of wood rats. Although Sorensen and Dearing (2003) did not see any difference in elimination rate of alpha-pinene in these two species, other results indicate they may detoxify compounds differently (S. Haley, unpublished data; Dearing et al., 2000, 2002). Could these species biotransform juniper toxins differently producing metabolites that differ in toxicity? Recently, more membrane-bound transport proteins related to Pgp have been described, and as many as 10 have been implicated in a cell's ability to defend against toxins (Taipalensuu et al., 2001; Scotto, 2003). If wood rats vary in Pgp activity and activity of phase I and II detoxifying enzymes, then they may vary in activity of other transporters or defense mechanisms that could result in the observed variability in juniper tolerance. Adding to the variation in the function of these mechanisms is a possible variation in the regulation of mechanisms that may be coordinated at the gene level (Scotto, 2003). A full understanding of the interplay between ingested toxins and the biochemical defense mechanisms in mammalian herbivores may require techniques that can simultaneously measure the activity of a myriad of proteins, or the corresponding genes. Efforts using DNA microarray analyses are currently underway. The difficulty in finding an explanation for the observed differences in alpha-pinene absorption and *J. monosperma* tolerance between *N. stephensi* and *N. albigula* highlights the challenges we face.

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Pharmacological Perspectives on the Detoxification of Plant Secondary Metabolites: Implications for Ingestive Behavior of Herbivores

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Abstract Plant secondary metabolites (PSMs) are a major constraint to the ingestion of food by folivorous and browsing herbivores. Understanding the way in which mammalian detoxification pathways are adapted to deal with PSMs is crucial to understanding how PSMs influence ingestive behavior of herbivores and hence their fitness and the impact that they have on vegetation. Pharmacological concepts can provide insights into the relationship between the absorption and metabolic fate of PSMs and ingestive behavior. Lipophilic PSMs will be absorbed into the bloodstream and must be removed fast enough to prevent their accumulation to toxic levels. Elimination depends on their metabolism, usually by cytochrome P450 enzymes, to more polar metabolites that can be excreted by the kidney. The concentration of PSM in blood (C) is a better measure of exposure to a toxin compared to the amount ingested because there can be great variability in the rate and degree of absorption from the gut. C rises and falls depending on the relative rates of absorption and elimination. These rates depend in part on metabolic and transport processes that are saturable and liable to inhibition and induction by PSMs, indicating that complex interactions are likely. Herbivores can use diet choice and the rate and amount of PSM consumption to prevent C from reaching a critical level that produces significant adverse effects.

Keywords Plant secondary metabolites · Mammalian herbivores · Pharmacological perspectives · Blood concentrations · Food choice · Pharmacokinetics · P450 enzymes · Cineole

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Introduction

Herbivory is a widespread feeding habit of terrestrial vertebrates. Among the Eutherian mammals, most of the Artiodactyls and Perrisodactyls including the Bovids, Equids, and Cervids are exclusively herbivorous. The Lagomorphs and some Rodents also subsist on a diet largely composed of plants (Williams and Kay, 2001). Among the marsupials, herbivory is also widespread within the Diprotodontia, including many examples of herbivorous mammals, such as the macropods and possums (Hume, 1999). Plant material is generally a diffuse source of nutrients, and large amounts of material need to be ingested to maintain the metabolism of herbivorous mammals (Iason and Van Wieren, 1999). As a result, these mammals can consume a high proportion of plant biomass production and hence have an important influence on ecosystem function (Hester et al., 2006).

Because of plants' immobile nature and vulnerability to herbivory, strong selection pressure exists to evolve antiherbivory adaptations (Rhoades, 1979). Antiherbivory strategies diverge along the lines of the major division within the plant kingdom. Monocotyledonous plants (e.g., grasses and sedges) tolerate herbivory; their basal meristems reduce the impact of grazing on plant survival by facilitating regrowth (McNaughton, 1983). Dicotyledonous plants tend to have chemical and physical defenses that discourage herbivory (Bryant et al., 1991). Chemical defense is manifest in the evolution of a large array of phytochemicals that appear to have minimal function in the primary metabolism of plants (Rosenthal and Berenbaum, 1991). These so-called plant secondary metabolites (PSMs) include alkaloids, terpenes, phenolics, glucosinolates, and cyanogenic glycosides. Mammalian herbivores that ingest PSMs are liable to suffer a variety of adverse effects including reductions in nutrient availability and toxicity.

In this review, we consider the effects of PSMs on the ingestive behavior of herbivores, by using a pharmacological perspective. We also review the mechanisms used by herbivores to overcome plant defenses and avoid PSM toxicity, and consider how the pharmacokinetics of PSMs may influence ingestive behavior.

Plant Secondary Metabolites as Drugs

Modern pharmacology arose from the traditional use of plant products as medicines, and even today many therapeutic drugs are plant secondary metabolites (e.g., morphine, digoxin, atropine, ephedrine, artemisinin, vincristine, paclitaxel) or PSM derivatives (e.g., codeine, buprenorphine, warfarin, docetaxel). The principles surrounding the absorption, distribution, metabolism, and excretion of phytochemicals are the same, whether they are considered in the context of the delivery of drugs to target tissues in humans, or the detoxification of PSMs by mammalian herbivores. These two views of PSMs, as drugs and as chemical defenses, have been independently pursued, and we argue here that an exchange of concepts will benefit both of these disciplines. In particular, pharmacological concepts can provide insights into the relationship between ingestive behavior and the absorption and elimination of dietary PSMs. Conversely, the study of the chemical ecology of plant/herbivore interactions can provide insights to pharmacologists by considering the evolutionary context in which physiological mechanisms for metabolism of modern drugs arose.

A Note on Terminology

A drug may be broadly defined as any chemical that produces a biological effect on a human or animal, including both therapeutic and toxic effects (which are often produced by the same substance at different doses). Many PSMs are known to have drug effects as medicines, toxins, or antifeedants, but others have not had any such effects characterized. A xenobiotic is a chemical that may be present in animal tissues following absorption from the environment, but that lacks nutritional value. Xenobiotics include PSMs, synthetic drugs, and agricultural and other industrial chemicals. Where possible we have referred to literature on PSMs, but for much of the discussion on pharmacology we found no PSM data available, and we have used findings on synthetic drugs and other xenobiotics.

Effects of Plant Secondary Metabolites on Herbivores

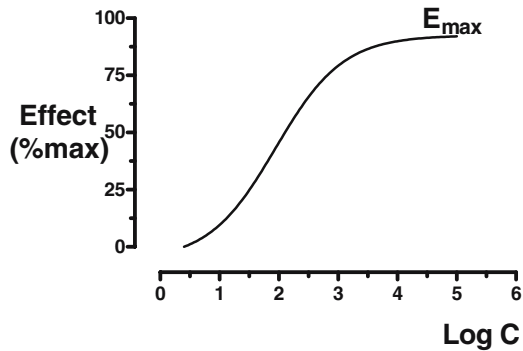
Gastrointestinal Effects

Some PSMs remain in the gut where they may have local adverse effects. Condensed tannins, for example, are large polar molecules that cannot cross the gastrointestinal epithelium and are, therefore, not absorbed (Terrill et al., 1994). Tannins have an astringent taste and can bind and reduce the absorption of dietary proteins. Formyl phloroglucinols (e.g., jensenone, sideroxydonal) are aldehydes that react so rapidly with amine groups on proteins and other cellular constituents that they are not absorbed from the gut (McLean et al., 2004). However, these reactions result in adverse effects, mediated through serotonin release, which deter herbivory (Lawler et al., 1998; Wiggins et al., 2005). These locally acting PSMs will not be further considered in this review.

Systemic Effects

Many PSMs are lipophilic in character and can pass readily through the cell membranes of the gastrointestinal epithelium and be absorbed into the bloodstream, in which they will be delivered around the body to produce effects at distant sites. For example, morphine and digoxin stimulate chemoreceptors in the *area postrema* of the brainstem, resulting in nausea (Saito et al., 2003). In some cases, vomiting ensues leading to removal of unabsorbed toxin. Emetic agents can also act in the gut, via the release of serotonin and substance P from enterochromaffin cells, exciting sensory neurons that project to the *area postrema* and other parts of the brainstem (Saito et al., 2003). Stimulation of the emetic center is also associated with the development of conditioned flavor aversions that deter herbivores from repeated ingestion of toxic plants (Zahorik et al., 1990). PSMs can target other organs with specific toxicity; for example, digoxin causes cardiac arrhythmias, pyrrolizidine alkaloids produce multiple organ toxicity and cancers (Fu et al., 2004), and terpenes cause depression of the central nervous system (Falk et al., 1991; Tibballs, 1995). PSMs can also target particular systems, such as cholinergic neurotransmission that is blocked by atropine (which occupies the muscarinic receptors of acetylcholine) and enhanced by physostigmine (which inhibits

Fig. 1 General shape of the log concentration–effect curve



acetylcholinesterase). Sublethal toxicity can deter future feeding through learned aversions (Provenza, 1995) and taste associations (Pass and Foley, 2000).

Pharmacodynamics: How Effect Depends on Concentration of Drug

The effect of a drug generally depends on the size of the dose taken and the consequent concentration produced at site(s) of action in the body (Wilkinson, 2001). The relationship between the log concentration of drug and its effect is sigmoidal rising to an asymptote (Fig. 1; Ross and Kenakin, 2001). Note that there is a dynamic range where the effect is sensitive to changes in concentration; a characteristic maximum effect (E_{\max}); and at low concentrations the effects are minimal or zero. We hypothesize that herbivore metabolic adaptations and ingestive behavior are directed toward preventing the concentration of PSMs in the body from exceeding tolerable levels.

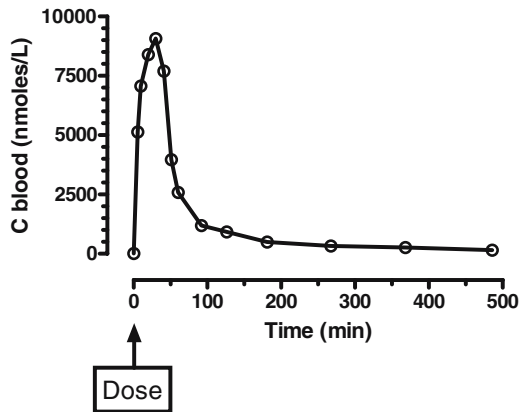
Potency

Drugs and other PSMs differ markedly in their potency, measured by the amount (or concentration) required to produce a particular effect. For example, digoxin and atropine act therapeutically in humans at submilligram doses, whereas the doses of salicin (once used as an analgesic) and quinine are 1000 times greater. Among antifeedant PSMs, terpenes and phenolics are present at high concentrations in *Eucalyptus* leaf (up to 12% and 40% dry matter, respectively; Cork and Sanson, 1991), but the major antifeedant effect against marsupial folivores is exerted by formylphloroglucinols, which are present at significantly lower concentrations (mostly below 4% dry matter; Moore et al., 2004; Moore and Foley, 2005).

Tolerance

Continued exposure to a drug can lead to tolerance: a reduced effect that is usually due to an adaptation by cells to the presence of the drug. This happens especially with drugs that act on the central nervous system, which for example becomes less sensitive to alcohol and nicotine in regular drinkers and smokers, respectively (Le Houezec, 1998; Nutt, 1999). Tolerance to alcohol can develop to such an extent that

Fig. 2 Blood concentration of cineole after an oral dose (100 mg/kg) administered to a brush-tail possum. Methods are given in Boyle et al. (2002)



individuals can survive normally lethal blood levels (Davis and Lipson, 1986), as well as tolerance to opioids in that addicts may take 50 times the normal dose, an amount which could be lethal for an opioid-naïve person (O'Brien, 2001). However, not all drugs elicit tolerance, as for example the doses of digoxin, warfarin, and atropine-like drugs that do not need to be increased with their continued therapeutic usage (Hardman et al., 2001).

Pharmacokinetics: How the Concentration of Drug Changes with Time After Dose

Absorption of Plant Secondary Metabolites

Absorption from the gut is usually by passive diffusion down a concentration gradient (Wilkinson, 2001). The speed of membrane permeation depends on the lipid solubility of the PSM. Some PSMs are substrates for active transporters, for example, glucosides that can be absorbed by the sodium-dependent glucose transporter SGL1, whereas glycosides of other sugars (e.g., rhamnose) are not transported (Manach et al., 2004). In general, glycosides must be hydrolyzed to be absorbed as the more lipophilic aglycone.

Most active PSM transport is by less specific efflux transporters such as P-glycoprotein (P-gp), which pumps foreign molecules out of the central nervous system and out of the body and into the gut, bile, and urine (Lin and Yamazaki, 2003).

Concentration in Blood

After an oral dose of a drug, its concentration in the body, which is usually measured in the blood or plasma (C), rises to a maximum (C_{\max}) and then falls (Fig. 2). The drug is distributed by the blood around the body and enters tissues where enzymes may convert it into metabolites that diffuse back into the blood and may be excreted in urine. Some metabolites that are formed in the liver are secreted into the bile and excreted in the feces (e.g., menthol glucuronide; Yamaguchi et al., 1994).

For many drugs, the time course of the effects following an oral dose will be described by a combination of the curves in Figs. 1 and 2. For example, the speed of onset of effects will depend on the rate of increase in concentration, and the maximum effect will be some function of C_{\max} . Pharmacokinetic–pharmacodynamic models incorporate change in concentration with time after a dose and the concentration–effect curve, and aim to describe the time–effect relationship (Meibohm and Derendorf, 1997). The overall scheme is illustrated below

$$\text{Dose rate} \xrightarrow{\text{PK}} C \text{ in body} \xrightarrow{\text{PD}} \text{Effects} \quad (1)$$

where PK is pharmacokinetics, PD is pharmacodynamics, and C is concentration.

The concentration at the tissue site of action usually cannot be directly measured, but the rise and fall in the concentration in blood plasma over time is a better measure of tissue exposure to a drug compared to the dose (which is the amount ingested over a given period of time, and gives far less data than a series of blood concentrations). Furthermore, there are many factors—such as body size and composition, and the rates of absorption and elimination—that affect the relationship between dose and concentration in the blood, which are often highly variable. Wilkinson (2001) gives a good introduction to pharmacokinetics, and there are specialist texts that give a detailed treatment (e.g., Rowland and Tozer, 1995; Shargel and Yu, 1999).

The concentration of a drug in blood relates best to its effect when it acts rapidly and reversibly. In other circumstances, there may not be a simple relationship between blood concentration and effect. For example, there can be delays if the equilibration between blood and tissue site of action is slow (as with some opioids; Lotsch et al., 2004), and the relationship between concentration and effect may be complicated if tissue sensitivity changes over time (as with levodopa; Deleu et al., 2002). Furthermore, the effects can persist after a drug has been eliminated from the blood if it is bound in tissues, or its actions are not reversible (e.g., irreversible enzyme inhibition or cell death). Sometimes the biological activity of a compound is attributable to its metabolite, as outlined below.

Elimination of Plant Secondary Metabolites

It is self-evident that herbivores must remove ingested PSMs from their body or risk their progressive accumulation to harmful levels. Metabolism and excretion are the processes used to eliminate PSMs from the body. Because the terrestrial kidney conserves water by extensively reabsorbing it from the renal tubules, lipophilic substances that can freely permeate the tubular epithelium are also extensively reabsorbed (Wilkinson, 2001). Thus, the more lipophilic a PSM, the more readily it will be absorbed from the gut and the less efficiently it will be excreted by the kidney. Animals possess a battery of enzymes that can transform lipophilic xenobiotics into water-soluble metabolites that are more readily excreted by the kidney.

Metabolism of Plant Secondary Metabolites

The enzymes that metabolize xenobiotics are considered to have evolved in herbivores in response to the formation of defensive chemicals by plants (Gonzalez and

Nebert, 1990; Lewis et al., 1998). Compared to the enzymes of intermediary metabolism, they are characterized by a remarkable lack of substrate specificity, enabling them to metabolize a chemically diverse range of PSMs and, incidentally, other xenobiotics. Thus, humans use the same group of enzymes to metabolize therapeutic drugs, whether of plant origin or the newly created products of synthetic chemistry.

The major type are the cytochrome P450 enzymes (CYPs), which are responsible for most oxidative metabolism of xenobiotics (Wilkinson, 2001). P450 genes are found in virtually all organisms, and the enzymes they code for are responsible for many different reactions: some plant P450 enzymes are involved in the synthesis of the plant defense chemicals that are subsequently catabolized in the animal by other related enzymes of the same superfamily (Werck-Reichhart and Feyerreisen, 2000).

The overall pattern of P450 genes is the same in humans and the puffer fish, and has been conserved despite 420 million yr of separate development (Nelson, 2003). Despite the similarities in P450 gene families among mammals, there is variation in the individual orthologous forms of P450 enzymes, probably driven by dietary differences, giving rise to marked differences between species in xenobiotic metabolism (Shimada et al., 1997; Lewis et al., 1998; Boyle et al., 1999, 2000, 2001; Machala et al., 2003). Within species, there can also be polymorphic and sex differences in P450 activity that, combined with individual differences in diet and associated induction processes, can result in significant individual differences in xenobiotic metabolism (Lewis et al., 1998; Ayrton and Morgan, 2001; Weaver, 2001; Weinshilbom, 2003).

The major sites of xenobiotic metabolism are the gastrointestinal endothelium, the liver, and the kidney (Parkinson, 2001; Doherty and Charman, 2002). In the case of ruminant herbivores, which have a bacterial fermentation chamber anterior to major sites of absorption, microbial biotransformation of PSMs in the rumen is an important mechanism for PSM metabolism (Carlson and Breeze, 1984). Because lipophilic PSMs are absorbed in the small intestine, they escape contact with the colonic bacteria of hindgut fermenters. However, many PSMs are polar (e.g., glycosides) and poorly absorbed, and are subject to bacterial metabolism in the colon (Manach et al., 2004). For example, quercetin-3-rutinoside (rutin) is hydrolyzed to the flavonol quercetin, which is partly absorbed intact and partly metabolized to hydroxy acids that are then absorbed (Olthof et al., 2003; Rechner et al., 2004).

Tissue level xenobiotic metabolism is divided into two phases: functionalization (Phase 1) and conjugation (Phase 2) (for a detailed account of xenobiotic biotransformation, see Parkinson, 2001). Phase 1 reactions serve to introduce or free a functional group, such as $-OH$, $-NH_2$, or $-COOH$. Most Phase 1 reactions involve a variety of oxidations catalyzed by P450 enzymes, but hydrolyses are also important for many PSMs such as esters, amides, and glycosides. In Phase 2, functional groups on xenobiotics or their metabolites are used to form adducts with endogenous substances, such as glucuronic acid, sulfate, glycine, or glutathione. The resulting conjugate is typically much more polar than its precursor, limiting its reabsorption from the renal tubules. Additionally, it may be a substrate for active transport into the renal tubules or bile duct, which can greatly increase the rate of elimination (Lin and Yamazaki, 2003; Lee and Kim, 2004).

Is Metabolism Detoxification?

In general, xenobiotic metabolism acts on lipophilic substrates that are converted into more polar, and, therefore, more readily excreted, metabolites. In changing the

chemical structure of a xenobiotic, metabolism usually changes the biological activity, but this does not always result in detoxification because there are many examples where the metabolite is active (Zhou et al., 2004a).

For example, P450 oxidation of pyrrolizidine alkaloids generates reactive metabolites that form adducts with protein and DNA, resulting in hepatotoxicity and carcinogenicity (Fu et al., 2004). Rumen bacterial biotransformation can also lead to activation of xenobiotics. For example, *S*-methyl cysteine sulfoxide, found in cruciferous plants, is metabolized in the rumen to dimethyl disulfide, which causes hemolytic anemia (Smith, 1980).

Induction and Inhibition of Metabolism and Transport

Competition between substrates can result in inhibition of metabolism (Lin and Lu, 2001; Weaver, 2001), especially with low-capacity pathways such as glycine conjugation (Moriwaki et al., 2005). Mechanism-based inhibition occurs when an enzyme produces a reactive product that binds to the enzyme and inactivates it (Weaver, 2001). The furanocoumarins in grapefruit juice, for example, inactivate enterocyte CYP3A, which can result in clinically significant increases in oral absorption of drugs (e.g., cyclosporin) that are extensively metabolized by this enzyme (Dresser and Bailey, 2003). Recovery depends on enzyme regeneration and takes a few days (Greenblatt et al., 2003).

Some xenobiotics bind to receptors in the cell nucleus leading to increased synthesis of certain xenobiotic-metabolizing enzymes, a process called induction (Lin and Lu, 2001; Weaver, 2001). Unlike enzyme inhibition, which is almost immediate, induction takes some days or weeks while the new protein is synthesized for the full effect to be seen (Hollenberg, 2002). The increased amount of enzyme enables faster metabolism of substrates, and is clearly a useful adaptation to a diet with changing PSM content. St John's wort (*Hypericum perforatum*) contains hyperforin, which activates the nuclear pregnane X receptor—thereby inducing the expression of CYP3A4, an enzyme that metabolizes most drugs (Moore et al., 2000). St John's wort also induces the intestinal efflux transporter P-gp, and both mechanisms contribute to the lowered blood levels of substrate drugs (Dresser et al., 2003). The full manifestation of the effects of St John's wort and other PSMs are complex, and depend on species, tissue, dose, timing, and PSM composition (Zhou et al., 2003, 2004b). Glucosinolates found in cruciferous plants also cause the induction of P450s (Bonnesen et al., 1999) following their hydrolysis in the digestive tract to active metabolites such as isothiocyanates.

Inducers are also substrates for the enzymes they induce (Hollenberg, 2002) and can act as competitive inhibitors of other reactions while present and bound to the active site. This is demonstrated in the brushtail possum, in which pretreatment for 10 d with a mixture of dietary terpenes induced hepatic P450 enzymes, resulting in faster *in vitro* metabolism of both the terpenes (1,8-cineole and *p*-cymene) and other substrates (Pass et al., 1999, 2001, 2002). When the inducing terpenes were added to liver enzyme preparations, they potently inhibited the metabolism of 1,8-cineole (Pass and McLean, 2002).

The consequences for PSM metabolism (and P-gp transport) by herbivores that consume large amounts of PSMs in their diet may be a complex result of induction (from recent exposure to inducing PSMs) and inhibition (by competitively inhibitory PSMs currently consumed and mechanism-based inhibitors recently consumed).

Dietary exposure of ruminant herbivores to PSMs can also lead to induced adaptation of the rumen microbial population, which can result in large changes in rates of metabolism and hence bioavailability. Such adaptation is generally presumed to depend on changes to microbial population structure in response to altered substrate availability. Thus, for example, the presence of oxalate salts in the diet of ruminant herbivores leads to the proliferation of *Oxalobacter formigenes*, a bacterial species capable of degrading oxalates (Allison et al., 1985). Adapted animals are tolerant to dietary oxalate exposure, and rates of degradation increase with increases in dietary exposure (Duncan et al., 1997).

Pharmacokinetic Factors Influence Plant Secondary Metabolite Concentration and Effects

The ingestive behavior of a herbivore effectively determines the PSM dosage rate (amount ingested per time), but the concentration in blood will depend as well on the relative rates of absorption and elimination, which are liable to considerable variability among species and individuals.

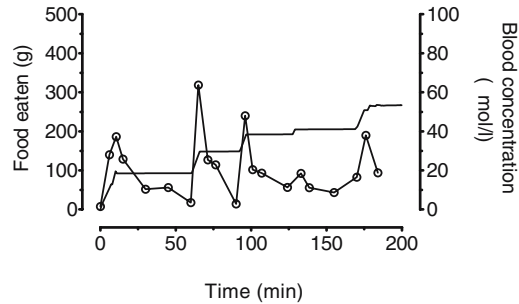
Some xenobiotics are extensively metabolized in the gut lumen, gut wall, and liver so that only a fraction of the oral dose reaches the systemic circulation (Wilkinson, 2001; Doherty and Charman, 2002). This fraction, termed the bioavailability (F), can be calculated from the ratio of the areas under the concentration–time curve after oral and intravenous dosing. Low values are inherently variable (Hellriegel et al., 1996), and a change in F from, say, 0.03 to 0.06 effectively doubles the amount of xenobiotics that reaches most of the body.

Limits to Consumption of PSMs

Herbivorous mammals typically eat in bouts separated by periods of not eating, and the total meal may take several hours (Arnold, 1981). The presence of PSMs in food plants presents a risk of toxicity to herbivores but, in most cases, it is presumed that they are able to limit consumption of toxic plants before they cause toxicity. Cases of clinical signs of toxicity among wild herbivores in their natural range are unusual. The means by which herbivores discern the toxicity of plants and avoid overconsumption are not well known, and we have developed a model system by using 1,8-cineole and the brushtail possum to address this. The monoterpene 1,8-cineole (cineole) is present in many species of *Eucalyptus* leaf as well as a number of conifer species (Von Rudloff, 1975). When brushtail possums were fed an artificial diet containing 2% cineole, the size of each bout was limited by the blood cineole concentration, since feeding stopped when this reached a critical level (Fig. 3; Boyle et al., 2005). The blood concentration depends on the amount of cineole ingested (i.e., the dose), its speed of absorption, and its bioavailability. Thus, the size and frequency of bouts, and the rate of eating during a bout may be governed by a negative feedback effect from the concentration of blood cineole or its metabolites.

Repeated ingestion of PSMs will lead to accumulation if some remains in the body after the previous feeding bout. The extent of accumulation depends on both the rate of ingestion (i.e., dose rate) and the rate of elimination. If an animal eats at

Fig. 3 Blood concentrations of cineole (○) in the brushtail possum in relation to eating a diet containing 2% cineole (—). From Boyle et al. (2005)



a constant rate, the PSM will accumulate to a steady state when the (usually first-order) elimination rate equals the intake rate. This is because the first-order rate of elimination will increase with the concentration of PSM (up to a concentration that saturates the enzymes).

$$\begin{array}{l} \text{PSM ingestion} \rightarrow C \rightarrow \text{Elimination rate} = k \cdot C \\ \text{(constant rate)} \qquad \qquad \qquad \text{(first order)} \end{array} \quad (2)$$

Because the elimination rate ($k \cdot C$) changes with C , the efficiency of elimination of a xenobiotic is better expressed by using the clearance concept, borrowed from renal physiology (Wilkinson, 2001). Clearance (CL) is the volume of blood or plasma from which a drug has been completely removed per unit time, and its value remains constant for any one individual and occasion so long as first-order conditions pertain. Clearance enables the steady-state concentration (C_{ss}) to be calculated for any constant dosing rate.

$$\text{Dosing rate} = \text{CL} \cdot C_{ss} \quad (3)$$

Continued accumulation of even low-potency PSMs will eventually lead to adverse effects. The consumption of PSMs on a daily basis will be limited by the capacity of the animal to eliminate them and their metabolites. This will depend on the rates of metabolism and renal excretion.

Implications of Pharmacokinetics of PSMS for Patterns of Ingestive Behavior

We have seen that, in a pharmacological context, circulating concentrations of drugs can serve as a useful approximation of drug concentrations at target tissues, and we argue that a similar approximation is useful in the context of herbivores and PSMs. Herbivores can avoid exceeding thresholds of plasma PSM concentrations by various means. First, spreading their feeding bouts on particular toxic plants throughout the day, or over longer periods, will reduce the probability of toxic thresholds being exceeded (Foley et al., 1999). There is evidence that consumption of toxic plants on 1 d influences intake rates on subsequent days, with cattle showing cyclical patterns of consumption to maintain circulating concentrations of toxins below toxic thresholds (Pfister et al., 1997). At shorter time-

scales, however, there is less evidence that herbivores alter patterns of ingestive behavior, including meal frequency, in response to PSM concentrations (Wiggins et al., 2003; Sorensen et al., 2005). A temporal distribution of feeding on a toxic plant species is likely to be facilitated by a dispersed spatial distribution of the plant. Large patches of toxic plants limit the potential for consuming alternative plants and may, therefore, limit intake potential. For example, extensive stands of invasive plants such as sagebrush (*Artemisia* spp.) are difficult to control by herbivory; smaller patches are more readily consumed presumably since alternative food sources may allow herbivores to maintain toxin concentrations below critical thresholds (Heady, 1964; Provenza et al., 2003).

Critical thresholds for particular toxins may also be avoided through diet mixing. The toxin dilution hypothesis (Freeland and Janzen, 1974) has been around for a long time, but never adequately tested (Iason and Villalba, 2006, this issue). It is certainly true that herbivores consume more food when offered a choice of foods containing different PSMs than when offered foods containing a single PSM (Burritt and Provenza, 2000; Wiggins et al., 2003), and Wiggins et al., 2006 (in this issue) have shown that leaf consumption is greater when two chemically dissimilar plants are offered together rather than either one alone. This phenomenon, however, has not yet been linked to circulating concentrations of PSMs that would demonstrate that animals are maintaining toxins below toxic thresholds through diet mixing.

Induction and inhibition of PSM metabolism also has implications for patterns of ingestion. Ruminant herbivores are generally cautious when they encounter novel foods, and this may be an adaptive mechanism to allow time for induction of enzymes responsible for PSM metabolism. Such induction may occur at the level of the gut microflora or the liver. Adapted animals consume more toxic plants than nonadapted ones (Duncan et al., 2000). Indeed, diet mixing, or occasional sampling of novel foods, may be an adaptive strategy for maintaining enzymes in an induced state to give maximum future protection against toxicity when encountering novel plants.

Pharmacological Model of Herbivory

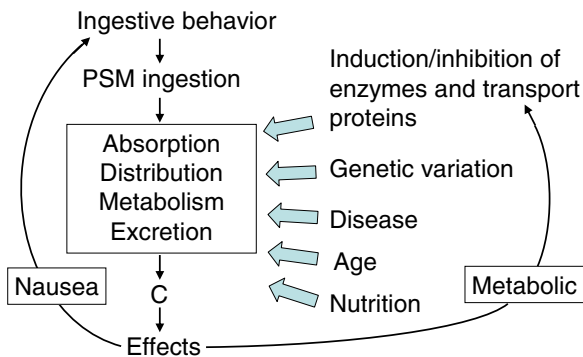


Fig. 4 A model showing the pharmacological factors that affect herbivory

Conclusion

A pharmacokinetic approach to the study of PSM metabolism has much to offer in terms of quantifying the impact of PSMs on large herbivores under different feeding scenarios and to suggest explanations for observed patterns of feeding behavior. Much of the research on the pharmacokinetics of PSMs derives from the pharmacological literature, and some of the methods and understanding of drug metabolism now need to be adopted by ecologists to advance the field of plant/animal interactions. A range of factors relating to the physiology of the subject influence the fate of drugs following their ingestion (Fig. 4). These include the state of enzyme induction/inhibition (Lin and Lu, 2001), genetic factors (Weinshilboum, 2003), disease states (Rodighiero, 1999), and the age of the animal (Woodhouse, 1992; Kinirons and O'Mahony, 2004) and its nutritional status (Walter-Sack and Klotz, 1996; Ioannides, 1999). Improved understanding of how such factors influence the metabolism of plant secondary compounds in herbivores would help to improve our understanding of the influence of PSMs on ingestive behavior. Of particular interest are the questions surrounding the kinetics of toxin elimination and how this relates to the kinetics of feeding behavior at a range of temporal scales.

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Application of Pharmacological Approaches to Plant–Mammal Interactions

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Abstract The dominant theory in the field of mammalian herbivore–plant interactions is that intake, and therefore tolerance, of plant secondary metabolites (PSMs) is regulated by mechanisms that reduce absorption and increase detoxification of PSMs. Methods designed by pharmacologists to measure detoxification enzyme activity, metabolite excretion, and most recently, drug absorption, have been successfully applied by ecologists to study PSM intake in a variety of mammalian study systems. Here, we describe several pharmacological and molecular techniques used to investigate the fate of drugs in human that have potential to further advance knowledge of mammalian herbivore–plant interactions.

Keywords Absorption · Distribution · Foraging behavior · Mammalian herbivores · Metabolism · Molecular ecological techniques · Pharmacology · Plant secondary metabolites

Introduction

The application of pharmacological techniques has significantly advanced the field of plant–animal interactions. Much of the success stems from the overlap between human–drug interactions and plant–animal secondary metabolite (PSM) interactions. Humans and other animals experience constant and unavoidable exposure to foreign chemicals, or xenobiotics. Xenobiotics include both manufactured and natural chemicals such as drugs, pollutants, and PSMs. Regardless of xenobiotic origin, the mechanisms by which humans and other animals process xenobiotics that enter the body are similar, and this overlap provides the platform from which these

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two fields can be integrated. Pharmacological techniques developed for human-drug studies have been most aggressively applied to insect–PSM interactions, and those reviews are found elsewhere (Rosenthal and Berenbaum, 1992; Berenbaum, 1999; Karban and Agrawal, 2002). Here, we focus on the less-explored aspects of pharmacology in understanding mammalian herbivore–PSM interactions.

In general, pharmacologists define the mechanisms by which animals process ingested xenobiotics by four major parameters: absorption, distribution, metabolism (detoxification), and excretion, or collectively ADME, each of which is described in detail elsewhere (Gibaldi and Perrier, 1982; Neubig, 1990; Boroujerdi, 2001; Hayes, 2001; Klaasen and Watkins, 2003). Tolerance to a particular xenobiotic is maximized in individuals that possess mechanisms to minimize absorption and distribution and maximize metabolism and elimination of xenobiotics. The relationship between ADME parameters and xenobiotic tolerance largely explains the variation in therapeutic efficacy of drugs in humans, and has the potential to account for the variation in the foraging ecology of mammalian herbivores. For humans, high tolerance to drugs afforded by low absorption, low distribution to target organs, and high metabolism and excretion results in lower therapeutic efficacy. For mammalian herbivores, high tolerance to PSMs afforded through these same mechanisms results in potentially greater intake of plants containing PSMs.

The general concepts of ADME have only recently been applied to study PSM tolerance and foraging behavior of mammalian herbivores, despite Freeland and Janzen's (1974) introduction of these concepts over 30 years ago. This delay is likely the result of numerous factors, including the cost of conducting pharmacological studies, the daunting task of searching the extensive pharmacological literature, and the complexity of investigating the complex mixtures of xenobiotics in plants. Despite these challenges, several ecologists have employed pharmacological techniques to investigate certain aspects of ADME in mammalian herbivores, primarily metabolism and excretion (McManus and Ilett, 1977; Bolton and Ahokas, 1997a,b; Boyle et al., 1999a,b; Pass et al., 1999, 2001, 2002; Stupans et al., 1999, 2001; Liapis et al., 2000; Ngo et al., 2000, 2003; Mangione et al., 2001; McLean et al., 2001; Pass and McLean, 2002; Lamb et al., 2004). Excretion of PSM metabolites has been largely used to measure metabolism qualitatively. However, to fully understand how PSMs influence the foraging behavior of mammalian herbivores, absorption and distribution (primarily in the blood) must also be considered. In this review, we provide summaries of several pharmacological techniques of ADME that can be used to understand the fate of PSMs in mammalian herbivores. Although ADME parameters are interconnected, absorption and metabolism receive the majority of attention, as they tend to guide the design of studies on distribution and excretion. Our goal is to introduce ecologists interested in studying interactions of PSMs and mammalian herbivores to pharmacological concepts and techniques that can advance the field. To that end, we outline the basic principles of absorption, metabolism, and distribution (as it relates to absorption and metabolism), and how they apply to several hypotheses important to studies of mammalian herbivores.

Absorption

Absorption is the first parameter of ADME that may significantly affect diet selection in mammalian herbivores. Mammalian herbivores may reduce absorption

of PSMs through various mechanisms, but here we focus on efflux transporters in cells lining the gut. Studies of efflux transporters, such as permeability glycoprotein (Pgp), are revolutionizing our understanding of the absorption of pharmaceuticals and other xenobiotics in humans (Bellamy, 1996; Hunter and Hirst, 1997; Sharom, 1997; Stein, 1997; Watkins, 1997; Benet et al., 1999; Schinkel, 1999; Silverman, 1999; Scheffer et al., 2000; Ayrton and Morgan, 2001; Benet and Cummins, 2001; Saier and Paulsen, 2001; Washington et al., 2001; Zhang and Benet, 2001; Melaine et al., 2002; Ambudkar et al., 2003; Dietrich et al., 2003; Fromm, 2003; Lin and Yamazaki, 2003a,b; Schinkel and Jonker, 2003; Sparreboom et al., 2003; Chan et al., 2004; Fromm, 2004; von Richter et al., 2004). Prior to the discovery of efflux transporters, absorption of compounds across the gut was thought to occur passively. We now know that transporters in the gut actively efflux xenobiotics, thereby preventing absorption. Research in this area is intensely active, with over 200 papers published on Pgp's impact on drug absorption and bioavailability in 2005. Despite the celebrity of efflux transporters in the areas of pharmacology and medicine, they have only recently been recognized in the area of herbivore–PSM interactions (Murray et al., 1994; Lanning et al., 1996a,b; Sorensen and Dearing, 2003; Green et al., 2004). An overview of the mechanisms and role of efflux transporters in effluxing PSMs and influencing plant–herbivore interactions is found in Sorensen and Dearing (2006, this issue). Here, we summarize specific assays that directly or indirectly investigate efflux transporters and their role in regulating absorption of PSMs in mammalian herbivores. Studies on efflux transporters as regulators of PSM absorption should first focus on quantifying these transporters in the gastrointestinal tract and testing PSMs as substrates for efflux transporters in enterocytes. However, efflux transporters are found in a variety of barrier tissues including the blood–brain barrier, liver, kidney, testes, and uterus.

Direct Quantification of Efflux Transporters

A combination of Western blots and quantitative polymerase chain reaction (qPCR) is used to determine the presence and quantify gene expression of efflux proteins in various tissue types. These assays can be used to test hypotheses of mammalian herbivore feeding preferences and tolerances. The exceptional tolerance to PSMs by dietary specialists may be, in part, attributed to reduced absorption afforded by efflux transporters. The most celebrated efflux transporter, Pgp, has been fully quantified by using monoclonal antibodies in a variety of organisms including herbivorous mammals (Dudler and Hertig, 1992; Murray et al., 1994; Sangster, 1994; Lanning et al., 1996a,b; Barnes, 2001; Doi et al., 2001; Keppler and Ringwood, 2001; Lee et al., 2001; Saier and Paulsen, 2001; Yazaki et al., 2001; Bard et al., 2002; Buss et al., 2002; Smith and Prichard, 2002; Sasaki et al., 2002; Green et al., 2004). An additional approach is to quantify mRNA of efflux transporters via qPCR (Wong and Medrano, 2005) by using species-specific primers. These primers are often designed from known sequence homology between species (for sequence information, see UCS Genome Bioinformatics website, <http://genome.ucsc.edu>). The benefits and constraints of each approach are described elsewhere (Sorensen and Dearing, 2006, this issue), but each has been applied to a variety of distantly related species and, therefore, holds promise for further investigation of efflux transporters in mammalian herbivores. Although the majority of assays designed to quantify efflux transporters focus on Pgp,

several exist, and new transporters are continuously being identified and should be considered as the field progresses.

Indirect Investigation of Efflux Transporters

Investigating the proportion of PSM ingested that is excreted unchanged in the feces is an indirect method to assess the role of efflux transporters. In general, a herbivore is fed a PSM or whole plant tissue, and the absolute amount of each PSM ingested is quantified (Sorensen et al., 2004). Feces are collected from the animal following intake of PSMs, and the quantity of unchanged (unmetabolized) PSM in the feces is determined. The assumption is that unchanged PSM in excreta represents PSMs that were not absorbed. Such data would indirectly indicate that some mechanism regulated the absorption of the ingested PSM. However, lack of unchanged PSM in the feces is not necessarily indicative of a limited role of efflux transporters. The PSM may indeed be a substrate for transporters, but is acted on by metabolizing enzymes or microbes prior to defecation. Another confounding factor associated with fecal studies is that positive results do not reveal the mechanism responsible for regulating absorption. A variety of mechanisms regulate absorption of ingested PSMs, and each is reliant on the physiochemical properties of the PSM. For example, the peritrophic membrane of insects (Lehane, 1997; Barbehenn, 2001) and tannin-binding salivary proteins (TBSPs) in mammals (McArthur et al., 1995; Skopec et al., 2004) act to regulate absorption of large complex PSMs, such as tannins. However, both peritrophic membranes and TBSPs are not effective regulators of the absorption of lipophilic compounds. Therefore, fecal excretion of unchanged lipophilic molecules (i.e., those with high partition coefficients), such as terpenes and alkaloids, may be a result of efflux transporters. Promising evidence that efflux transporters may play a role in regulating absorption of PSMs is that several PSMs (e.g., alkaloid vinblastine from periwinkle, *Vinca rosea*, Sharom, 1997) and diterpenes from spurges (*Euphorbia* spp., Hohmann et al., 2002; Appendino et al., 2003) are substrates for Pgp. A list of Pgp substrates, inhibitors, and inducers from plants is given in Sorensen and Dearing (2006, this issue) and Zhou et al. (2004).

Testing PSMs as Efflux Transporter Substrates

A variety of *in vitro* assays are available to test whether PSMs are substrates for efflux transporters. Monolayer cell systems have been developed to positively identify xenobiotics as substrates, inhibitors, and inducers of efflux transporters (Brayden, 1997; Bjornsson et al., 2003; Weiss et al., 2003; Fröhlich et al., 2004). Although many cell lines are specifically designed to overexpress Pgp, they can be selected to express a variety of different efflux transporters. Results from established cell lines and a particular PSM are compared with known transporter substrates (Sharom, 1997; Seelig and Landwojtowicz, 2000; Polli et al., 2001; Bjornsson et al., 2003). Although efflux monolayer systems are regarded as the standard for identifying transporter substrates, other techniques include ATPase activity assays (Scarborough, 1995; Litman et al., 1997; Schmid et al., 1999) and calcein-AM fluorescence assays (Liminga et al., 1994; Tiberghien and Loor, 1996). Each has advantages and disadvantages compared to efflux monolayer systems that

are reviewed elsewhere (Polli et al., 2001). Although these *in vitro* assays provide a noninvasive approach to screen PSMs as substrates for efflux transports, results from such studies can be confounded. First, results may not necessarily translate to the organism of interest. Identifying which efflux transporter a PSM interacts with in cell-based systems containing multiple transporter proteins is another complication. In addition, *in vitro* studies may not correspond to *in vivo* studies. Therefore, follow-up studies need to isolate cells and tissues from the herbivore of interest (Green et al., 2004, 2005), and/or researchers should conduct *in vivo* experiments (described below in [Blood Distribution](#)) to confirm the role of efflux transporters in mammalian herbivores.

Metabolism

Once a PSM enters the blood stream, metabolism becomes the most important factor in ADME, and directly affects distribution and excretion. In general, the faster an herbivore metabolizes a given PSM, the more quickly it clears it from the body and, thus, the greater its tolerance should be for the PSM. In the following paragraphs, we outline the various techniques that can be utilized to study the metabolism or detoxification of PSMs by mammalian herbivores. Before discussing pharmacological techniques, we briefly touch on the overall process of detoxification in mammals and the general groups of enzymes involved.

Detoxification Pathways

Mammalian detoxification is split into a two-phase process. Phase I, or functionalization, contains a suite of enzymes that act on xenobiotics by adding functional groups. Oxidation and reduction are two of the most common types of chemical reactions carried out by functionalization enzymes. The family of enzymes called cytochrome P450s (CYPs) is one of the largest and best studied groups of functionalization enzymes that carries out oxidation reactions in mammals. The functionalization of xenobiotics has three possible outcomes: (1) the metabolite becomes sufficiently water-soluble to be excreted in bile or urine; (2) the metabolite becomes more reactive and more toxic than the parent compound; or (3) the metabolite is further detoxified by phase II enzymes. Phase II, or conjugation, contains a suite of enzymes that add a water-soluble conjugate, often a sugar or amino acid moiety. These conjugates make the xenobiotic more water-soluble so that it can be excreted in urine or bile. The detoxification system of vertebrate herbivores is reviewed in Dearing et al. (2005), and a complete description can be found in Casarett and Doull's Toxicology (Klaassen and Watkins, 2003). Although the overall outcome of the detoxification system is relatively straightforward, i.e., to make xenobiotics more water-soluble for excretion, regulation of the detoxification system is complicated and dynamic. Up- and down-regulation of enzyme content and activity occur frequently and rapidly, on the order of days, in response to the presence of xenobiotics in the blood (Hollenberg, 2002; Bock and Köhle, 2004). Therefore, carefully designed studies are needed to identify the importance and relative capacity of specific detoxification pathways that mammalian herbivores use to metabolize ingested PSMs.

Analysis of Metabolites

Several studies have documented the metabolites produced and excreted when either whole plant material or purified PSMs are fed to mammalian herbivores (Boyle et al., 1999a,b; Mangione et al., 2001; McLean et al., 2001). Depending on the chemical nature of the PSM, gas chromatography, mass spectrometry, or high-performance liquid chromatography can be used to analyze the metabolites produced. Metabolites can be analyzed in blood or urine. Depending on the size and nature of the herbivore, one type of sample may be easier to collect than the other. Metabolite studies are an excellent first step in studying mammal–PSM interactions. Whereas characterizing metabolites gives potential clues as to which pathways may be involved in the detoxification of PSMs, these types of studies are more qualitative than quantitative with respect to rate and route of detoxification.

Enzyme Assays

Another technique frequently used in pharmacology that lends itself well to studying nonmodel species is measuring the amount of liver detoxification enzymes. Total CYP content can be measured in a spectrophotometric assay (Hayes, 2001) that yields an overall view of one of the most important groups of phase I enzymes in mammalian herbivores. To measure the CYP content, fresh liver tissue is required, making this type of study usually a lethal endpoint one. However, if the mammalian herbivore were large enough, a liver biopsy may be possible. The liver tissue is homogenized and differentially centrifuged to separate the microsomal (endoplasmic reticulum) fraction from the cytosolic fraction. The CYP content is measured in the microsomal fraction of the liver. A number of labs studying mammalian herbivores have used total CYP content as an assay, and these results are summarized in Table 1. Total CYP content can be used to determine the detoxification ability of a single species (Bolton and Ahokas, 1997a,b;

Table 1 Total cytochrome P450 contents in the livers of mammalian herbivores

Species	Status	Diet	P450 content	Reference
Koala ($N = 4$) (<i>Phascolarctos cinereus</i>)	Wild	Eucalyptus	0.87 ± 0.18	(Stupans et al., 1999)
Tammar wallaby ($N = 6$) (<i>Macropus eugenii</i>)	Wild	Grazer	0.24 ± 0.04	(Stupans et al., 1999)
Quoakka ($N = 8$) (<i>Setonix brachyurus</i>)	Captive	Lab diet	0.95 ± 0.08	(McManus and Ilett, 1977)
Kangaroo ($N = 10$) (<i>Macropus fuliginosus</i>)	Wild	Browser	0.47 ± 0.05	(McManus and Ilett, 1977)
Brushtail possum ($N = 4$) (<i>Trichosurus vulpecula</i>)	Captive	Control lab diet	0.27 ± 0.04	(Pass et al., 1999)
Brushtail possum ($N = 4$) (<i>Trichosurus vulpecula</i>)	Captive	Terpene lab diet	0.41 ± 0.02	(Pass et al., 1999)
Brushtail possum ($N = 20$) (<i>Trichosurus vulpecula</i>)	Wild	Eucalyptus	0.90 ± 0.06	(Bolton and Ahokas, 1997a)
Stephens woodrat ($N = 4$) (<i>Neotoma stephensi</i>)	Wild	Juniper	1.74 ± 0.25	(Lamb et al., 2004)

Lamb et al., 2004), and also for comparative studies among species on different diets (McManus and Ilett, 1977; Stupans et al., 1999), as well as species on different diets (Pass et al., 1999).

In addition to measuring total CYP content, a number of researchers have measured the activity of specific detoxification enzymes in wild herbivores (Bolton and Ahokas, 1997a,b; Pass et al., 1999; Stupans et al., 1999, 2001; Liapis et al., 2000; Ngo et al., 2000, 2003; Lamb et al., 2004). Enzyme-specific substrates are used to determine the activity of individual enzymes of interest. The disappearance of the substrate or the appearance of metabolites is generally measured spectrophotometrically. Enzyme activity assays are robust methods that lend themselves well to nonmodel species studies and interspecies comparisons. Hayes (2001) is an excellent reference for enzyme activity assays. A table containing liver CYP enzyme activities in selected species and associated references can be obtained from Xenotech, Lenexa, KS, USA (<http://xenotechllc.com>).

Although enzyme assays are robust in nature, they require expensive equipment (e.g., homogenizers, spectrophotometers for turbid solutions, high-speed centrifuges) that might not be a part of a standard ecology lab. Careful selection of the detoxification enzymes to analyze is important because the activity assays can be time consuming and expensive. Another limitation of enzyme activity assays is that they exist for only a subset of the more than a hundred detoxification enzymes. The most thorough studies typically measure the activities of about 10 detoxification enzymes. A carefully designed study with well-chosen enzymes can lead to useful intraspecific or interspecific comparisons. Studies on urinary or blood metabolites can be used to guide the selection of enzyme assays. Comparing the detoxification enzyme activities of mammalian herbivores on different diets will allow ecologists to understand not only how the PSMs are detoxified, but also potentially how the herbivore regulates detoxification enzymes.

Researchers can also use PSMs directly in microsomal assays. The PSM can be added to the microsomal fraction, and its disappearance or the appearance of metabolites can be measured over time (Pass et al., 2001, 2002; Pass and McLean, 2002). This assay measures the combined activities of all detoxification enzymes present in the microsomal fraction but does not elucidate the particular enzymes by which detoxification occurs. However, this approach will give an idea of the speed and capacity of a herbivore's detoxification system for a particular PSM.

A noninvasive approach to rapidly screen PSMs of interest as substrates for various metabolizing enzymes and guide future enzyme studies is to use commercially available hepatocytes or recombinant enzymes and specific probe substrates (LeCluyse, 2001). The approach can be used to test which metabolic pathways are most likely to metabolize a specific metabolite and also which are produced without the use of live mammalian herbivores. Hepatocytes are advantageous in that they represent the "true" physiological environment of the organism from which they are derived (e.g., human, rat, insect, and frog). Recombinant enzymes, on the other hand, are more readily available, less expensive, and the results are not confounded by metabolic processes other than those from the enzyme of interest. One limitation of using hepatocytes and recombinant enzymes is that they only indicate whether the PSM is a substrate for an enzyme from the organism from which the enzyme was isolated. Enzymes in the herbivore of interest may differ significantly in their interaction with the same PSM. These studies are useful as an initial screen to predict which enzymes may be

responsible for PSM metabolism in the herbivore of interest prior to whole animal studies. Although time and resource intensive, researchers can isolate, culture, develop hepatocytes, and design recombinant enzymes directly from mammalian herbivores (Mankowski et al., 1999; Gardmo et al., 2005; Jung'a et al., 2005).

Gene Expression

An alternative strategy to determine how PSMs are processed by mammals is to quantify expression of detoxification enzyme genes. The three major techniques used to analyze gene expression are Northern blots, qPCR, and microarrays. Northern blots involve running mRNA samples on a gel and using specially designed probes to identify and quantify the mRNA of interest on the gel (Belin, 1998; Sabelli, 1998). Northern blots have been used successfully for the genus *Neotoma* with probes designed for laboratory rats (Lamb et al., 2001, 2004). Ecologists studying herbivores closely related to a model species may also be able to take advantage of predesigned probes. However, for herbivores not closely related to a model species, genes of interest will need to be sequenced to design appropriate probes. Quantitative PCR is a relatively new technique that can also be used to study gene expression of detoxification enzymes. As in quantification of efflux transporters, specific primers are needed for the reactions to ensure that the only double-strand DNA produced is from the gene of interest (Wong and Medrano, 2005). Both Northern blots and qPCR can be used to study how PSMs affect the regulation of the detoxification system by quantifying changes in gene expression of detoxification genes. Depending on quantity of sample, equipment access, budget, etc., ecologists can decide between Northern blots or qPCR.

Microarrays

Microarrays hold promise for investigating detoxification systems on a much larger scale (Gibson, 2002; Meyer and Gut, 2002; Gracey and Cossins, 2003; Klaper and Thomas, 2004; Thomas and Klaper, 2004; Tittiger, 2004). Microarrays are a cutting-edge technology in the field of genomics that allow for the parallel analysis of the expression of thousands of genes. Representative sequences of genes are printed in high density onto a solid surface, often a glass microscope slide. A number of commercial arrays made for model organisms are available, and cross-species hybridization works well between closely related species (Gonzalez and Nebert, 1990; Enard et al., 2002; Moody et al., 2002). Therefore, the commercial rat, mouse, or human arrays may be acceptable for a number of mammalian herbivores. To perform microarray analysis, extracted RNA is reverse-transcribed, fluorescently labeled, and hybridized to the array. The arrays are then scanned by specialized scanners, and the intensity and color of the spot at each gene are related to the abundance of the gene transcript in the sample. The popularity of microarrays as a technique for studying gene expression has led most major research universities to develop core facilities that perform microarray experiments for a fee; therefore, specialized equipment is not needed. Even with a core facility, the cost of microarray experiments is considerable. Arrays cost ~\$200–400; a single experiment with 10 individuals and two treatments (i.e., 20 samples) can easily cost between \$2000–8000 depending on the experimental design and the array. Although the laboratory methods for running a microarray are straightforward, the data analysis

can be overwhelming because microarrays produce data for thousands of genes. Therefore, careful planning of the experimental design and analysis is essential before starting a microarray experiment (Churchill, 2002; Reiner et al., 2003; Yang et al., 2003; Woo et al., 2004; Khatri and Drăghici, 2005). A well-designed microarray experiment will not only give a fairly complete picture of gene regulation occurring in response to PSMs, but also provide a number of candidate genes for further studies.

Gene Sequencing

Whereas quantitative differences in gene products produce detoxification differences within and between species, qualitative differences in gene products because of sequence differences may also play a large role in the variation of detoxification abilities. The field of pharmacogenomics examines the relationships between polymorphisms in detoxification genes and their effects on drug detoxification and disease susceptibility in humans (Puga et al., 1997; Nebert and Roe 2001; Meyer, 2004). Polymorphisms within human populations can lead to 30- to 40-fold differences in detoxification of drugs and xenobiotics (Nebert and Dieter, 2000). The plant–insect interaction community has also studied the effects of differences in sequences in detoxification genes on herbivore detoxification capacity (e.g., Li et al., 2004). With the advent of automated sequencing facilities at most research universities, comparative sequencing is a relatively straightforward procedure. Sequencing projects will lead to increased understanding of detoxification differences both within and between different species of mammalian herbivores as well as to the discovery of novel detoxification genes. For example, a novel CYP has been reported in koalas, CYP4A15 (Ngo et al., 2000; Stupans et al., 2001).

Hypnotic State Assays

Although the application of the assays described in the previous sections holds promise for exploring the metabolism of PSMs in mammalian herbivores, they often require a lethal endpoint and are, therefore, not feasible for many researchers working on mammals. Several nonlethal assays have recently been borrowed from pharmacology by ecologists that allow for repeated measures of detoxification capacity in mammals. Hypnotic state assays measure the length of time an animal spends in a drug-induced hypnotic state (or loss of righting reflex) as a proxy for detoxification capacity for a specific enzyme. This assay is often used in the investigation of new pharmaceuticals (Sasaki, 1994; Konishi et al., 2002; Kim and Shin, 2005) to determine whether detoxification of a novel compound will cause interactions with other drugs through competition for the same detoxification pathways. In such studies, a dose of the compound is administered prior to the hypnotic agent, and the length of the hypnotic state is measured. Drug interactions are considered possible if administration of the compound increases the length of time the animal spends in a hypnotic state compared to the control treatment, i.e., when only the hypnotic agent is delivered. Because the detoxification pathways of the hypnotic agents have been established, but those of the novel compound typically have not, the results can be used to infer putative detoxification pathways for the novel compound.

Sleep time under hexobarbital and paralysis time under zoxazolamine are two of the more common agents used for hypnotic state assays (Sasaki, 1994; Koizumi et al., 2001). For either assay, the compound is injected intraperitoneally. After losing the ability to right itself, the animal is placed on its back with its head elevated, and the length of time (typically 20–300 min) the animal remains in this state is measured. As there is considerable variation among individuals and sexes, sample sizes greater than 8 for each sex are advisable for comparative studies.

Hypnotic state assays can easily be applied to detoxification of PSMs by mammalian herbivores. The potential safety of PSMs for use as pharmaceuticals has been assessed with hypnotic state assays (Wada et al., 1993; Oliveira et al., 2005). Two significant benefits of this assay compared to other pharmacological approaches (e.g., microsomal assays) are that it is relatively noninvasive and nonlethal. Because different sleep agents are acted on by different detoxification enzymes (e.g., hexobarbital by CYP2B; zoxazolamine by CYP1A), these assays can be used to measure the capacity or role of particular pathways. The assays can also be performed under field conditions, as well as repeatedly to permit comparative studies between animals on different diets, treatments, or from various populations.

Blood Distribution

Pharmacokinetic Studies

Pharmacokinetics (PK) is a nonlethal approach that evaluates the distribution of PSM in the blood (or plasma). In general, PK is the study of the time course of xenobiotic absorption, distribution, metabolism, and excretion and can be used to compare these attributes among mammalian herbivores under various conditions. PK studies require collection of serial samples of blood, tissues, urine, or feces, as well as strong analytical chemistry skills or access to radiolabeled PSMs for quantification. In general, the animal is given an intravenous and/or oral administration of a PSM, and the subsequent increase and decrease of its concentrations in body compartments are measured over time. Blood is the most commonly used and informative compartment investigated, but organs and excreta can also be evaluated to provide information on the time course and fate of PSMs and their metabolites. PK experiments require that the herbivore be catheterized or that researchers have sufficient sample sizes to generate a blood concentration–time curve from single blood collections from the orbital sinus, tail vein, or venous punctures from different animals. Pharmacokinetics in blood provides information on clearance, half-life, bioavailability, total exposure [i.e., area under the curve (AUC)], and apparent volume of distribution of the PSM (Fig. 1). The detailed calculations and relationships among these parameters are available elsewhere (Gibaldi and Perrier, 1982; Neubig, 1990; Boroujerdi, 2001). Recent PK studies have illustrated the link between PSM concentrations in the diet, feeding behavior, foraging strategy, and metabolism and absorption in mammalian herbivores (Sorensen and Dearing, 2003; Boyle and McLean, 2004). Here, we outline additional hypotheses that can be generated and tested by using PK studies.

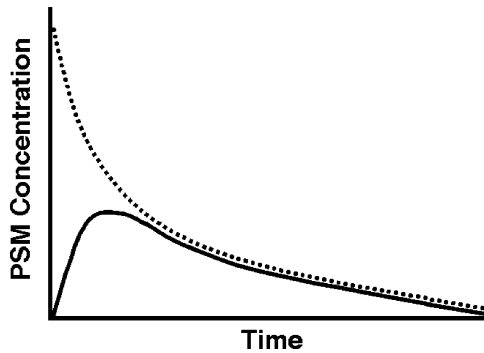


Fig. 1 Schematic representation of possible pharmacokinetic (PK) results following intravenous (IV, dashed line) and oral (solid line) administration of a plant secondary metabolite (PMS) to an animal in a linear scale (see Gibaldi and Perrier 1982; Neubig 1990; Boroujerdi 2001). Data are based on first-order kinetics and a one-compartmental model. Resultant PSM concentrations over time may be derived from blood, plasma, tissue, bile, feces, or urine. The time profile is dependent on feasibility, but reliable PK data should adhere to the following criteria: (1) initial time points should be collected more frequently than latter time points; (2) blood collections should be collected out to at least five half-lives; and (3) total blood collection should be <10% of total blood volume of the herbivore. PK parameters obtained from IV and oral dosing are defined in Table 2

Absorption

Pharmacokinetic studies can be used to assess absorption. For example, if oral half-life ($t_{1/2}$, hr) is longer than intravenous half-life and/or if bioavailability is low, then the distribution of the PSM may be influenced by the absorption process. Ecologists can take advantage of the established list of substrates for efflux transporters as positive controls, inhibitors, and inducers (Sharom, 1997; Seelig, 1998; Seelig and Landwojtowicz, 2000; Polli et al., 2001; Bjornsson et al., 2003) to investigate regulated absorption of PSMs. Studies can be designed that administer a PSM in the diet and then compare food intake, PSM blood concentrations, and fecal excretion

Table 2 Pharmacokinetic parameters that can be determined from intravenous (IV) and oral dosing of a PSM to a mammalian herbivore (Gibaldi and Perrier, 1982; Neubig, 1990; Boroujerdi, 2001)

PK parameter	Symbol	Definition	Calculation
Clearance (mL/min/kg)	Cl	Efficiency of drug elimination from the blood	$\text{Dose}_{\text{IV}} / \text{AUC}_0^\infty$
Half-life (hr)	$t_{1/2}$	Time it takes for concentration of PSM to decline 50%. This is referred to as the “terminal half-life”	$1.0693 \times V_d / \text{Cl}$
Area under the curve (min*mg/mL)	AUC_0^∞	Area under the plasma concentration–time curve from time 0 to infinity. Considered as an index of exposure to the PSM	$\int_0^\infty C \cdot dt$, where C is PSM concentration
Apparent volume of distribution (mL/kg)	V_d	Hypothetical volume of body fluid dissolving the xenobiotic at the same concentration as that in the plasma	$\text{Dose}_{\text{IV}} / C_0$, where C_0 is the concentration of PSM at time 0
Bioavailability (%)	F	The fraction of the dose absorbed	$(\text{AUC}_{\text{oral}} \cdot \text{Dose}_{\text{IV}}) / (\text{AUC}_{\text{IV}} \cdot \text{Dose}_{\text{oral}})$

of the PSM with and without the administration of a known inhibitor or inducer of the efflux transporter. If the PSM is a substrate for a transporter, then coadministration of the metabolite with a transporter inhibitor should result in decreased intake of diet containing the PSM, increased blood concentrations of the PSM, longer half-life, and decreased excretion of unchanged PSM in the feces.

Metabolism

Pharmacokinetic studies can also be used to assess metabolism. Faster clearance (Cl, in ml/min/kg) indicates faster rates of metabolism. Therefore, clearance rates can be evaluated to test whether dietary specialists or animals with evolutionary history with PSMs have higher detoxification capacity than dietary generalists or animals naive to PSMs (Sorensen and Dearing, 2003). The use of inhibitors of detoxification enzymes can also be employed in PK studies to evaluate the role of specific detoxification pathways on PSM clearance. Parameters can be compared between herbivores administered a PSM with an inhibitor (or inducer) of the putative metabolizing enzyme to that of herbivores administered the PSM without the inhibitor. Such studies are currently underway in brushtail possums (McLean, personal communication) and take advantage of the litany of enzyme substrates as positive controls, inhibitors, and/or inducers (<http://medicine.iupui.edu/flockhart/table.htm>; Newton et al., 1995; Tucker et al., 2001; Bjornsson et al., 2003; Sorensen and Dearing, 2006, this issue). PK studies can also be used to determine if a PSM interacts with a specific metabolizing enzyme that uses known substrates. For example, the CYP3A inhibitory properties of a PSM can be evaluated by comparing *in vivo* blood concentrations of a well-known CYP3A substrate, midazolam, orally administered with and without oral administration of a PSM (Bjornsson et al., 2003). If plasma levels of midazolam are higher with the PSM compared to without it, then the metabolite would be considered a strong inhibitor of CYP3A. Inhibition can be a result of either competitive or noncompetitive interactions with the enzyme of interest, and further studies are then required to elucidate the type of inhibition (Bjornsson et al., 2003). In contrast, if plasma concentrations of midazolam are lower with the PSM than without it, this suggests that it may induce CYP3A and therefore aid in midazolam clearance.

Tissue Affinity

Pharmacokinetic studies can also provide information on PSM affinity for tissues by evaluating the apparent volume of distribution (V_d , in L/kg) following intravenous PSM administration. A V_d that is higher than the water volume of the animal (0.7 L/kg, Davies and Morris, 1993) suggests high tissue affinity and is usually associated with a long half-life of the PSM. Potentially, a higher V_d may be a characteristic of PSMs that bind with tissues by interacting with specific receptors. For example, cannabinoids from *Cannabis sativa* have a high V_d (Samara et al., 1988), are distributed in the brain, and have high affinity and selectivity for G-protein-coupled cannabinoid receptors (Grotenhermen, 2005). In addition, the phenolics from *Eucalyptus jensenii* are thought to mediate the feeding behavior of mammalian herbivores via serotonin action on 5HT3 receptors (Lawler et al., 1998). We predict that these phenolics will also have high brain distribution and a large V_d . Distribution studies could guide research into which receptors are targeted by

PSMs and help identify the mechanisms responsible for PSM-related changes in behavior and physiology in mammalian herbivores.

Summary

Over three decades ago, Freeland and Janzen (1974) proposed that the feeding strategies of mammalian herbivores were governed by their detoxification systems. Since then, surprisingly few researchers have examined metabolism of PSMs in wild herbivores, and even less have examined the role of absorption or distribution. The objective of this review was to provide a brief overview of assays typically used in pharmacology that can be applied to research on mammalian herbivores. We would like to point out that as in pharmacology, no single assay can uncover the complexities of the fate of xenobiotics in mammalian herbivores. Each technique has benefits and limitations. The most informative future studies will integrate several of these *in vitro* and *in vivo* techniques to yield a comprehensive understanding of how mammalian herbivores process PSMs. The particular suite of techniques that will result in the most useful information is dependent on the particular questions asked and the study system. Lastly, because many of the techniques described herein require expensive equipment and/or reagents as well as technical expertise, we encourage novices to visit a laboratory that regularly performs the technique and to establish collaborations with pharmacologists and toxicologists.

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The Detoxification Limitation Hypothesis: Where Did it Come From and Where is it Going?

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Abstract The detoxification limitation hypothesis is firmly entrenched in the literature to explain various aspects of the interaction between herbivores and plant toxins. These include explanations for the existence of specialist and generalist herbivores and for the prevalence of each of these. The hypothesis suggests that the ability of mammalian herbivores to eliminate plant secondary metabolites (PSMs) largely determines which plants, and how much, they can eat. The value of the hypothesis is that it provides a clear framework for understanding how plant toxins might limit diet breadth. Thus, it is surprising, given its popularity, that there are few studies that provide experimental support either for or against the detoxification limitation hypothesis. There are two likely reasons for this. First, Freeland and Janzen did not formally propose the hypothesis, although it is implicit in their paper. Second, it is a difficult hypothesis to test, requiring an understanding of the metabolic pathways that lead to toxin elimination. Recent attempts to test the hypothesis appear promising. Results suggest that herbivores can recognize mounting saturation of a detoxification pathway and adjust their feeding accordingly to avoid intoxication. One strategy they use is to ingest a food containing a toxin that is metabolized by a different pathway. This demonstrates that careful selection of food plants is a key to existing in a chemically complex environment. As more studies characterize the detoxification products of PSMs, we will better understand how widespread this phenomenon is.

Keywords Detoxification limitation hypothesis · Mammalian herbivores · Plant secondary metabolites · Diet mixing · *Trichosurus vulpecula* · Chemical defense · Food choice · Foraging

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Introduction

Free-ranging mammalian herbivores can usually feed from a wide variety of plants, but most do not forage at random. Rather, they make careful choices about what they eat. Ultimately, these choices need to provide the animal with its nutritional requirements for maintenance, growth, and reproduction, without causing harm. It is well known that captive herbivores can select a balanced diet if given a variety of foods containing different individual nutrients (Wang and Provenza, 1997; Aranda et al., 2000; Scott and Provenza, 2000) or different concentrations of a given nutrient (Shariatmadari and Forbes, 1993; Gous and Swatson, 2000). No doubt, free-ranging herbivores can do the same. There is an important difference, however, between captive and wild animals: the latter must obtain their nutrients from plants that often have potentially toxic chemicals. Put simply, herbivores need to eat plants, but plants have developed many chemicals, known as plant secondary metabolites (PSMs), that act as defensive agents. Not surprisingly, given the diversity of plants, PSMs are extremely diverse and widespread and often occur in sufficient quantities to harm, or even kill, any animal that might eat it. As Freeland and Janzen (1974) point out, however, it is much easier to find animals that eat plants known to contain toxins than to find animals that can avoid them entirely. It seems that herbivores cannot reliably avoid ingesting PSMs, and instead have evolved a suite of mechanisms to counter their effects (Fig. 1).

There is no doubt that PSMs can influence feeding by mammalian herbivores, reducing food intake, altering dietary preferences, and sometimes even killing the animal (Foley et al., 1999). However, because of the difficulties in studying these interactions with wild animals, most of the evidence comes from domestic or captive animals. For example, cattle ingesting large amounts of alkaloids from rangeland plants or cardiac glycosides from oleander can die (Galey et al., 1998; Pfister et al., 2001). A less extreme example is of lambs that choose to eat less of a diet containing terpenes than one free of terpenes (Villalba and Provenza, 2005). Another scenario is of researchers feeding natural diets, but with only a partial understanding of the PSMs that the animal faces. *Eucalyptus* foliage, which is well known as a rich source of tannins, phenolics, and terpenes (Moore et al., 2004), to list just a few PSMs, is a prime example. Increasing concentrations of formylated phloroglucinol compounds (FPCs) in eucalypt foliage restrict feeding by marsupial folivores, but at any concentration of FPCs, there is typically wide variation in food intake (Wallis et al., 2002; Marsh et al., 2003; Moore et al., 2005). This implies that compounds other than FPCs also influence feeding.

The depression of food intake by PSMs is not uniform. There are other instances where animals have shown almost no response to extremely high dietary PSM concentrations. For example, common brushtail possums (*Trichosurus vulpecula*) maintained their food intake when the dietary concentration of rutin (a glycoside of quercetin) increased from 0 to 27% of dry matter (Marsh et al., 2006). Perhaps even more bizarre is the decline in feeding when brushtail possums are given a diet containing small amounts of free amino acids (DeGabriel et al., 2002; Marsh et al., 2005). In this case, the nutrients (amino acids) appear to be behaving as toxins.

The seemingly impossible complexity of plant–animal interactions may explain why the detoxification limitation hypothesis, emanating from a review by Freeland and Janzen (1974) some 30 years ago, has become so popular with so little conclusive testing. The hypothesis seems reasonable on an intuitive level, but

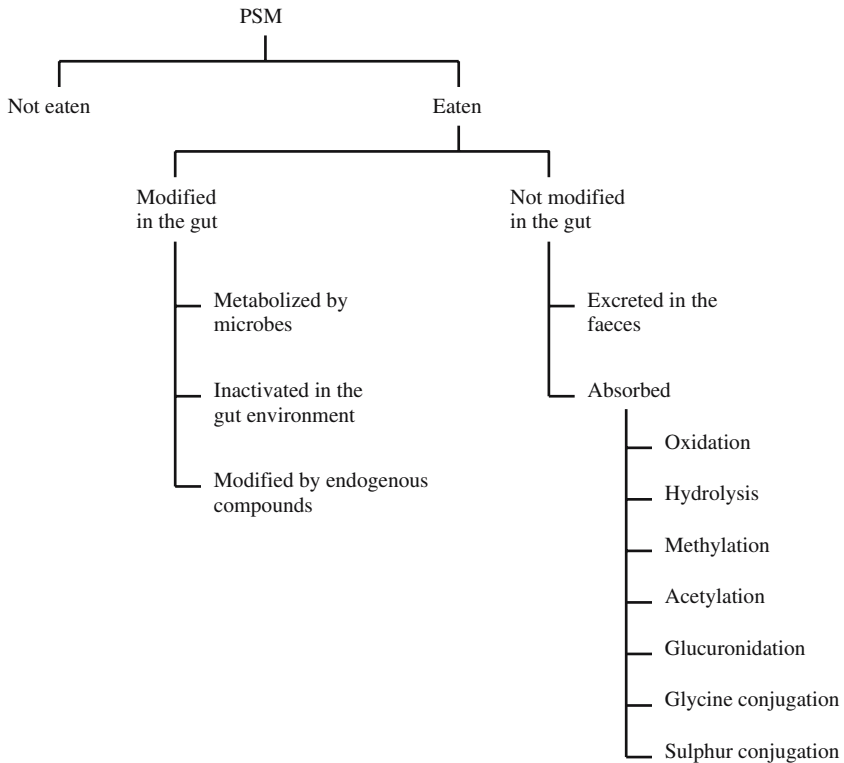


Fig. 1 Schematic of the possible fates of PSMs encountered by herbivores. Availability of alternative foods and the current detoxification capacity of the herbivore are some examples of factors that may influence whether a herbivore chooses to ingest a particular PSM. Because these factors can change, it may choose differently at different times. However, once a PSM has been ingested, its fate will depend on the particular PSM–herbivore combination

further understanding of the processes involved, as well as more specific testing, will further strengthen and refine it.

The Fate of Plant Secondary Metabolites

Animals have many defenses against PSMs (Fig. 1), the first being avoidance of plants that contain them. This may be a learned response (Provenza, 1996) or an initial reaction to the aversive sensory properties of the PSM (Foley et al., 1999). The second line of defense is to retain the PSM in the gastrointestinal tract and perhaps modify it there. This strategy might be a physiological response of the animal, or it might occur through a synergistic association with microbes. The responses of animals that ingest tannins provide examples of both mechanisms. Mule deer (*Odocoileus hemionus*) respond to tannins by producing proline-rich salivary proteins that bind them (Robbins et al., 1991), whereas koalas (*Phascolarctos cinereus*) have a symbiotic relationship with tannin-degrading bacteria (Osawa et al., 1993).

However, a great many PSMs have features that enable ready absorption from the gut—they are lipid-soluble, nonpolar, and nonionic at physiological pH (McLean and Duncan, 2006). Unless detoxified and eliminated from the body, these compounds will eventually reach harmful concentrations, although there are instances where metabolites are more toxic than the original compound (Tamási et al., 2003). For excretion in the urine—the typical mode of removal for metabolites with a molecular weight less than 500—detoxification processes convert the molecule to one that is water-soluble, contains polar groups, and is ionic at physiological pH (McLean and Duncan, 2006). Larger molecules are usually excreted in the bile and may even be reactivated to the original compound in the gut (Dearing et al., 2005).

Biotransformation (usually leading to detoxification) of PSMs occurs mainly in the liver in two phases that may occur independently or simultaneously and involve many enzymes. Phase I enzymes typically catalyze reactions such as oxidation, reduction, or hydrolysis, whereas those in phase II conjugate PSMs to polar molecules, known as conjugates. In the context of the detoxification limitation hypothesis, it is important to realize that any herbivore will potentially have many metabolic pathways in both phases to cope with the chemical diversity it might encounter (Dearing et al., 2005; McLean and Duncan, 2006). For example, there are more than 17 families of cytochrome P450 enzymes involved in oxidation reactions (Lin and Lu, 2001) and a similarly diverse range of enzymes that conjugate compounds to glucuronic acid (Radomska-Pandaya et al., 1999).

The particular sequence of reactions leading to the elimination of a compound is called the detoxification pathway. As with any chemical reaction, a pathway may become saturated for various reasons, including a depletion of enzyme or cosubstrate, resulting in the accumulation of the PSM. Thus, the time required for detoxification depends on the rate of detoxification, the concentration of PSM in the plant, the amount ingested, the proportion absorbed, and the interactions between PSMs and the physiological state of the animal, to name just a few (McLean and Duncan, 2006).

What is the Detoxification Limitation Hypothesis?

The detoxification limitation hypothesis has its origins in the seminal paper by Freeland and Janzen (1974). The paper emphasizes the complexity of the interactions between animals and PSMs, but, interestingly, the authors do not propose a hypothesis that they specifically call “the detoxification limitation hypothesis.” Instead, they finish their review with a section entitled “Hypothetical feeding behavior” in which they summarize their expectations as follows:

Generalist herbivores should: (1) treat new foods with extreme caution; (2) be able to learn quickly to eat or reject particular foods; (3) have the capacity to seek out and eat plants containing highly specific classes of nutrients; (4) have to ingest a number of different staple foods over a short period of time; (5) preferentially feed on the foods with which they are familiar, and continue to feed on them for as long as possible; (6) prefer to feed on foods that contain only minor amounts of toxic plant secondary compounds; and (7) have searching strategies and a body size that neither maximize the number of types of foods

that are potentially available nor maximize the total amount of food eaten, but rather compromise between these two functions.

The hypothesis as we know it was more formally delivered in a later review. Freeland (1991) suggested that:

Feeding behavior of herbivorous mammals is thus dependent on the individual mammal's capacity to detoxify and/or tolerate the biological effects of particular individual or combinations of plant secondary metabolites. Metabolites that cannot be detoxified/tolerated are likely to be avoided, while those that can be successfully detoxified are likely to be consumed. The rate at which a particular plant metabolite is detoxified is of obvious importance in determining how much a mammal eats per unit time.

The hypothesis itself has been given different names by different researchers. Some mention its predictions without giving it a name (Provenza, 1995; Foley et al., 1999; Burritt and Provenza, 2000), whereas others call it “Freeland and Janzen's hypothesis” (Iason, 2005), the “toxin dilution hypothesis” (Behmer et al., 2002; Singer et al., 2002; Duncan et al., 2003; Miura and Ohsaki, 2004), the “toxin avoidance hypothesis” (Dearing and Schall, 1992; Cassini, 1994), or the “detoxification limitation hypothesis” (Dearing and Cork, 1999; Sorensen and Dearing, 2003; Wiggins et al., 2006). It appears that the first study to propose the name “detoxification limitation hypothesis” was Dearing and Cork (1999).

Although it is more than 30 years and more than 500 citations since Freeland and Janzen, few studies have provided conclusive experimental support either for or against the hypothesis. Many researchers acknowledge that the detoxification limitation hypothesis can potentially explain aspects of the feeding behavior of herbivores (e.g., Freeland and Winter, 1975; Freeland et al., 1985; Freeland and Saladin, 1989; Dearing and Cork, 1999; Foley et al., 1999; Hagele and Rowell-Rahier, 1999; Burritt and Provenza, 2000; Behmer et al., 2002; Singer et al., 2002; Wiggins et al., 2003; Miura and Ohsaki, 2004; Rogosic et al., 2006). None of these studies, however, had the information required to rigorously test the hypothesis. This aside, if we are to understand the influence of PSMs on animal feeding, and thus their ecology, there is need for a hypothesis, and by far the most popular is the detoxification limitation hypothesis.

What Does the Detoxification Limitation Hypothesis Predict about Animal Feeding?

There appear to be three main ways that detoxification limitations might influence an animal's feeding behavior. The first suggests that feeding rates depend on the rates at which herbivores can detoxify PSMs. If rates of ingestion exceed rates of detoxification, then PSMs will accumulate. The consequences of a link between feeding and detoxification rates are twofold. First, the herbivore will need to alter its rate of feeding with changing concentrations of a PSM in the food to keep the rate of ingestion of the PSM stable (Fig. 3). Second, because detoxification systems are dynamic, an animal must adjust its rate of feeding to suit its current detoxification state, which depends on a myriad of factors discussed by Freeland and Janzen (1974)

and elsewhere (e.g., Foley et al., 1999; Dearing et al., 2005). For example, exposure to a PSM often induces the production of specific enzymes involved in detoxification (Pass et al., 1999; Boyle and McLean, 2004). An animal should alter its feeding behavior whenever the rate of detoxification changes (Fig. 2).

The second prediction of the detoxification limitation hypothesis is that specialist herbivores should be better at detoxifying the PSMs they encounter than should generalists. Specialist herbivores rely on few plants for most of their food, whereas generalist herbivores tend to eat many different plants, even when one is abundant. Generalist herbivores are much more common than specialists, probably because they do not have to rely on a limited food source for all of their lives (Freeland and Janzen, 1974). Where specialist and generalist herbivores occur sympatrically, however, such as koalas and possums in the eucalypt forests of southeastern Australia, the dietary niches of each may indicate their differing abilities to detoxify the PSMs in their food.

Finally, the detoxification limitation hypothesis suggests that because generalist herbivores are less efficient than specialists at detoxifying the PSMs that are found in a group of related plants, they must obtain their nutrients from a wider variety of plants (Sorensen and Dearing, 2003). This variety of foods, which we will refer to as “diet mixing,” is thought to increase the amount that a generalist herbivore can eat. For diet mixing to succeed, the detoxification limitation hypothesis assumes that the PSMs ingested from different plants are metabolized by separate pathways. However, even the detoxification of an individual PSM is complex, with many using several pathways for detoxification. This is illustrated in Table 1, showing the detoxification pathways that brushtail possums use for six PSMs. It shows that some PSMs share pathways, and so, when ingested simultaneously, may interact. For example, two PSMs partially overlap when they are both absorbed into the bloodstream, transformed by different processes during phase I, but then conjugated with the same molecule, such as glucuronic acid, in phase II. In contrast, an animal may ingest a food rich in benzoic acid, which is rapidly absorbed and detoxified by conjugation with glycine. It might simultaneously ingest a food rich in tannins, which combine with salivary proteins and remain in the gut pending excretion (Robbins et al., 1991). Discounting any interaction in the gut, there appears little competition between the two PSMs for detoxification. The response in feeding should

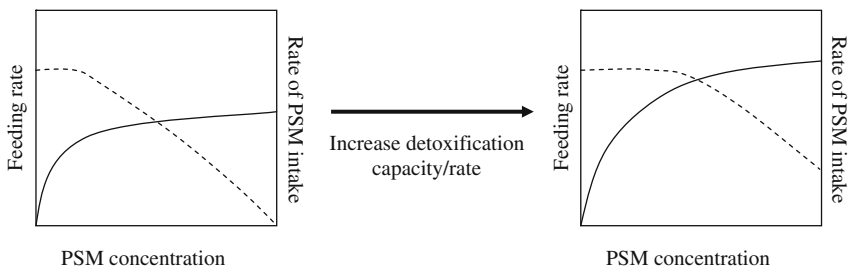


Fig. 2 The hypothetical rate of feeding and of PSM ingestion by a herbivore, for which food intake is limited by the rate of PSM detoxification. If the detoxification rate increases, we would expect that the rate of PSM ingestion would also be able to increase, allowing food intake (or the rate of feeding) to be maintained at the maximum to a higher PSM concentration. The dashed lines show changing feeding rates as PSM concentrations increase, and the solid lines show how this is linked to rates of PSM intake

Table 1 The percentage of PSMs ingested by brushtail possums that were metabolized by a given pathway in 24 hr

	1,8-Cineole	<i>p</i> -Cymene	Benzoic acid	Salicin	Orcinol	Rutin
Hydrolysis (I)				80		71
Oxidation (I)	33	51		22		71
Glycine conj. (II)		trace	90	10		
GA conj. ^a (II)	16	28	5	63	66	69

Most of the tested PSMs were metabolized by multiple mechanisms, and in some cases, both phase I and II reactions occurred (reaction type indicated in parentheses). Some columns add to more than 100% because the same molecule underwent multiple modifications. Adapted from Marsh et al., (2006).

^a GA = glucuronic acid.

presumably correlate with the degree of competition for detoxification between the PSMs.

Can Detoxification Limitations Influence Feeding Rates?

There is little evidence either supporting or refuting any of the predictions of the detoxification limitation hypothesis. This does not discount the importance of PSMs and detoxification limitations in the interactions between herbivores and plants, but

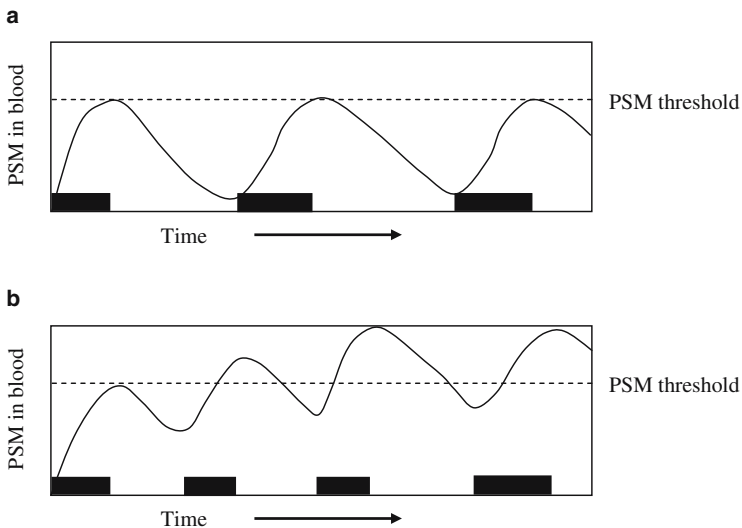


Fig. 3 (a) The detoxification limitation hypothesis suggests that the feeding behavior of a herbivore should aim to keep the amount of unmetabolized PSM in the blood below a threshold amount (dashed line). (b) If a herbivore ingests a PSM faster than it can detoxify it, then the concentration in the blood will exceed the threshold and poisoning could occur. The amount of unmetabolized PSM present at any time is a result of a combination of the rate of ingestion, the degree and rate of absorption, and the rate of detoxification. The black bars indicate times at which the herbivore is feeding, and the solid line tracks the hypothetical amount of unmetabolized PSM in its blood as it ingests a PSM with its food, and subsequently detoxifies it

rather demonstrates that it is difficult to elucidate their contribution to feeding. For example, slow rates of detoxification may explain why many mammalian herbivores ingest threshold amounts of PSMs (e.g., Pfister et al., 1997; Wang and Provenza, 1997; Lawler et al., 2000; Mangione et al., 2000; Stapley et al., 2000). In order to show that the feeding decisions of herbivores depend on the rate of detoxification, however, it is necessary to know how the herbivore of interest, in a certain physiological state, detoxifies a particular compound.

The “drink-driving” laws in many countries illustrate clearly the idea that the rate of ingestion of a substance determines toxicity (Fig. 3). It is possible to imbibe alcoholic drinks and drive a motor vehicle as long as the rate of metabolism of the alcohol equals the rate of ingestion. Ingesting alcohol at a faster rate, so that the blood alcohol concentration exceeds 50–80 mg per 100 ml, makes one unfit to drive, whereas raising the blood alcohol content to 300–400 mg per 100 ml may be lethal. This system appears simple because it involves a single toxin for which the pathway of detoxification is well known. However, recommended alcohol intakes differ for males and females, and tolerance to alcohol can differ widely among individuals in a population and among populations (Norberg et al., 2003). Other enzymes for drug metabolism show similar interindividual variability (Mulder, 1995; Lin and Lu, 2001), and different mammalian species may metabolize a particular drug at different rates and sometimes in differing ways (Walton et al., 2001), illustrating the complexity of detoxification. It is apparent that a defined experimental system is necessary to test the detoxification limitation hypothesis. Only then can we answer simple questions about interactions between herbivores and toxins, before tackling ones that are more complex. These might include questions about how toxins interact with each other and with other systems, such as the gut microbes.

Study Systems for Testing the Detoxification Limitation Hypothesis

A system for testing an animal’s feeding response to any toxin has several requirements. First, it requires a species that is easy to keep in captivity, usually in a metabolism cage, and is easy to maintain on an artificial diet. The diet should provide all of the animal’s nutrient requirements, be free of plant toxins, and be easily manipulated to provide, for example, different concentrations of nutrients or plant cell walls. Finally, the toxin should be a substance that the species typically encounters in the wild. It is necessary to know its mode of detoxification in the animals in question, and it must be possible to obtain enough toxin for well-replicated feeding experiments. The factor that usually prevents researchers from proving the detoxification limitation hypothesis is not knowing detoxification pathways.

Proof of the Detoxification Limitation Hypothesis

There appears to be only one study of mammalian herbivores that satisfies the requirements for a study system and shows that the rate of detoxification influences the rate of feeding. In this study, Marsh et al., (2005) fed common brushtail possums a basal diet consisting mainly of fruit and cereals, and they manipulated the concentrations of both benzoate and glycine. Benzoate is eaten by wild brushtail possums, and it is also known that a variety of animals, including brushtail possums, detoxify it by conjugation with glycine (Bridges et al., 1970; Awaluddin and

McLean, 1985). Furthermore, the rate of conjugation of benzoate depends on the supply of glycine (Griffith and Lewis, 1923; Amsel and Levy, 1969; Gregus et al., 1993). The key finding of Marsh et al., (2005) was that possums eating a diet containing both benzoate and glycine detoxified the benzoate faster and ate more than when eating a similar diet without the glycine. In other words, the rate of detoxification determined the rate of feeding. Thus, plant toxins do not have a fixed effect on feeding, but rather, their effect will depend on the animal's current detoxification state.

There is also at least one study in invertebrates showing that feeding depends on the rate of detoxification. In this study with tobacco hornworms (Snyder and Glendinning, 1996), piperonyl butoxide slowed the detoxification process by inhibiting cytochrome P450 enzymes, which are important in phase I reactions. The hornworms responded by eating less of a diet containing the plant toxin nicotine.

Both of the previous examples illustrate extremely simple cases that show a link between rates of detoxification and feeding rates. There is a need for more manipulative studies to examine a wide range of apparently simple interactions, but also to delve into complexities, such as interactions among plant toxins, and to separate effects that are not attributable to the rates of detoxification. By this, we mean that rates of feeding may depend on the concentrations of PSMs in plants but be independent of the rates of detoxification. One example is of plants that contain bitter or spicy compounds, for which the main consequence of ingestion is the stimulation of capsaicin receptors that cause a burning sensation (Jakubas and Mason, 1991; Pass and Foley, 2000). Another is of plants containing compounds that stimulate nausea or satiety receptors causing feeding to stop. For example, McLean et al., (2004) propose that some of the compounds found in eucalypt foliage are not absorbed from the gut, but cause the enterochromaffin cells to release serotonin, which leads to nausea and the cessation of feeding.

Another important condition that we know little about is the interaction between physiological state and detoxification limitations. In particular, animals with higher nutrient requirements, such as those living in the cold or reproducing, must eat more, but avoid physiological damage. This may be complicated. For example, a nocturnal animal lactating during the summer may have to eat much more in a short night, with less opportunity to spread feeding to aid detoxification. Stapley et al., (2000) examined the tolerance to a plant toxin in the face of higher nutrient requirements by comparing feeding in brushtail possums housed at 4°C or at 18°C. As expected, those in the cold ate more of a basal diet than did those living in the warm environment. However, both groups of possums ate the same amount of a diet containing jensenone, a toxin from eucalypt foliage. This indicates that possums habituated to the cold did not acquire any mechanisms for detoxifying this particular PSM faster, suggesting that similar animals in the wild would need to seek different options.

How Do Animals Overcome the Limitations Imposed by Plant Toxins?

If plant toxins can reduce feeding and even kill certain animals, then it is clear that herbivores must have mechanisms to circumvent them. The strategies of specialist and generalist herbivores may differ, but still be equally effective ways of obtaining the required nutrients without succumbing to intoxication. The detoxification

limitation hypothesis suggests that the ability to detoxify large quantities of PSMs from a single source defines a specialist herbivore. No studies, however, have shown that a specialist herbivore is able to detoxify the PSMs in its food faster than a generalist herbivore that also eats that food. Although specialist wood rats (*Neotoma* spp.) ingest more juniper PSMs than do generalists (Dearing et al., 2000), they cope with them by absorbing less from the gut, rather than detoxifying them faster (Sorensen and Dearing, 2003). But the overall result is the same—the specialist herbivore can ingest more PSMs without toxic effects. The folivores of *Eucalyptus* provide another example. The specialist koala oxidizes the terpenes, 1,8-cineole and *p*-cymene, more than does the generalist possum (Boyle et al., 1999, 2001), but it is unknown whether this speeds the rate of detoxification. Instead, what it may do is keep other pathways free for the detoxification of PSMs ingested simultaneously (Boyle et al., 2001) and thus minimize competition.

Because limitations of detoxification may force generalist herbivores to obtain their nutrient requirements from a variety of plants and plant parts, these animals should be skilled at selecting diets that spread the PSM load over their repertoire of detoxification pathways. As a consequence, presenting a herbivore with several foods containing PSMs that use different detoxification pathways should allow them to eat more. Although Freeland and Janzen (1974) specifically targeted mammalian herbivores in their predictions, the detoxification limitation hypothesis has attracted as much attention from those explaining why generalist invertebrate herbivores mix their diets (e.g., Hagele and Rowell-Rahier, 1999; Behmer et al., 2002; Singer et al., 2002; Miura and Ohsaki, 2004).

This appealing field of research has been the most common way in which researchers have attempted to test the detoxification limitation hypothesis (Freeland and Winter, 1975; Freeland et al., 1985; Freeland and Saladin, 1989; Dearing and Cork, 1999; Hagele and Rowell-Rahier, 1999; Burritt and Provenza, 2000; Behmer et al., 2002; Singer et al., 2002; Wiggins et al., 2003; Miura and Ohsaki, 2004; Marsh et al., 2006; Rogosic et al., 2006). However, it has not proved particularly fertile, probably because many experiments do not satisfy the stringent requirements needed for the study system. Although most of these studies show that generalist herbivores can eat more when offered a choice between two distinct diets, it is difficult to interpret them because they generally feed PSMs whose detoxification remains undescribed. Simply providing animals with foods with a variety of flavors or textures stimulates them to eat more than they would if given just one of these foods (Rolls et al., 1981; Treit et al., 1983; Clifton et al., 1987; DiBattista and Sitzer, 1994; Ginane et al., 2002). This indicates that different sensations alone can influence feeding behavior and reiterates the importance of devising robust systems when testing diet mixing aspects of the detoxification limitation hypothesis.

The importance of understanding modes of detoxification is borne out in the results of Burritt and Provenza (2000). Lambs did not eat more when offered two diets containing sparteine (a quinolizidine alkaloid) and saponins (triterpenoid glycosides extracted from *Quillaja* bark) or quebracho condensed tannins and saponins than when offered a single diet with one of the additives. The obvious interpretation is that all compete directly for detoxification, leaving no scope for a change in feeding behavior. This interpretation, however, requires knowing the detoxification pathways involved.

We set forth to test diet mixing in relation to limitations on detoxification (Marsh et al., 2006), taking advantage of pharmacological studies of PSM metabolism in

common brushtail possums (Table 1). By knowing the detoxification pathways, we could roughly predict the degree of competition for detoxification that might occur when characterized compounds were ingested simultaneously. Thus, we could predict how possums should respond when offered a choice between two PSMs, compared to the amount they ate of a diet containing only one of the compounds. For example, we expected possums to eat more if allowed to choose between diets containing PSMs metabolized by distinct pathways than if offered two diets with the same PSM or containing different PSMs that compete directly for detoxification (Marsh et al., 2006). The results were appealing in that the feeding responses of possums usually matched our predictions. For instance, possums did not eat any more than when given the choice between diets containing either 1,8-cineole or *p*-cymene (Marsh et al., 2006), which are both detoxified by oxidation followed by conjugation with glucuronic acid (Table 1). In contrast, giving possums a choice between the basal diet supplemented with 1,8-cineole or with benzoate (no competition for detoxification; Table 1) enabled them to eat significantly more (Marsh et al., 2006), indicating the benefits of diet mixing.

Problems with Understanding the Role of Detoxification in Diet Mixing

Although many of the predictions in the diet mixing experiment with possums were correct, others did not match the experimental findings. For example, possums offered a choice between salicin and orcinol, two compounds that are excreted as conjugates with glucuronic acid (Table 1), ate more food than possums offered either of the compounds singly (Marsh et al., 2006). Thus, there did not appear to be competition for detoxification. Do these findings refute the detoxification limitation hypothesis, or do they indicate a poor understanding of the system? We feel that the latter explanation is probably true.

There are many difficulties in understanding the links between diet mixing and detoxification limitations. First, it is necessary to understand the meaning of “competition,” which implies competition for a limiting resource. This means that there can be no competition when unlimited supplies of a resource, such as an enzyme or cosubstrate, exist. Brushtail possums detoxify *p*-cymene mainly by oxidizing it, but conjugate some with glucuronic acid and a trace with glycine (Boyle et al., 1999). This implies that *p*-cymene would compete strongly for detoxification with compounds that also undergo oxidation, but would have little influence on the rates of detoxification of compounds that are conjugated with glycine. However, if there is a shortage of glycine, then this conjugation step may become limiting. Thus, it may be difficult to measure whether two PSMs compete for detoxification because a series of processes eliminate most PSMs, and the limiting step needs to be identified for each.

Further complicating the picture is that there may be limited competition for detoxification between two compounds that appear to be metabolized by the same process. This is because a finer-scale definition of detoxification pathways would focus on the individual enzymes and enzyme families that carry out a broad class of reactions. For example, more than 17 families of cytochrome P450 enzymes have been characterized (Lin and Lu, 2001). Thus, oxidation reactions may involve quite separate cytochrome P450 enzymes and thereby reduce competition. If one knows the chemical pathways that detoxify a PSM, then *in vitro* experiments, such

as those of Pass and McLean (2002), can probably convey much information about competition.

Another problem is that some compounds can inhibit the detoxification of others, without ever competing for enzymes or cosubstrates. For example, furocoumarins inhibit cytochrome P450 enzymes (Baumgart et al., 2005) and can thus inhibit the detoxification of compounds requiring oxidation by these specific enzymes.

On an even broader scale, all pathways are fuelled by energy, and depletion of cellular energy could act as a limit to detoxification. Gregus et al., (1996) showed that a depletion of liver ATP reserves can slow the rate of detoxification of benzoate. Thus, even compounds that do not compete for any common enzymes or cosubstrates might still influence the rates of detoxification of other PSMs. Detoxification pathways may never truly be independent of others, but instead may be separated by differing degrees. This might explain why possums fed with PSMs that did not compete for detoxification still ate less than they did of a basal diet free of PSMs (Marsh et al., 2006).

How Are Detoxification Limitations Detected?

Measuring changes in food intake is a simple way of showing that a toxin influences feeding. However, this finding tells us nothing about the workings of the PSM—why it reduces feeding and how the animal knows it is there. One crucial area that previous discussions of the detoxification limitation hypothesis do not address is how animals detect excessive intake of one toxin, and then make the decision to eat food containing a different toxin. If an animal ingests a PSM at a rate that exceeds its detoxification capacity, it must trigger a feedback signal that stops the animal eating that food. Presumably, other signals exist that tell an animal whether it has met its caloric or protein needs. These signals interact so that the animal obtains its nutritional needs without experiencing toxicosis.

The details of these signals are not well known. Although several have been proposed, experimental evidence is mostly lacking. One likely signal is nauseous feedback, stimulated by PSM-linked increases in neurotransmitter activity in the gut or brain. Provenza et al. have drawn attention to this possibility through an extensive series of papers suggesting that aversive learning is a basis to herbivores choosing their diets (e.g., Provenza et al., 1992, 1998; Provenza, 1995, 1996). In support of this concept, Aldrich et al., (1993) found that a nonselective dopamine antagonist, metoclopramide (which in humans attenuates nausea and vomiting), allowed sheep to eat more endophyte-infected tall fescue. Further support comes from the study of Lawler et al. (1998) with marsupials fed with the *Eucalyptus* PSM jensenone. Injecting the human antinausea/antivomiting drug, ondansetron, a selective 5-HT₃ receptor antagonist, enabled these animals to ingest more jensenone, suggesting that the signal monitoring the consumption of jensenone involved the release of serotonin (5-HT). Both Lawler et al., (1998) and McLean et al., (2004) speculated that damage to cells in the stomach or small intestine led to the release of serotonin from enterochromaffin cells, which bound to 5-HT₃ receptors locally, initiating a cascade of events that triggered nausea.

Because nausea is effective at conditioning aversions to foods or flavors (Provenza, 1995), it might signal impending toxicoses in other situations. If so, it might be a more widespread signal that lets animals recognize their limits and make

appropriate feeding decisions. Marsh et al., (2005), however, showed that ondansetron failed to attenuate the reduction in feeding by brushtail possums fed with benzoate-rich diets. This suggests that other mechanisms, apart from nausea, signal detoxification limitations in that system. One possible mechanism is a change in blood pH because of the formation of organic acids from conjugation reactions (Foley et al., 1995).

However, before placing too little, or too much, emphasis on the emetic system, it is important to note that the emetic system is complex, and little is known about the neurochemistry of nausea. Nausea and vomiting can be triggered by input to the emetic center from the chemoreceptor trigger zone (CTZ), neurotransmitters in the gastrointestinal tract, the cerebrocortical pathway (responsible for learned associations), or the vestibular pathway (body positional changes such as in motion sickness; Rhodes and McDaniel, 2001). Furthermore, serotonin acting at the 5-HT₃ receptor is only one of many neurotransmitter–receptor combinations that can lead to nausea and/or vomiting. Other combinations that elicit emetic responses to stimuli include substance P and tachykinin NK₁ receptors (Gardner et al., 1996), acetylcholine and muscarinic receptors (Takeda et al., 1993), dopamine and D₂ receptors (Harrington et al., 1983), as well as histamine and H₁ receptors (Takeda et al., 1993). The gastrointestinal tract, the CTZ, and the emetic center are all rich in these receptors (Rhodes and McDaniel, 2001). New selective drugs that target specific receptors are appearing as knowledge increases. We suggest that more studies, targeting a variety of potential receptors, will help us to understand how widespread nausea might be in regulating the ingestion of PSMs by animals.

The Cost of Detoxification

Presumably, if there were no costs of detoxification, then there would be few limitations to the processes. Instead, arguments about limitations of detoxification imply that it is costly to animals. There is good evidence that this is true. Cork (1981) calculated that koalas use about 25% of their fasting glucose entry rate to excrete glucuronide conjugates. In research with sheep, Lowry et al., (1993) showed that the conjugation of phenolic acids with glycine, to form hippuric acid, resulted in them losing almost 20% of their digestible nitrogen intake in urine. These detoxification costs are a cost of foraging, little different from increases in search times. Thus, from an optimal foraging perspective, one might predict that animals should eat a food only when the nutritional benefits outweigh the costs of detoxification.

If there is a cost of detoxification that influences an animal's fitness, then there needs to be a currency to measure it. The complexities of detoxification mean that researchers have largely avoided calculating the energy exchanges in individual chemical reactions, in favor of integrative measures, such as whole-body energy expenditure. For example, Iason and Murray (1996) gave sheep intraruminal infusions of orcinol and found that their basal metabolic rate (BMR) increased by 5%. This was rather small compared with voles (*Microtus pennsylvanicus*) consuming diets containing gallic acid, whose BMR increased by up to 22% (Thomas et al., 1988). More recently, Sorensen et al., (2005) used wheel-running behavior and measures of energetics to quantify detoxification costs in two species of wood rats (*Neotoma* sp.). Both the BMR and locomotor activity of the specialist

species of wood rat dropped as they ate more juniper, whereas the generalist reduced only locomotor activity.

These studies illustrate that detoxification consumes energy that the animal could use for another function. They also illustrate the strengths and the weaknesses of using energy as the currency for measuring the cost of detoxification. The obvious advantage of using energy is that it allows the integration of the costs of many different processes. The main disadvantage is that free-living animals can make subtle metabolic changes that are difficult to measure and may obscure the costs of detoxification. The drop in BMR and activity by wood rats is one example. Another is the research of White and Lawler (2002), who used the change in the heat increment of feeding on browse, relative to that of feeding on hay, to estimate the energy cost of detoxification for muskoxen (*Ovibos moschatus*). For some species of browse, they found that reductions in ruminal methane production (and its subsequent loss by eructation) partially compensated for the energy cost of detoxifying secondary compounds. The opportunity for tradeoffs and compensations in energy metabolism across different species of browse makes it a poor currency for measuring the cost of detoxification.

We suggest that measuring whole-body protein turnover might be a better way of estimating the costs of detoxification. Whole-body protein turnover measures the cost of the synthesis and turnover of proteins and is strongly related to metabolic rate because protein synthesis is energetically expensive (Waterlow, 2006). Whole-body protein turnover is more integrative of the range of processes that occur during detoxification than simple measurements of nitrogen or energy balance because it includes intermediary metabolism, amino acid activation, and associated processes such as RNA turnover (Waterlow, 2006). For example, protein is required for synthesis of cytochrome P450 and other detoxification enzymes. That many of the enzymes involved in detoxifying PSMs are inducible suggests that they are costly to maintain.

Changes in protein turnover should translate directly into changes in protein requirements and, so, are easier to incorporate into models of diet selection than are measurements of energy loss. They can also integrate many different detoxification processes. Animals can excrete some phenolic glycosides with a minimal apparent energy cost (McLean et al., 2001), but there may be costs that are not immediately obvious and need to be paid for later. For example, these compounds may increase protein requirements through damage to the kidney's filtration processes (Garner et al., unpublished data), through uncoupling of oxidative phosphorylation (Singleton and Kratzner, 1969), and through acid–base adjustments arising from the excretion of organic acids of detoxified metabolites (Foley et al., 1995).

Changes in whole-body protein turnover during various types of immune challenges suggest major costs of maintaining the immune system (Klasing and Calvert, 2000). These appear to have an evolutionary basis because there are direct links between the increased protein requirements of these challenges and compelling models of diet selection (Lee et al., 2006). PSMs have complex effects on the immune systems of mammals (Allen et al., 2003). This indicates that we need to view detoxification costs more broadly, and that separating detoxification costs from other potential costs, such as immune responses, is probably artificial. Accordingly, we recommend that effort be given to measuring whole-body protein turnover of animals fed with diets containing either purified PSMs or, more usefully, complex mixtures typical of those found in most browse plants.

Applying the Detoxification Limitation Hypothesis to Wild Herbivores

This review of the detoxification limitation hypothesis has so far considered the case of the captive animal given a limited choice of foods. In contrast, wild animals face situations that are not nearly so simple. The feeding behavior of wild herbivores depends on many factors in addition to forage quality. These include social interactions, nutritional status, and the need for a home range with other qualities, such as a den or shelter. Clearly, determining whether wild herbivores feed according to the predictions of the detoxification limitation hypothesis is difficult. Even so, in this section, we outline some of the factors that researchers might consider when studying how free-living herbivores might mix several plant toxins in their diet. Foremost among them is the spatial distribution of plant defenses, which are presumably important because they influence costs, such as search times.

There is growing evidence that the spatial distribution of defense variation affects feeding choices (Hjalten et al., 1993; Alm Bergvall and Leimar, 2005). Nevertheless, few studies provide evidence that the spatial patterns required for such behaviors exist in nature (Covelo and Gallardo, 2004; Brenes-Arguedas and Coley, 2005). In the case of diet mixing, it is essential for variation to occur at a relevant scale for herbivores—presumably within a home range. A patchy distribution could increase the cost of diet mixing because animals would be unlikely to encounter chemically different food plants in the same patch or stand. Herbivores might prefer areas where diverse food is available, such as between patches, or where different food species overlap.

Another interesting difference in the environments of captive and wild herbivores is that those of the latter may provide mechanisms that enable the animal to cope with toxins. For thousands of years, humans have used a variety of methods to render plant toxins harmless. Herbivorous animals sometimes do the same. Perhaps the most obvious is removing toxic plant parts before ingestion. Other techniques include eating substances that are not normally considered food to negate the effects of plant toxins. One of the best examples is of parrots practicing geophagy—the ingestion of soil. By carefully selecting soils rich in particular clays, with a high capacity for binding plant toxins, the birds avoid absorbing the toxins from the gut (Gilardi et al., 1999). The abundance and distribution of such resources clearly impinge on the nutritional ecology of the herbivores that can use them.

Although the amount eaten of a plant should ultimately depend on an animal's ability to detoxify PSMs, its initial selection may be driven by other factors. The amount of feeding an individual plant attracts probably depends on the characteristics of the surrounding matrix of plants (Atsatt and O'Dowd, 1976; Milchunas and Noy-Meir, 2002; Alm Bergvall et al., 2006). Thus, the nutritional and toxic characteristics of a plant may influence how herbivores use neighboring plants (Villalba and Provenza, 2005). From a plant's perspective, the benefits and costs of defense need to consider both the individual and the community it inhabits (Tuomi and Augner, 1993). Likewise, the foraging strategy of the animal should account for this. One example is that sheep were more likely to graze a relatively unpalatable shrub when the surrounding plants were palatable (Rousset and Lepart, 2003).

To understand the role of detoxification in nature, there is a need for detailed studies of the feeding behavior and population dynamics of wild animals, in relation to the distribution of PSMs in their food plants. One approach might be to use data from feeding studies with captive animals to predict the palatability of wild plants

and then to follow this up by comparing the expected patterns of use with the observed patterns. Recently developed methods for studying spatial autocorrelation (Double et al., 2005) can detect the scale and intensity of patchiness in PSM distributions (Andrew et al., unpublished data), which can then be related to herbivore behavior.

In summary, the initial testing of the detoxification limitation hypothesis on wild herbivores requires a simple system consisting of a specialist herbivore eating a diet whose PSMs are well characterized. An example of this approach is that used by Marsh et al. (unpublished data), who fitted koalas with radiocollars equipped with microphones, enabling the constant monitoring of both the animals' location and the amount that they ate. By sampling foliage from all of the trees in the area, they could then examine a koala's feeding pattern in relation to the chemistry of its environment. They found that the chemistry of foliage influenced the choice of trees by koalas, as well as the amount of food eaten.

Conclusions

Some 30 years after Freeland and Janzen's seminal paper on the interactions between herbivores and plant toxins, the hypothesis it gave rise to, detoxification limitations, is entrenched in the literature but remains inadequately tested. There are thousands of plant toxins and a wide variety of metabolic systems to render them less potent, making the study of the interactions complex. Some PSMs require similar modes of detoxification and are said to compete, whereas the animal metabolizes others by pathways that appear to be independent. In between these modes, however, are detoxification pathways that compete to differing degrees and are much harder to understand. In fact, because all are fueled by energy, it may be that no pathway is truly independent of another. It is impossible to study all of the systems of detoxification, and there is little to gain from trying. However, further investigations of the detoxification limitations of herbivores feeding on PSM-rich diets, and how they detect these, are likely to advance our understanding of the interactions between animals and plants.

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Resource Availability and Quality Influence Patterns of Diet Mixing by Sheep

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Abstract In grazing systems, forage availability is a function of herbivore density, which can influence an animal's ability to be selective. In turn, the influence of food availability on selectivity has the potential to influence plant biodiversity. We hypothesized that the ability of herbivores to mix toxin-containing foods in their diets is a function of the availability of nontoxic foods and the nutritional characteristics of the toxin-containing foods. We evaluated this hypothesis in two trials simulating different diet qualities (high-quality foods in trial 1, low-quality foods in trial 2). Within each trial, the four treatment groups were offered with different amounts of nutritious, familiar foods—10, 30, 50, and 70% of *ad libitum* intake—but were offered with *ad libitum* access to toxin-containing foods. Each lamb was presented with five foods, including three toxin-containing unfamiliar foods (terpenes, tannins, and oxalates) and two nutritious familiar foods (alfalfa and barley). In trial 1, as the availability of nutritious familiar foods decreased, animals ate more of all three toxin-containing foods. As the availability of nutritious alternatives increased, the pattern of selection shifted from terpenes to tannins and oxalates. In trial 2, animals also ate more toxins as the availability of nutritious alternatives decreased, but their pattern of diet mixing changed. Low availability of nutritious alternatives resulted in the animals eating more oxalates. During preference tests when all five foods were offered *ad libitum*, animals fed with 10, 30, 50, and 70% of *ad libitum* intake from trial 1 ate 49, 47, 41, and 38% of the three toxin-containing foods, respectively. The lower diet quality in trial 2 affected intake of the toxin-containing foods such that animals fed with 10, 30, 50, and 70% of *ad libitum* intake ate 37, 36, 29, and 27%, respectively, of the three toxin-containing foods. Thus, the quality of toxin-containing foods and the availability of nutritious alternatives interacted to modify the pattern of diet mixing by lambs.

Keywords Toxins · Biodiversity · Foraging · Behavior · Sheep · Diet mixing

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Introduction

Herbivores modify environments by selectively consuming foods (Palo and Robbins, 1991), and the process of diet selection is shaped by intricate interactions between nutrients, toxins, and animal experience (Hanley, 1997; Provenza et al., 2003a; Villalba et al., 2004). Nutrients and toxins both influence the kinds and amounts of foods eaten as animals strive to meet nutritional needs (Westoby, 1978), and regulate their intake of toxins (Freeland and Janzen, 1974; Villalba et al., 2002a). Experiences early in life, of both the social and physical environments, shape behavior, influence gene expression (McCormick et al., 2000; Duffy et al., 2002), and improve performance by inducing neurological, morphological, and physiological changes in animals (Piersma and Lindstrom, 1997; Schlichting and Pigliucci, 1998). Such plasticity enables adaptation to different environmental conditions and implies that different conditions influence how an animal perceives diet and habitat quality (Davis and Stamps, 2004). These dynamics influence the structure and diversity of plant communities (Provenza et al., 2003a,b).

Herbivores learn to forage (goats, Distel and Provenza, 1991; lambs, Green et al., 1984; and cattle, Wiedmeier et al., 2002), but how they learn to mix their diets given a variety of alternatives is poorly understood (Provenza et al., 2003b). The availability of alternative plants likely increases the difficulty of distinguishing beneficial or deleterious relationships involving toxin–toxin, nutrient–toxin, and nutrient–nutrient interactions. Such interactions influence the amount of nutrients and toxins an animal can ingest (Freeland and Janzen, 1974; Illius and Jessop, 1995; Banner et al., 2000), as well as preferences for foods that differ in nutrients and toxins (Villalba et al., 2002a,b). In addition, interactions among toxins can either increase or decrease consumption of combinations of foods that contain toxins (Schmidt et al., 1998; Dearing and Cook, 1999; Burritt and Provenza, 2000; Provenza et al., 2003a; Villalba et al., 2004).

We hypothesized that the ability of herbivores to mix toxin-containing foods in their diets is a function of the availability of nontoxic foods and the nutritional characteristics of toxin-containing foods. Based on these hypotheses, we predicted that young animals exposed to high availability of nutritious alternatives would consume less novel toxin-containing foods, thus impeding their acquisition of diet-mixing behavior. In contrast, we predicted that young animals exposed to low availability of nutritious alternatives would learn to consume more of the novel toxin-containing foods.

Methods and Materials

The objective of these studies was to determine the degree to which sheep mix an array of unfamiliar toxin-containing foods with familiar high-quality foods when the familiar foods were available in different amounts: 10, 30, 50, and 70% of the animal's intake capacity. The toxins were present in a high-quality (trial 1) or a low-quality (trial 2) base food. Trial 1 was conducted from June 24, 2002 to July 31, 2002, and trial 2 from August 6, 2002 to September 12, 2002. The studies were conducted at the Green Canyon Ecology Center, located at Utah State University in Logan, UT, USA, and were approved by the Animal Care and Use Committee at Utah State University.

We used a different set of 32 lambs in each trial. Lambs were commercial crossbreds of both sexes, 4–5 mo old. In trial 1, they averaged 40 kg (SE = 0.73) body mass, and in trial 2, they averaged 37 kg (SE = 1.03) body mass. They were inexperienced with the toxins at the start of each trial, as well as with the ingredients of the base food that contained the toxins. Lambs were individually penned with free access to trace mineral salt blocks and freshwater. They were individually penned 10 d prior to initial exposure, during which they received alfalfa pellets *ad libitum* and barley (300 g/lamb/d).

High-Quality Foods

We used five foods in this study: three unfamiliar and two familiar to the lambs. The three unfamiliar, high-quality test foods were formulated with the same ingredients, but 0 isoenergetic (2.9 ± 0 Mcal/kg, NRC 1985) and isonitrogenous (126 g CP/kg; NRC, 1985). The ingredients and compositions of the diets were as follows: (1) tannin diet (76% beet pulp, 9% soybean meal, and 15% quebracho tannin); (2) terpene diet (52.8% beet pulp, 26% grape pomace, 14% soybean meal, 1.82% camphor, 1.1% 1-8-cineole, 0.12% methacrolein, 0.06% *p*-cymene, and 4.1% vegetable oil); and (3) oxalate diet (67.5% beet pulp, 20% grape pomace, 11% soybean meal, and 1.5% oxalic acid). The remaining two familiar, high-quality foods were (4) a 50:50 mix of ground alfalfa and ground barley (3.2 Mcal/kg and 14% crude protein) and (5) ground alfalfa (2.5 Mcal/kg and 15% crude protein).

Low-Quality Foods

Three low-quality foods also were formulated to be isoenergetic (2.3 ± 0 Mcal/kg; NRC, 1985) and isonitrogenous (63 g CP/kg; NRC, 1985), with the same ingredients and different concentrations and types of toxins. The ingredients and compositions of the diets were as follows: (1) tannin diet (58.5% beet pulp, 25% grape pomace, 1.5% soybean meal, and 15% quebracho tannin); (2) terpene diet (35.8% beet pulp, 51% grape pomace, 6% soybean meal, 1.82% camphor, 1.1% 1-8-cineole, 0.12% methacrolein, 0.06% *p*-cymene, and 4.1% vegetable oil); (3) oxalate diet (50.5% beet pulp, 45% grape pomace, 3% soybean meal, and 1.5% oxalic acid). The other two foods were the same familiar foods (alfalfa/barley; alfalfa) offered in the previous trial.

Food ingredients in both test diets were ground to 2- to 3-mm particle size to facilitate mixing. Toxins were in crystalline (oxalic acid, camphor), powder (tannin), or liquid (1,8-cineole, methacrolein, *p*-cymene) form. Volatilization of the terpenoids was minimized by daily mixing in vegetable oil prior to mixing them with the remaining ingredients. The concentrations of toxins in the test diets were selected according to the amount of toxins sheep can ingest in 1–4 hr (Villalba et al., 2004).

Trial 1: Toxins in a High-Quality Food

This trial consisted of 7 d of preconditioning and 21 d of conditioning. During the 7 d of preconditioning, lambs were offered with the two familiar foods *ad libitum* from 0800 to 1200 hr, after which refusals were collected and weighed. Animals then were stratified into four groups of eight animals according to their individual total intake of the two familiar foods. Animals from these groups were then randomly

allocated to one of the four treatments. For the next 21 d, animals in treatments 1, 2, 3, and 4 received, respectively, 10, 30, 50, and 70% of the average total intake during preconditioning. The proportions of these two familiar foods were based on the average intakes of each animal during the pretrial. For example, a lamb in treatment 1 that ate on average 1000 g of ground alfalfa/barley and 500 g of ground alfalfa received 100 g of the alfalfa/barley mix and 50 g of the ground alfalfa during the trial. Lambs were offered with the three unfamiliar (toxin-containing) foods *ad libitum*.

During the 21 d of conditioning, all five foods were offered from 0800 to 1200 hr. No other foods were offered for the remainder of the day. At 1200 hr, refusals were gathered and weighed, and intake of each of the five foods was recorded for each animal. Lambs were weighed individually at the beginning and end of the 21-d conditioning period.

Trial 2: Toxins in a Low-Quality Food

Trial 2 occurred immediately after trial 1 with a new set of 32 lambs. Conditioning for trial 2 followed the same protocol as for trial 1, the only difference being the reduced quality of the test diets that contained the toxins.

Preference Tests for Trials 1 and 2

For 2 d immediately after conditioning in each trial, animals had *ad libitum* access to all five foods from 0800 to 1200 hr. Refusals were collected and weighed, and individual intake was recorded. Following that preference test, animals were fed with alfalfa pellets (*ad libitum*) and barley (300 g/lamb/d) for 7 d. This period allowed all animals to be on a comparable plane of nutrition. Following this period, another 2-d preference test was conducted.

Statistical Analyses

The statistical design for the analysis of variance (ANOVA) during the conditioning period and the preference tests was a split-plot with day as a repeated measure. At the whole-plot level, there were four treatments (10, 30, 50, and 70% restriction) with eight lambs nested within each of the four treatments. At the subplot level, there were five foods and their interaction with treatment. Day ($N = 21$ during conditioning and 2 during preference tests) was the repeated measure. Intake was converted to grams of food ingested/kg MBW ($\text{kg}^{0.75}$) to account for differences in body mass within and between trials. When F values were significant ($P < 0.05$), differences in means were analyzed with the least significant difference test. ANOVA was performed using the MIXED procedure in SAS (SAS; Littell et al., 1996).

Results

Trial 1 Conditioning

The availability of alternatives influenced the diet selection of lambs. The total amount of toxin-containing foods ingested by lambs in treatments 1 and 2 (61 and

59 g/kg^{0.75}) was greater than for treatments 3 and 4 (43 and 46 g/kg^{0.75}; $P < 0.005$). As the availability of nutritious alternatives—alfalfa and barley—increased, the total amount of toxin-containing foods consumed decreased.

Intake of the individual toxin-containing foods also varied among treatments (Fig. 1). Consumption of the terpene-containing food was higher for lambs in treatment 1 than for any of the other treatment ($P < 0.005$). The intake of oxalate-containing food also was higher in treatment 1 than in treatments 2 and 3 ($P < 0.05$;

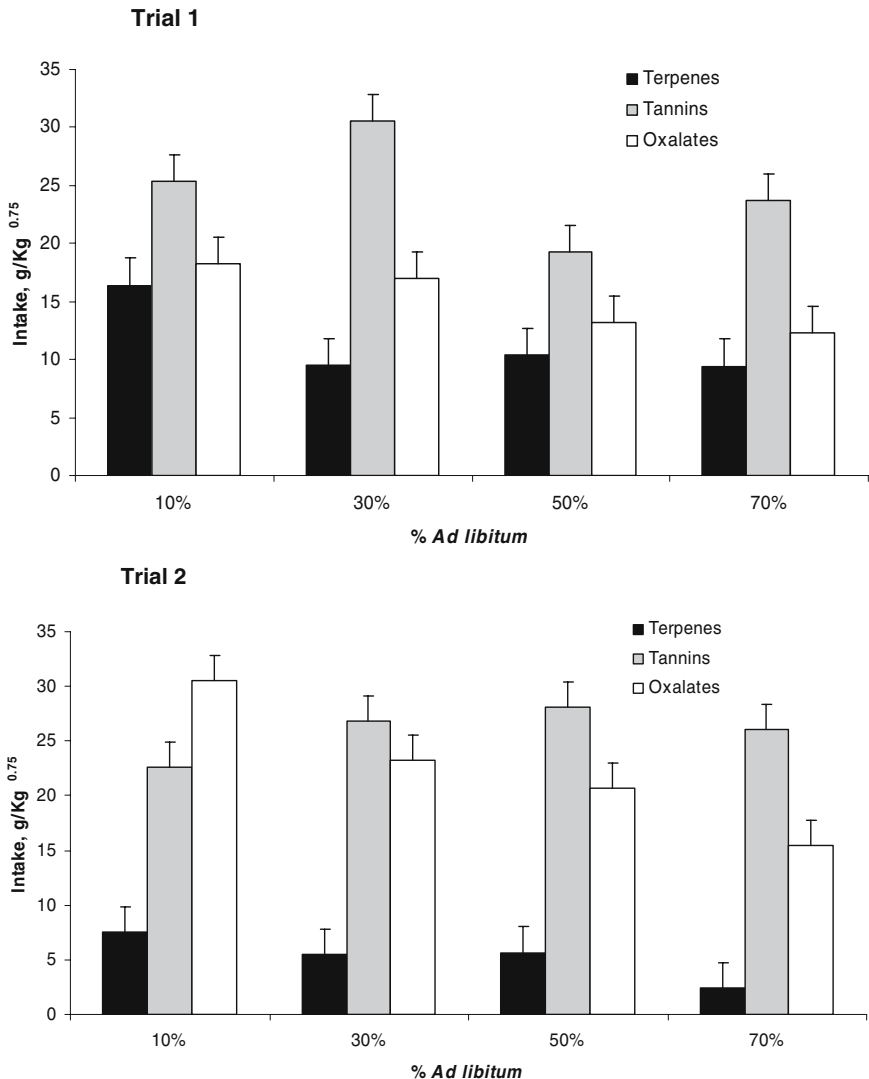


Fig. 1 Intake [means and standard errors (2.3)] by lambs of foods containing terpenes, tannins, or oxalates during conditioning in trial 1 (high quality) and trial 2 (low quality). Treatments 1–4 received, respectively, 10, 30, 50, and 70% of the *ad libitum* average consumed during preconditioning in alfalfa and alfalfa/barley

Fig. 1). Lambs in treatment 2 ate more of the tannin-containing food than lambs in treatments 1, 3, and 4 ($P < 0.05$; Fig. 1). Within each of the four treatments, intake of the tannin-containing food was higher than intake of the terpene or oxalate-containing foods ($P < 0.005$). For treatment 2, intake was higher for oxalates than terpenes ($P < 0.005$; Fig. 1).

The average daily weight gain did not differ among treatments as the availability of alternatives increased ($P > 0.05$). The trial average increase was 0.102 kg/lamb/d.

Trial 1 Preference

Restriction during conditioning increased preference for the toxin-containing foods. Given *ad libitum* access to all five foods, lambs showed higher preference for the three toxin-containing foods in treatments 1 (49%) and 2 (47%) than in treatments 3 (41%) and 4 (38%; $P < 0.05$). Total intake of the toxin-containing foods did not differ from that of the nutritious familiar alternatives for any of the groups ($P > 0.05$).

Intake of the terpene-containing food was greater in treatments 1 and 3 than in treatments 2 and 4 ($P < 0.05$; Fig. 2). Lambs in treatment 2 consumed more tannin-containing food than lambs in treatments 3 and 4 ($P < 0.05$). Lambs in treatment 1 consumed more tannin-containing food than those in treatment 3 and more oxalate-containing food than those in treatment 4 ($P < 0.05$; Fig. 2). The intake of alfalfa and alfalfa/barley did not differ among treatments ($P > 0.05$; Fig. 2).

Preference was greater in all treatments for the tannin-containing food than for the terpene or oxalate-containing foods ($P < 0.05$; Fig. 2). In treatments 1 and 4, intake was greater for the oxalate than the terpene-containing food ($P < 0.05$; Fig. 2). The alfalfa/barley mix was preferred by all treatments ($P < 0.005$), whereas the intake of alfalfa was less than the tannin-containing food in treatments 1, 2, and 4 ($P < 0.005$).

Trial 2 Conditioning

Lambs were influenced by the availability of alternatives and the poorer diet quality. The amount of total toxin-containing foods ingested decreased as the availability of alternatives increased, such that lambs in treatments 1–3 (61, 56, and 54 g/kg^{0.75}, respectively) ate more total toxin-containing foods than lambs in treatment 4 (44 g/kg^{0.75}; $P < 0.005$). Lambs in treatment 1 also consumed more total toxin-containing foods than those in treatment 3 ($P < 0.05$).

Consumption of individual toxin-containing foods also varied among treatments (Fig. 1). The intake of the oxalate-containing food was greater for lambs in treatment 1 than treatments 2–4 ($P < 0.05$) and greater in treatments 2 and 3 than in treatment 4 ($P < 0.05$). Lambs in treatment 1 also consumed more terpene-containing food than lambs in treatment 4 ($P < 0.05$), whereas those in treatment 3 ingested higher amounts of the tannin-containing food than lambs in treatment 1 ($P < 0.05$).

The intake of individual toxin-containing foods also varied (Fig. 1). Lambs ate the least of the terpene-containing food ($P < 0.005$). In treatment 1, intake of the oxalate-containing food was highest, whereas in treatments 3 and 4, consumption of the tannin-containing food was highest ($P < 0.005$). Lambs in treatment 2 ate similar amounts of the tannin and oxalate-containing foods ($P > 0.05$).

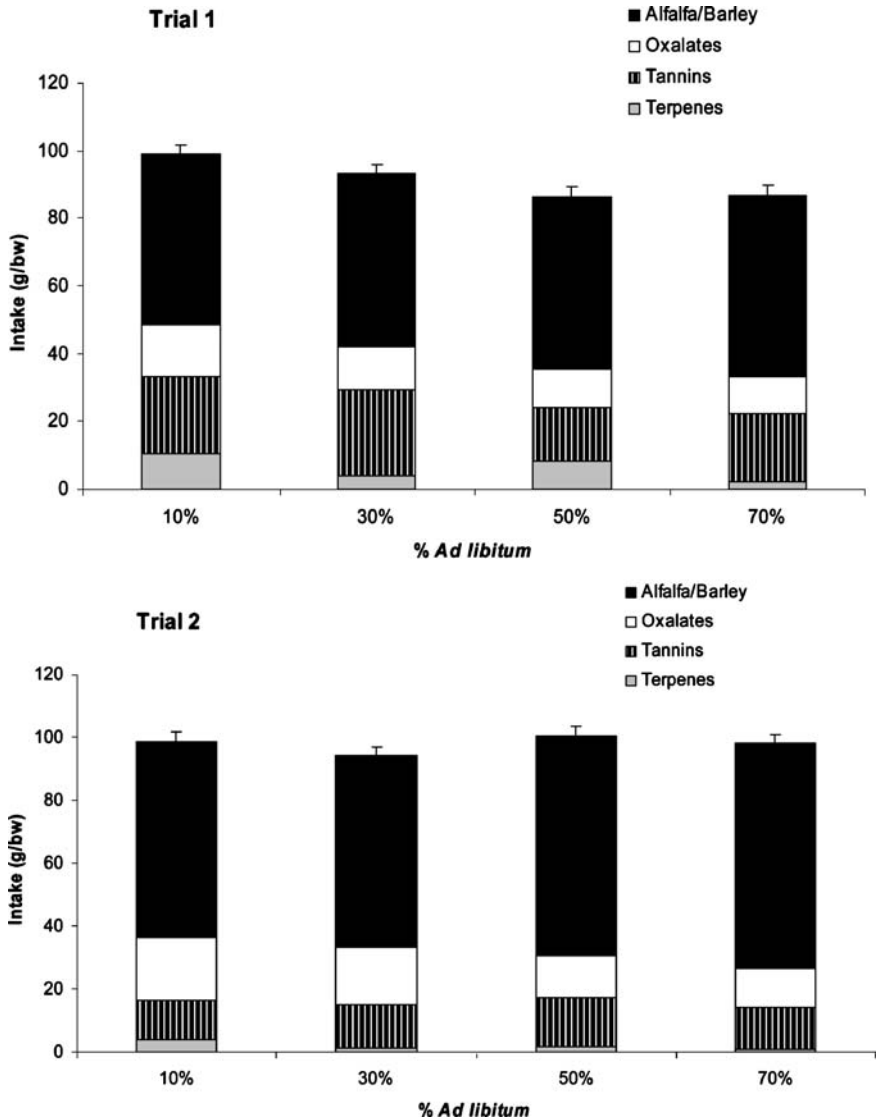


Fig. 2 Intake [means and standard errors (2.9)] of four diets—terpenes, tannins, oxalates, and alfalfa/barley (50:50) plus alfalfa—offered simultaneously and *ad libitum* to lambs during preference tests that occurred immediately after lambs were conditioned with toxin-containing foods of high quality (trial 1) and low quality (trial 2)

Patterns of use of toxin-containing foods varied between trials 1 and 2 (Fig. 1). Animals in trial 1 ate large amounts of tannins, whereas those in trial 2 ate large amounts of tannins and oxalates. Lambs in treatments 1–3 all ate more oxalates in trial 2 than in trial 1 ($P < 0.005$). All lambs ate more oxalates than terpenes in trial 2, and this pattern was most pronounced in lambs in treatment 1 that ate more oxalates than tannins or terpenes ($P < 0.005$; Fig. 1). Finally, intake of terpenes by treatments 1, 3, and 4 decreased from trials 1 to 2 ($P < 0.05$).

The average daily weight gain in trial 2 did not differ among treatments ($P > 0.05$). However, lambs gained less in trial 2 (0.015 kg/lamb/d) than in trial 1 (0.102 kg/lamb/d; $P < 0.005$).

Trial 2 Preference

While preference continued to be influenced by the availability of alternatives, the lower diet quality in trial 2 affected intake of the toxin-containing foods. Lambs in treatments 1 (37%) and 2 (36%) showed higher preference for the toxin-containing foods than lambs in treatments 3 (29%) and 4 (27%; $P < 0.05$). The total amount of toxin-containing foods consumed and the total amount of safe foods consumed during the preference tests did not differ ($P > 0.05$).

Intake of individual toxin-containing foods varied among treatments. Lambs in treatment 1 consumed more oxalate than those in treatments 3 and 4 ($P < 0.05$), and lambs in treatment 2 consumed more oxalates than those in treatment 4 ($P < 0.05$; Fig. 2). Preferences did not differ among treatments for the terpene and tannin-containing foods ($P > 0.05$; Fig. 2). Preference for the alfalfa/barley mix was lower in treatment 1 than treatments 3 and 4 and lower in treatment 2 than in treatments 1, 3, and 4 ($P < 0.05$; Fig. 2).

Within each of the four treatments, preference for the terpene-containing food was lower than for the other two toxin-containing foods ($P < 0.005$). Within treatment 1, preference was higher for the oxalate-containing food than for the tannin-containing food ($P < 0.005$; Fig. 2). As in trial 1, all lambs preferred the alfalfa/barley mix to other foods ($P < 0.005$). Alfalfa intake was higher than the tannin-containing food in treatment 2 ($P < 0.05$), higher than the oxalate-containing food in treatment 3 ($P < 0.05$), and higher than both tannin and oxalate-containing foods in treatment 4 ($P < 0.05$).

The preference for toxin-containing foods also varied between trials (Fig. 2). Lambs ate more oxalate and less terpene during conditioning in trial 1 than in trial 2, and this pattern persisted during the preference trial. Treatments 1 and 2 consumed more oxalate and less terpene than they did during trial 1 ($P < 0.05$). Additionally, lambs in treatments 1, 2, and 4 had a lower preference for tannin in trial 2 than in trial 1 ($P < 0.05$). Lambs in trial 2 also ate more alfalfa/barley mix (treatments 1, 3, and 4, $P < 0.05$) and more alfalfa (treatments 2 and 4, $P < 0.05$; and treatment 3, $P < 0.05$).

Discussion

We hypothesized that the ability of herbivores to mix toxin-containing foods in their diets is a function of the availability of nontoxic foods and the nutritional characteristics of toxin-containing foods. Based on this hypothesis, we predicted that young lambs exposed to high availability of nutritious alternatives would consume less novel toxin-containing foods, thus impeding their acquisition of diet-mixing behavior. In contrast, we predicted that young lambs exposed to low availability of nutritious alternatives would learn to consume more of the novel toxin-containing foods.

In our studies, both the quality of the toxin-containing food and the availability of alternatives affected the acquisition and retention of diet-mixing behavior. Animals consistently ingested more of the three toxin-containing foods during both conditioning and preference trials, as the availability of high-quality foods decreased and the quality of the foods containing the toxins increased (Figs. 1 and 2). Given an adequate level of nutrition, the toxin-containing diets we used complement one another in that lambs eat much more when offered a meal of three foods (tannins, terpenes, and oxalates) than they do in a meal of two foods (tannins–terpenes, tannins–oxalates, or terpenes–oxalates), and they eat much more in a meal of two foods than when they are offered only one of the foods (Villalba et al., 2004). According to optimal foraging theory, herbivores should forage on plants that maximize energy intake per unit effort (Stephens and Krebs, 1986), and they should either eat or ignore food depending on the density of other more profitable foods (the zero–one rule, Stephens and Krebs, 1986; or the none-or-all rule, McNamara and Houston, 1987). According to this rule, animals should not exhibit partial preferences for poorer quality foods. However, generalist herbivores typically do exhibit partial preferences (Stephens and Krebs, 1986; Belovsky and Schmitz, 1991; Belovsky et al., 1999), and several explanations have been proposed (McNamara and Houston, 1987; Illius et al., 1999; Provenza et al., 2003b). Our study provides evidence that partial preferences depend on prior foraging experience and nutrient–toxin interactions.

Experience and the availability of alternative foods both influence diet mixing, as illustrated in trials where lambs with 3 mo of experience mixing tannin, terpene, and oxalate-containing foods were compared with lambs with no previous experience with the same foods (Villalba et al., 2004). As in the present study, all lambs were offered five foods, including the toxin-containing foods and two nutritious alternatives—ground alfalfa and barley. However, in contrast to this study, which limited the availability of familiar foods to 10, 30, 50, or 70%, that study provided half of the lambs with 200 g (restricted to approximately 20–25% of *ad libitum*) and half with *ad libitum* access to the familiar foods. Lambs lacking experience with the toxin-containing foods, and given the nutritious alternatives *ad libitum*, ate much less of the toxin-containing foods than lambs with restricted alternatives (66 vs. 549 g/d). Experienced lambs also ate less of the toxin-containing foods if they were given *ad libitum* rather than restricted access to alternatives (809 vs. 1497 g/d). In both cases, the availability of nutritious alternatives and previous mixing experience influenced how much of the toxin-containing foods were consumed. In both trials, animals learned to mix toxin-containing foods with nutritious alternatives if nutritious alternatives were heavily restricted.

In this study, as diet quality decreased from trials 1 to 2, animals with the lowest availability of nutritious alternatives switched from tannin- to oxalate-containing foods (Fig. 1). This may be caused in part by detoxification processes, which impose nutritional costs by depleting the body of protein and glucose (Illius and Jessop, 1995, 1996) and inhibiting nitrogen utilization (Robbins et al., 1987a,b, 1991). These costs can deter feeding by herbivores (Dziba et al., 2006; Marsh et al., 2005). While knowledge of the effects of most toxin-containing foods in the body and their elimination pathways is incomplete (Foley et al., 1999; Villalba et al., 2002b), there is evidence that many terpenes are metabolized in the liver (Foley et al. 1999), that tannins bind to proteins in the diet (Robbins et al., 1991), and that oxalates are detoxified primarily by rumen microbes (Cheeke and Schull, 1985). Thus, we

speculate that lambs may have relied more on hepatic and dietary mechanisms of detoxification when their diets were of higher quality, consuming more terpenes and tannins. Additionally, lambs may have been less sensitive to protein-binding tannins when they ate the higher diet quality. When diet quality was reduced, lambs consumed more food with oxalates, evidently relying more on ruminal detoxification and adaptation to consume oxalates (Cheeke and Schull, 1985). Finally, a decrease in diet quality reduced total intake of the three toxin-containing foods regardless of the availability of alternatives. Presumably, the low-quality diet reduced the supply of nutrients needed for both ruminal and hepatic detoxification processes, which, in turn, reduced the amount of toxin-containing foods the animals could ingest.

Our findings suggest that different grazing regimes may influence how animals forage. Herbivore density influences forage availability, which can influence the way animals select foods (Provenza et al., 2003a,b; Villalba et al., 2004). Animals in this study with greater access to nutritious alternatives ate less of the toxin-containing foods, regardless of diet quality (Figs. 1 and 2). These densities increased selective foraging and prevented animals from discovering complementarities among toxin-containing plants. In contrast, higher herbivore densities may decrease selectivity and facilitate the use of less palatable species, which can lead to herbivores learning about complementary relationships among toxin-containing species (Provenza et al., 2003a). During preference tests in these trials, animals restricted to the lowest levels of nutritious alternatives ate the toxin-containing foods despite having *ad libitum* access to the two safe familiar foods (Fig. 2). Use of chemically defended species could alter successional momentum, preventing less palatable species from increasing in dominance (Villalba et al., 2004) and potentially influencing plant biodiversity (Provenza et al., 2003a).

How animals learn about a foraging environment can influence diet breadth. Herbivores that experience toxin-containing foods while on a high plane of nutrition show a higher preference for them than animals on a low plane of nutrition (Baraza et al., 2004). Consequently, an animal's physiological state, which is influenced by biochemical diversity, including nutrient availability, is a context further shaping foraging behavior. This biochemical diversity, which includes less palatable species, may allow herbivores to discover and utilize suboptimal but complementary food alternatives, offering multiple foraging pathways from which to meet their nutritional requirements while consuming less palatable species (Singer et al., 2002; Villalba et al., 2004). In this study, animals conditioned with low levels of nutritious alternatives and high diet quality ate significantly more of the three toxin-containing foods than animals conditioned with high levels of nutritious alternatives and lower diet quality (Fig. 1).

Finally, diet-mixing behavior did not adversely influence animal performance. Animals that experienced the lightest restrictions ate more safe foods, but did not gain more weight than animals forced to consume higher amounts of toxin-containing foods (trials 1 and 2). Thus, above a threshold of restriction, animals did not eat other alternatives when the amounts of food were high enough to survive. In contrast, below a threshold of restriction, animals consumed toxin-containing foods. Possibly, because complementarities were present, they developed new foraging strategies. However, when the quality of the toxin-containing food declined (trial 2), the amount of nutrients ingested may not have been enough to allow for detoxification and growth. Thus, intake and preference for the toxin-containing foods declined as did animal performance.

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Role of Species Diversity and Secondary Compound Complementarity on Diet Selection of Mediterranean Shrubs by Goats

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Abstract Goats foraging on Mediterranean shrubs containing secondary compounds (toxins) may consume a variety of shrubs that contain different phytotoxins, thereby increasing shrub intake and avoiding toxicosis. We conducted eight experiments to examine whether goats offered different mixtures of shrubs containing different phytotoxins (tannins and saponins) would consume more shrub biomass than goats offered one shrub a single phytotoxin (tannin or saponin). In the first three experiments, goats fed a mixture of three tannin-rich shrubs (*Quercus ilex*, *Arbutus unedo*, and *Pistacia lentiscus*) ate more foliage than goats offered only one shrub (23.2 vs. 10.7 g/kg BW; 25.2 vs. 13.4 g/kg BW, and 27.9 vs. 7.9 g/kg BW), regardless of tannin concentration in individual shrub species. Goats also consumed more foliage when offered the same three tannin-rich shrubs than when offered the saponin-rich shrub *Hedera helix* (25.4 vs. 8.0 g/kg BW). However, goats offered a mixture of the same three tannin-rich shrubs consumed less foliage than goats offered a mixture of two shrubs containing tannins and saponins: *Quercus* and *Hedera* (21.6 vs. 27.1 g/kg BW), *Arbutus* and *Hedera* (21.8 vs. 27.1 g/kg BW), and *Pistacia* and *Hedera* (19.7 vs. 22.0 g/kg BW). Comparison of intake of shrubs containing only tannins or saponins to intake of shrubs containing both tannins and saponins indicated that goats consumed more total biomass when fed with shrubs with both classes of compounds than with either tannins or saponins alone. Our

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results suggest that goats can increase intake of Mediterranean shrubs high in secondary compounds by selecting those with different classes of phytotoxins. Simultaneous ingestion of shrubs containing tannins and saponins may promote chemical interactions that inhibit toxic effects of these phytotoxins in the intestinal tract. In addition to complementary interactions between tannins and saponins, biological diversity within Mediterranean maquis vegetation also plays a positive role in increasing shrub intake by goats.

Keywords Mediterranean shrubs · Maquis · Biodiversity · Secondary compounds · Tannins · Saponins · Goats

Introduction

Goats are primary consumers of the Mediterranean maquis vegetation and thereby shape the diversity, structure, and dynamics of these extensive ecosystems (Naveh, 1972; Hodgson and Illius, 1996; Kaiser, 2000). Vegetation of these plant communities usually consists of 20–25 shrubby species (Rogosic, 2000), and its diversity, function, and stability depend on biological/biochemical links between plant and animal components of these ecosystems (Dziba et al., 2005). Plants possess a variety of flavor intensities and nutritional qualities that influence diet selection by herbivores (Villalba and Provenza, 2000). Olfactory, gustatory, visual, and tactile stimuli are important sources of information that herbivores use to detect potentially nutritious or toxic plants (Augner and Bernays, 1998). Collectively, the phytochemicals in a plant give rise to the characteristic flavor of a shrub species. Traditionally, food selection has been attributed to the innate ability of animals to sense specific flavors, nutrients, and toxins in plants through taste and smell (Rhoades, 1979), but food preference is a learned process involving complex relationships between flavor and postingestive effects of the plant (Provenza et al., 1998).

Given the diversity of plant cues that herbivores typically encounter, their ability to learn to recognize food and to generalize over food cues are important traits (Augner et al., 1998). Herbivores learn to prefer food flavors associated with macronutrients (Ralphs et al., 1995; Villalba and Provenza, 1999) and to avoid flavors paired with toxins (Ralphs, 1992). Once flavor–postingestive feedback associations have been learned, herbivores use visual and olfactory cues to recognize specific plants (Launchbaugh and Provenza, 1993), and they generalize preference and aversion across familiar cues for nutrients and toxins (Launchbaugh et al., 1993).

Goats foraging on Mediterranean shrubs that contain secondary compounds may consume a variety of foods to avoid toxicosis (Freeland and Janzen, 1974). Different toxins have different physiological effects on herbivores (Cheeke, 1998), and herbivores are equipped with various physiological mechanisms to counter toxins (McArthur et al., 1991). A varied diet composed of plant species containing different kinds of nutrients and toxins may increase food intake and animal production by enabling individuals to select plants that are biochemically complementary (Freeland et al., 1985; Rogosic et al., 2003). The objective of this study was to examine the effect of feeding Mediterranean shrubs containing varying concentrations of tannins and/or saponins on intake by goats.

Methods and Materials

Study Shrubs and Animals

The four major shrub components of goat diets in the Mediterranean maquis plant community *Orno-Quercetum ilicis* (Horvatić, 1958) were examined in these experiments. These shrubs include *Quercus ilex* L. (Fagaceae), *Arbutus unedo* L. (Ericaceae), *Pistacia lentiscus* L. (Anacardiaceae), and *Hedera helix* L. (Araliaceae). *P. lentiscus* has the highest tannin index (1.50), followed by *A. unedo* (1.26) and *Q. ilex* (0.96). Other secondary compounds in these shrubs include a cyclic diterpene alcohol and quinic acid (*Q. ilex*), arbutoside and ethyl gallate (ethyl 3,4,5-trihydroxybenzoate; *A. unedo*), terpenes [including α -pinene, β -pinene, camphene, *trans*-caryophyllene, cubebene (or a similar sesquiterpene), and cadinene], and fatty acids (*P. lentiscus*; Rogosic and Clausen, unpublished data). *H. helix* is reported to contain a mixture of pentacyclic terpenoids (Burrows and Tyrl, 2001) classified as genins, monodesmosides (α -hederin and β -hederin), or bidesmosides (hederacosides C and B). This complex mixture is often referred to as saponins.

We conducted eight trials at an experimental station 25 km from Split, Croatia, in the central area of the Adriatic seaside. Experimental animals were Alpine goats (mean body weight = 24.2 kg) with experience on grazing on Mediterranean shrublands near the Croatian coast. Animals were penned (1.5 × 2 m) individually. All shrubs offered were hand-harvested each week in the same location. Leaves and 1-yr old twigs, 10 cm long, were clipped and placed in bags. Within an hour, the plant material was ground to 1-cm length with a chipper, mixed for uniformity, placed in woven, polyethylene feed sacks, and refrigerated at 4°C. Every morning before the trial, bags of shrubs to be fed that day were removed from cold storage and offered immediately to animals.

Conditioning

Prior to the experiments, baseline intake of alfalfa pellets was determined for each animal on days 1–5. After baseline intake was established, all goats ($N = 12$) were offered all four shrubs simultaneously from 0900 to 1300 hr for 5 d. Shrub intake was monitored, and goats were stratified into two experimental groups ($N = 6$) based on total shrub intake. The same 12 goats were used in all experiments, and treatment groups were not changed between experiments. Between experiments, goats were fed with alfalfa pellets and barley at maintenance level for 3 d.

During experiments, goats received 100 g of barley from 0800 to 0830 hr and were offered 200 g of each shrub species simultaneously in separate feeders from 0900 to 1500 hr daily for 7 d. Feeders were monitored at 30-min intervals, and additional shrub biomass was placed in empty boxes. At 1500 hr, feed refusal was weighed and shrub consumption was calculated, and goats were fed with 550 g of alfalfa pellets. Throughout experiments, animals had free access to trace mineral blocks and freshwater.

Experiments 1–3

The objective of the first three experiments was to determine if goats consuming three high-tannin shrubs varying in nutrients and flavor would consume more biomass than goats offered only one shrub. Total intake when offered *Q. ilex*, *A.*

unedo, and *P. lentiscus* was compared to intake of only *Q. ilex*, *A. unedo*, or *P. lentiscus* in experiments 1–3, respectively.

Experiment 4

The objective of experiment 4 was to determine if goats offered the same three high-tannin shrubs would consume more biomass than goats offered one shrub (*H. helix*) containing saponins.

Experiments 5–7

The objective of experiments 5–7 was to determine if goats offered three shrubs containing tannins would consume more foliage than goats offered two shrubs containing two different classes of toxins (tannin and saponins). Intake of three shrubs (*Q. ilex*, *A. unedo*, and *P. lentiscus*) was compared to intake of two shrubs (*Q. ilex* + *H. helix*, *A. unedo* + *H. helix*, or *H. helix* + *P. lentiscus*) in experiments 5–7, respectively.

Experiment 8

The objective of experiment 8 was to determine the influence of saponins in *H. helix* on biomass intake of the three high-tannin shrubs. Intake of the three high-tannin shrubs was compared to intake of the same three shrubs + *H. helix*.

Statistical Analyses

The total daily shrub intake in each experiment was used as the dependent variable in the analysis. The experimental design for all eight experiments was a completely random design. Animals were a random factor in the mixed model analysis (SAS, 2000). The model included the two experimental groups (group 1 vs. group 2), shrub species (in different experimental groups), and the species \times group interaction. The model also used days as a repeated measure with all other interactions included. All analyses on shrub intake were adjusted for body weight (g/kg BW). Data were analyzed by using analysis of variance (SAS, 2000), and means were separated using least significance differences ($P < 0.05$).

Results

In the first three experiments, goats fed with a mixture of three tannin-rich shrubs ate more foliage ($P < 0.01$) than goats offered only one shrub, regardless of concentration of tannins in the individual shrub (Fig. 1; experiments 1–3). In experiment 1, goats offered shrubs *Quercus*, *Arbutus*, and *Pistacia* ate more foliage ($P < 0.01$) than goats offered only *Q. ilex* (23.18 ± 2.62 vs. 10.66 ± 1.52 g/kg BW). Intake of *Q. ilex* did not differ between the two groups (9.49 ± 0.99 vs. 10.66 ± 1.52 g/kg BW). Goats offered the same three shrubs in experiment 2 also ate more foliage ($P < 0.01$) than goats offered only *A. unedo* (25.23 ± 2.53 vs. 13.36 ± 2.3 g/kg BW). Likewise, *Arbutus* biomass intake did not differ between groups (12.75 ± 2.7 vs. 13.36 ± 2.3 g/kg BW). In experiment 3, consumption was again greater ($P < 0.01$) for goats

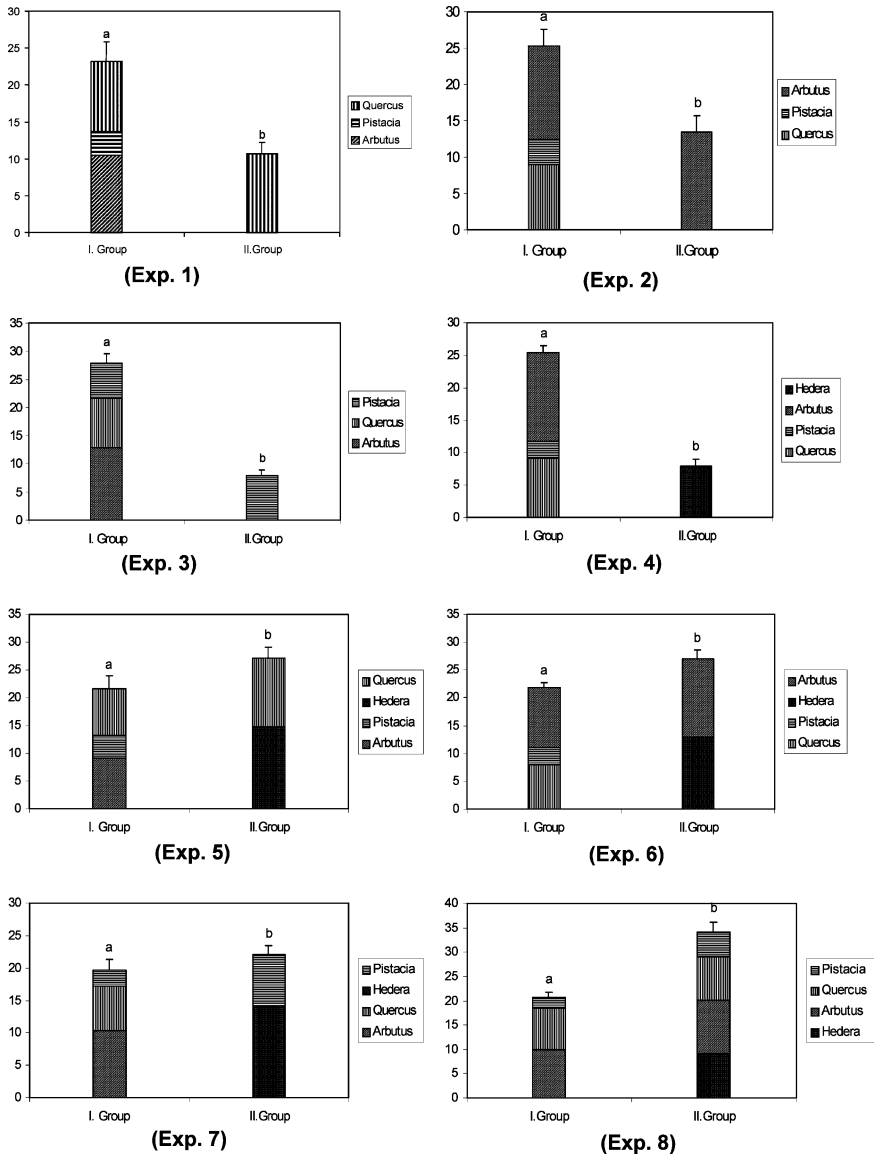


Fig. 1 Intake of goats (g/kg BW) fed with different combinations of high-tannin (*Quercus ilex*, *Arbutus unedo*, and *Pistacia lentiscus*) and/or high-saponin *Hedera helix* shrubs; N = 6 in each group. Standard error bars refer to total intake

fed the mixture of tannin-rich shrubs than for goats fed *P. lentiscus* (27.88 ± 1.72 vs. 7.85 ± 1.05 g/kg BW). In contrast to experiments 1 and 2, intake of *P. lentiscus* differed ($P < 0.01$, Fig. 1; experiment 3) between groups (6.22 ± 0.64 vs. 7.85 ± 1.05 g/kg BW). Goats with a choice of the same three tannin-rich shrubs also consumed more foliage ($P < 0.01$) than those receiving the saponin-rich shrub *H. helix* (Fig. 1; experiment 4; 25.37 ± 1.16 vs. 7.99 ± 0.93 g/kg BW).

Goats provided the mixture of three tannin-rich shrubs consumed significantly less foliage than those offered only two shrubs containing tannins or saponins (Fig. 1; experiments 5–7). In experiment 5, goats consumed more biomass ($P < 0.01$) when fed *Quercus* and *Hedera* than the group offered the three high-tannin shrubs (27.11 ± 2.03 vs. 21.64 ± 2.37 g/kg BW). Likewise, in experiment 6, goats receiving *Arbutus* and *Hedera* ate more ($P < 0.01$) than those offered *Arbutus*, *Quercus*, and *Pistacia* (27.05 ± 1.59 vs. 21.77 ± 0.89 g/kg BW). In experiment 7, goats fed the high-tannin mixture again consumed more foliage ($P < 0.05$) than those fed *Pistacia* and *Hedera* (22.03 ± 1.37 vs. 19.68 ± 1.6 g/kg BW). Cumulatively, the results of experiments 5–7 indicate that combinations of two shrubs containing both classes of phytotoxins resulted in a complementary interaction and greater intake than three shrubs containing only tannins. Goats ate more *Quercus* ($P < 0.01$) in experiment 5 when fed in combination with a saponin-containing shrub than in experiment 1 when fed either by itself or in combination with other high-tannin shrubs (12.29 ± 1.23 vs. 10.66 ± 1.52 or 8.49 ± 1.15 g/kg BW). Goats ate more *Arbutus* when fed in conjunction with *Hedera* (experiment 6) than when fed with two other high-tannin shrubs (experiment 2; $P < 0.01$; 14.01 ± 1.14 vs. 10.82 ± 0.76 g/kg BW), although intake of *Arbutus* in experiment 6 did not differ from experiment 2 when offered alone (14.01 ± 1.14 vs. 13.36 ± 2.3 g/kg BW; $P < 0.01$). Goats ate more *Pistacia* in experiment 7 in conjunction with *Hedera* than in experiment 3 when fed two other high-tannin shrubs (7.89 ± 0.64 vs. 6.22 ± 1.72 g/kg BW; $P < 0.01$), but intake of *Pistacia* in experiment 7 with *Hedera* did not differ ($P < 0.01$) from experiment 3 when *Pistacia* was fed alone (7.89 ± 1.05 vs. 7.85 ± 0.64 g/kg BW). Intake of *H. helix* was much lower ($P < 0.01$) in experiment 4 than in experiments 5–7 when fed in conjunction with a tannin-containing species (7.99 ± 0.93 vs. 14.82 ± 2.03 ; 13.04 ± 1.59 ; 14.14 ± 1.37 g/kg BW). In experiment 8, goats offered all three tannin-rich shrubs plus the saponin-rich *H. helix* ate more foliage ($P < 0.01$) than goats offered only the tannin-rich shrubs (34.06 ± 2.06 vs. 20.78 ± 0.99 g/kg BW). Tannin-rich shrubs increased the intake of *H. helix*, and conversely, *H. helix* positively influenced the consumption of tannin-rich shrubs.

Discussion

Plant phytochemicals play important ecological roles in plant defenses (Bryant et al., 1991), plant classifications (Levin, 1971), palatability (Provenza et al., 1992), phytotoxicity (Pfister et al., 1994), and perhaps in fire ecology (Naveh, 1974). Tannins and saponins are two of the major groups of phytochemicals in Mediterranean shrubs. Tannins suppress shrub intake by reducing macronutrient digestibility and causing illness (Pritchard et al., 1992; Reed, 1995). Moreover, tannins bind to proteins, cell walls, and cell solubles (Kumar and Vaithiyathan, 1990) and adversely affect rumen microbial and intestinal enzyme activity (Silanikove et al., 1996; Rogosic et al., 2005a), and consequently reduce ruminal volatile fatty acids (Makkar et al., 1995). Clinical signs of saponin toxicity include vomiting, excess salivation, and diarrhea, indicating both liver and digestive tract irritation (Harshberger, 1920). *H. helix* has also been reported to cause abortions in sheep and goats (Bondi et al., 1973). Specific roles of saponins involve modification of gut microbes, particularly in ruminants (Gee et al., 1993). Saponins suppress rumen protozoa by binding to cholesterol in the protozoa cell membrane, causing the

organisms to lyse and die (Makkar et al., 1998). Likewise, saponins can damage intestinal mucosal cells by altering cell membrane permeability and interfering with active transport (Gee et al., 1989), an effect that is dependent on the structure of the individual saponin molecule (Johnson et al., 1986).

The first four experiments were designed to determine if goats offered a combination of three shrubs containing different concentration of tannins and presumably differing nutritional profile and flavor would consume more foliage than goats offered only one of those shrubs (experiments 1–3) or a shrub containing saponins (experiment 4). Goats offered a choice of three shrubs (experiments 1–3) containing different concentrations and types (molecular weights) of tannins consumed more foliage than goats offered each of these shrubs individually, regardless of tannin concentration in shrubs. Mule deer also consumed more sagebrush (*Artemisia tridentata*) and juniper (*Juniperus osterosperma*; plants high in different kinds of terpenes) than when consuming sagebrush or juniper alone (Smith, 1959). Phytochemicals such as tannins and terpenes apparently differ in their physiological effects and/or are detoxified by different means. The concentrations and specific molecular composition of toxins in Mediterranean shrubs fed to goats probably influences toxin satiation, which is not fixed but dynamically determined by the physiological state of an animal relative to the quality and quantity of nutrients and toxins available (Villalba et al., 2002). In essence, toxins cause herbivores to eat a variety of plants to meet their needs for nutrients because the pathways of detoxification are saturable (Freeland and Janzen, 1974; Foley et al., 1999). *H. helix* contains significant levels of saponins; these bitter, toxic compounds protect this shrub from browsing herbivores by affecting their feeding behavior (Rogosic et al., 2003). Goats offered three tannin-rich shrubs in experiment 4 ate more biomass than goats fed only *H. helix*. These results are consistent observations that a greater number of shrub species in the diet increase intake (Smith, 1959; Rogosic et al., 2005a,b).

The complementarity of tannin-rich shrubs and a saponin-rich shrub positively influenced biomass intake in experiments 5–7. Goats provided with a choice between shrubs containing tannins and saponins were able to consume combinations of the shrubs that may have reduced aversive feedbacks associated with consumption of either toxin alone. Our results are consistent with this hypothesis. Goats offered two shrubs containing different classes of secondary compounds (tannins and saponins) consumed more foliage than goats offered three shrubs that all contained only tannins. Complementary interactions between shrubs containing tannins and saponins (experiment 8) further support the idea that biochemical diversity (higher number of shrubs species in the diet) has a positive influence on shrub intake, enabling animals to better meet nutritional needs and avoid toxicity. Tannins, saponins, and other allelochemicals may form complexes within the intestinal tract, given that they form chelation complexes in *in vitro* systems (Freeland et al., 1985). Intestinal binding of tannins with saponins may minimize toxic effects by reducing absorption. Simultaneous consumption of plants containing chemical chelators (e.g., tannins) and those containing other toxins may provide a mechanism for reducing both pre- and postabsorption toxicity. If herbivores use phytochemical interactions as a means of neutralizing toxins, this mechanism could be important regarding management of ruminants on Mediterranean rangelands.

As each of these shrubs presumably exhibits different flavors, as well as different concentrations of nutrients and toxins, the flavor–nutrient–toxin interaction may also

explain why the efficacy of plant defenses varies with the mixture of plants, and why chemical attributes of a single shrub species must be considered within a larger context of the plant community. For a given plant compound, preference may range along a continuum from strongly aversive if nutrients and toxins are not complementary to strongly positive if nutrients and toxins (e.g., tannins and saponins) are complementary. Understanding the role of plant phytochemicals and their interactions will lead to a greater understanding of the influence of biochemical diversity on grazing herbivores and the effect of herbivores on plant diversity, both of which could have important implications for management of natural grazing ecosystems.

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Ethanol and Methanol as Possible Odor Cues for Egyptian Fruit Bats (*Rousettus aegyptiacus*)

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Abstract Frugivorous bats from the Old and New World use odor cues to locate and assess fruit condition. We hypothesized that Egyptian fruit bats (*Rousettus aegyptiacus*) use as odor cues those volatile compounds that increase in emission rate as fruit ripens. We examined whether the smell of fermentation products may indicate the degree of ripeness to fruit bats. We analyzed volatile compounds in the headspace (the gas space above a fruit in a closed container) of dates (*Phoenix dactylifera*) and rusty figs (*Ficus rubiginosa*), both of which are consumed by fruit bats, to elucidate which compounds originate from fermentative pathways and to determine which change in emission rate during ripening. Ethanol, acetaldehyde, and acetic acid were the only volatile compounds detected as products of fermentation in both fruits. In dates, emission rates of these compounds increased during maturation, whereas in rusty figs, they decreased or remained constant. Methanol, although not a fermentation product, increased in emission rate during ripening in both fruits. We found that *R. aegyptiacus* was neither attracted nor deterred by the smell of methanol at any of the concentrations used. Although the odor of ethanol emanating from food containing concentrations similar to those found in ripe fruit did not attract the bats, at relatively high concentrations ($\geq 1\%$), the smell of ethanol deterred them. Thus, ethanol at high concentrations may serve as a signal for bats to avoid overripe, unpalatable fruit.

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Introduction

Frugivory has evolved separately in two lineages of bats: Pteropodidae from the Old World and Phyllostomidae from the New World. Olfaction is an important sense for fruit-eating bats in both families, and several studies suggest that both groups use odors emitted from fruits to locate and evaluate their palatability (Laska and Schmidt, 1986; Rieger and Jakob, 1988; Acharya et al., 1998; Luft et al., 2003). However, little is known about the chemical compounds used by frugivorous bats to find fruits and to assess their quality.

Fruit-eating bats generally prefer ripe fruit to unripe or overripe unpalatable fruit, as defined by using anthropomorphic criteria (Fleming et al., 1977; Kalko et al., 1996; Korine and Kalko, 2005). This implies that, in nature, frugivorous bats choose fruit emitting volatile compounds within a particular range of concentrations. If frugivorous bats use these compounds as odor cues, they may be able to discern variation in their emission rates to assess fruit condition.

As fruit ripens, a variety of chemical and structural changes take place, including either synthesis or degradation of carbohydrates, lipids, proteins, and mRNAs and the production of pigments and flavor-imparting compounds (Sharaf et al., 1989; Tucker, 1993). Given that the perception of flavor involves the senses of touch, smell, and taste (Rohan, 1972), the flavor of fruit depends on the complex interaction of sugars, organic acids, phenols, and more specialized flavor compounds, including a wide range of volatiles. The profile of volatile compounds for a fruit is usually complex, and may include alcohols, aldehydes, esters, and other chemical groups (Nursten, 1970).

Several fruit-eating primates and one Neotropical fruit bat are sensitive to the odor of aliphatic alcohols (Laska, 1990; Laska and Seibt, 2002). Moreover, Laska and Seibt (2002) suggested that fruit-eating mammals are specifically sensitive to aliphatic alcohols that are products of microbial fermentation in fruit, and these may serve as indicators of fruit ripeness. Indeed, several studies have shown that fruit flies (Hoffmann and Parsons, 1984), fruit-eating beetles (Rochat et al., 2000), butterflies (Utrio and Eriksson, 1977), and moths (Cosse et al., 1994) use the smell of ethanol and other products of fermentation, alone or combined, to find food.

We hypothesized that fruit-eating bats use volatile compounds that change in emission rate during ripening as odor cues to discern fruit palatability. Accordingly, we predicted that food emitting volatile compounds at concentrations close to those emitted by ripe fruit will attract fruit bats. Fruit bats will not respond to food emitting volatile compounds at concentrations similar to unripe fruit, and will be deterred by emissions from overripe, unpalatable fruit.

Methods and Materials

Experimental Animal

The Egyptian fruit bat *Rousettus aegyptiacus* (Pteropodidae) (mean adult body mass = 145.3 ± 2.6 SE g) is the only fruit bat found in Israel. It feeds mainly on fleshy

fruit, in particular on figs (*Ficus* spp.), as well as on leaves and pollen (Korine et al., 1999). We used Egyptian fruit bats from a colony maintained on the Sede Boqer Campus of Ben-Gurion University of the Negev. Bats were kept in an outdoor flight cage ($5 \times 4 \times 2.5$ m) that could be ventilated and cooled with an evaporative swamp cooler. The cage's chicken-wire sides were covered with cloth that provided 80% shade. Diet consisted of locally purchased, seasonal fleshy fruit, such as melons, watermelons, apples, mangos, and bananas that were provided *ad libitum*.

Plant Material and Collection of Volatile Compounds

We used two species of fruit consumed by Egyptian fruit bats in the wild: the rusty fig *Ficus rubiginosa* (Family Moraceae) and the date *Phoenix dactylifera* (Family Areaceae) (Korine et al., 1999). Based on skin color, fruits were classified as unripe or ripe. Unripe fruit of both species were either green or yellow. Ripe rusty figs were red to dark red, and ripe dates were brown. After collection, fruits were sealed in plastic bags and cooled to 2–5 °C and transported to the Life Science Trace Gas Facility of Radboud University of Nijmegen, where the volatiles were measured within 5 d of collection by proton-transfer reaction mass spectrometry (PTR-MS).

PTR-MS is based on the soft ionization of volatile substances by proton transfer reactions from H_3O^+ ions (Lindinger et al., 1998). PTR-MS measures volatile organic compounds at concentrations down to 0.1 ppbv in real time (~1 sec). We used a modified version of the system described by Boamfa et al. (2004). Signals generated by the PTR-MS are proportional to the trace gas concentrations in the sample air. After calibration of the PTR-MS, we determined acetaldehyde, ethanol, and methanol concentrations (in ppbv) at 1 atm pressure and room temperature. By accounting for the flow rate (1.0 l hr^{-1}), the molar mass of each compound, and the fresh mass of the fruit, we calculated the emission rates.

Glass cuvettes containing the fruit samples were continuously flushed (1.0 l hr^{-1}) with clean, dry air, or with nitrogen gas containing a low oxygen concentration. The outlet flow was connected directly to the PTR-MS. Measurements were made in air and 22 °C to simulate the natural conditions in which bats would smell the volatiles. We switched from air to nitrogen gas for 3–5 hr to induce fermentation and to identify the gasses emitted. Identification of the gasses was based on the mass of the compounds, isotope ratios, and solubility in water. In addition, we used the difference in compound fragmenting behavior when the drift field (i.e., the kinetic energy of the reactant ions in the proton-transfer reaction cell) was varied (Steeghs et al., 2004). To further examine the emitted gasses, several fruits were sealed in vials for 2–6 hr, and the headspace was analyzed with a GC-MS (Polaris Q, Thermo Finnigan).

Ethanol and Methanol as Odor Cues

In the flight cage, we exposed bats to ethanol or methanol in either distilled water or commercial mango juice (Prigat®) presented in a six-way food-choice device. We used distilled water, which provides a neutral background not related to fruit, as a control. Mango juice is attractive for bats and contains little methanol (<0.001%) or ethanol (0.01–0.015%). Six identical feeders were suspended from the corners of a hexagon of cardboard (40-cm side) that was secured to the ceiling of the flight cage. The apparatus was designed to mimic conditions in which a bat forages for fruit on the same branch of a fruiting plant or on branches of different plants that are close together.

To enable bats to learn to use the feeders, we habituated them for 1–3 d during which we filled a single, randomly chosen feeder with mango juice. During the experiments, two randomly chosen feeders were filled with treatment mixtures. They were filled with 15 ml of fluid, to a level that did not allow the bats to see the contents while flying in the cage. After the bat had inspected the cage, or had flown or hovered around the feeders, we recorded the first feeder on which it chose to alight. Because the food was not visible to the bats, we assumed that they selected the feeder by its odor. If the bat went directly to one of the feeders after being released, we disqualified the trial. Trials lasted 1–10 min but usually for less than 6 min. In total, we used 20 bats in the experiments; however, if a bat did not fly around the cage within 10 min of being freed into it, we replaced the animal with another. The observations were performed under dim red light between 18:00 and 02:00 hr.

We made the following experimental comparisons: (1) ethanol in water vs. water; (2) ethanol in fruit juice vs. fruit juice; (3) methanol in water vs. fruit juice; and (4) methanol in fruit juice vs. fruit juice. Comparisons 1 and 3 were designed to evaluate whether ethanol or methanol by themselves could act as odor cues. Comparisons 2 and 4 tested whether ethanol or methanol either encouraged or discouraged a bat's food choice relative to a constant background odor of a particular fruit. We prepared mixtures of distilled water or mango juice with ethanol or methanol a few minutes before the start of an experiment.

Preliminary experiments (Sánchez et al., 2004) showed that the amount of ethanol in wild fruits eaten by fruit bats varies between 0.02 and 0.2% in unripe fruit and between 0.1 and 0.7% in ripe fruit. In the present study, we used v/v mixtures of ethanol in water or mango juice at concentrations of 0, 0.01, 0.1, 0.3, 0.5, 0.7, 1, and 2%. The concentrations of methanol in wild fruit are unknown, although the methanol concentration in ripe melon, *Cucumis melo*, a fruit readily eaten by bats, is ~0.03% (our own measurement). Therefore, we mixed methanol in water or mango juice at 0.001, 0.01, 0.1, and 1%. We did two sets of trials, one with ethanol and the other with methanol. In each set, the order of presentation of the treatments was randomly chosen.

Statistical Analyses

Spearman's rank correlation method (Zar, 1999) was used to examine the relationships between the emission rates of the volatile compounds and stage of ripening. In addition, we used binomial tests (Zar, 1999) to analyze the results of the odor experiments, assuming that trials were independent. $P < 0.05$ was chosen as the lowest acceptable level of significance.

Results

Volatile Compounds Emission under Low Oxygen Conditions

Under aerobic conditions, 14 volatile compounds emitted from dates and rusty figs were detected, with atomic mass units between 30 and 103. Of these, only ethanol, acetaldehyde, and acetic acid increased in the headspace of both dates and rusty figs at low $[O_2]$ (Fig. 1A–D). In both species, acetaldehyde and ethanol were considerably more abundant than acetic acid.

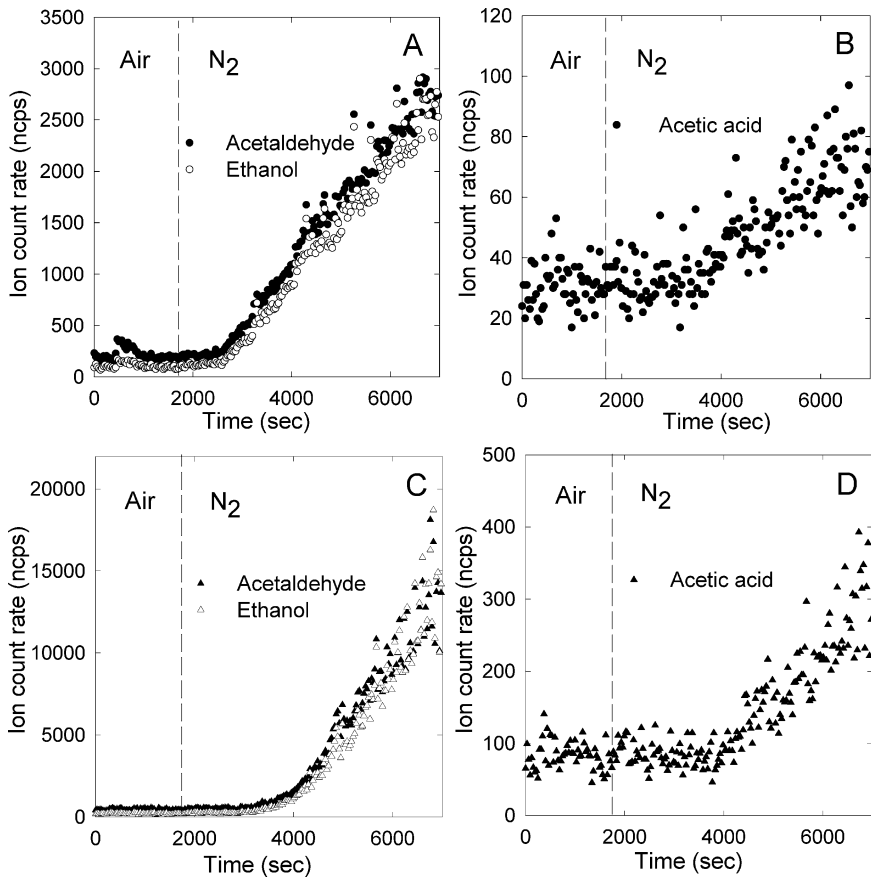


Fig. 1 The change from ambient air to low oxygen conditions (N₂ treatment) increased the emission of acetaldehyde and ethanol (A), and acetic acid (B) from dates (*Phoenix dactylifera*), as well as from rusty figs (*Ficus rubiginosa*) (C, D). Ion count rate is expressed in normalized counts per second (ncps), i.e., the count rate referenced to a fixed number of primary H₃O⁺ ions

Changes in Ethanol, Acetaldehyde, Acetic Acid, and Methanol Emission during Ripening

The emission rates of ethanol, acetaldehyde, and acetic acid in the headspace of dates were positively correlated with stage of ripening. In contrast, the emission rates of ethanol and acetaldehyde in rusty figs significantly decreased during ripening (Table 1). The emission of acetic acid was not significantly correlated with stage of ripening (Table 1). In fruit of both species, the emission rate of methanol was positively correlated with stage of ripening (Table 1).

Ethanol as an Odor Cue

Bats were not attracted to any of the mixtures of ethanol in water compared to water alone (Fig. 2A). At low concentrations of ethanol (0.01, 0.1, 0.3, 0.5, and 0.7%), they had no preference for mango juice mixed with ethanol over mango juice alone (two-

Table 1 Correlations between the emission rates of ethanol, acetaldehyde, acetic acid, and methanol and the stage of ripening of dates and of rusty figs

Dates	Emission rates for dates (ng hr ⁻¹ g ⁻¹ fresh fruit) (mean ± SE)				Rank-Spearman correlation coefficient	<i>P</i>
	Unripe green (<i>N</i> = 3)	Unripe yellow (<i>N</i> = 4)	Ripe brown (<i>N</i> = 5)			
Ethanol	14.80 ± 3.37	22.64 ± 6.74	117.96 ± 18.90	0.83	<0.01	
Acetaldehyde	21.00 ± 7.40	31.18 ± 11.45	160.58 ± 31.02	0.83	<0.01	
Acetic acid	4.41 ± 1.01	4.08 ± 0.67	10.98 ± 1.68	0.66	0.02	
Methanol	29.65 ± 13.89	27.92 ± 14.23	204.54 ± 28.25	0.78	<0.01	

Figs	Emission rates for rusty figs (ng hr ⁻¹ g ⁻¹ fresh fruit) (mean ± SE)				Rank-Spearman correlation coefficient	<i>P</i>
	Unripe green (<i>N</i> = 4)	Unripe yellow (<i>N</i> = 6)	Ripe red (<i>N</i> = 6)			
Ethanol	1580.67 ± 162.51	1346 ± 362.26	596.61 ± 276.69	-0.58	0.02	
Acetaldehyde	1210.90 ± 117.56	1022.13 ± 255.52	424.32 ± 186.39	-0.63	<0.01	
Acetic acid	82.48 ± 8.04	61.28 ± 14.94	49.42 ± 9.76	-0.41	0.12	
Methanol	333.49 ± 92.46	1731.46 ± 73.80	8233.07 ± 1080.20	0.94	<0.01	

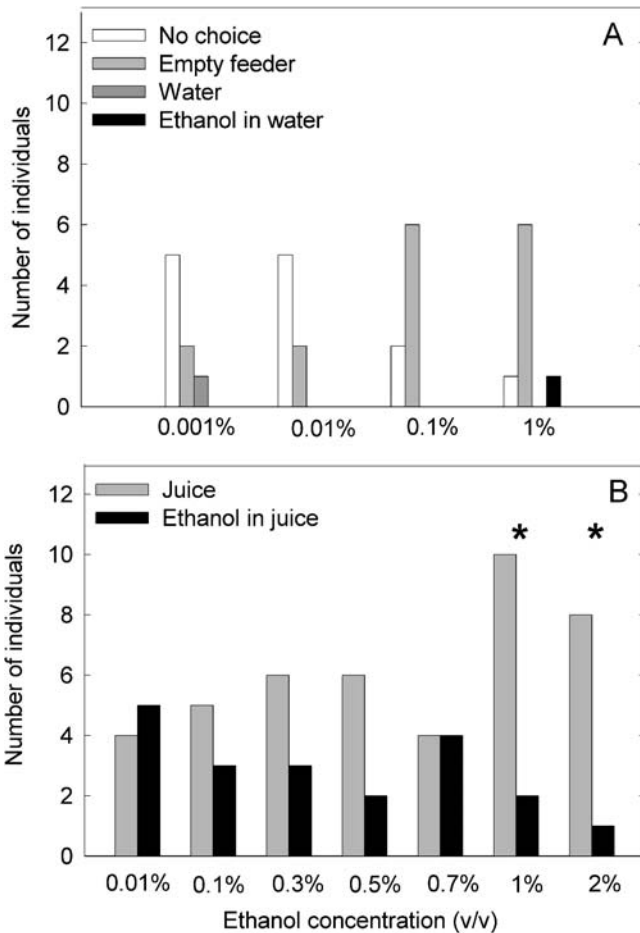


Fig. 2 Comparison of the responses of Egyptian fruit bats in odor experiments to (A) ethanol in water vs. water and (B) ethanol in mango juice vs. mango juice. In (A), *no choice* means that the bat inspected the feeders but did not settle on any of them, and *empty feeder* means that after inspecting the feeders and the bat settled on an empty feeder. Asterisks denote significant differences ($P < 0.05$) from binomial tests

tailed binomial test, $P > 0.05$ in all cases, Fig. 2B). However, they preferred the mango juice alone over mango juice mixed with ethanol at 1 and 2% (two-tailed binomial test, both $P < 0.05$). These results indicate that Egyptian fruit bats might use the smell of ethanol to assess food quality by recognizing it when present in relatively high concentrations and also that alone it does not serve as a short-distance attractant.

Methanol as an Odor Cue

Bats visited feeders with mango juice more often than feeders containing methanol in water (one-tailed binomial test, all $P < 0.05$, Fig. 3A). They had no preference for mango juice mixed with different amounts of methanol compared to mango juice alone (two-tailed binomial test, all $P > 0.05$, Fig. 3B). Thus, they were neither at-

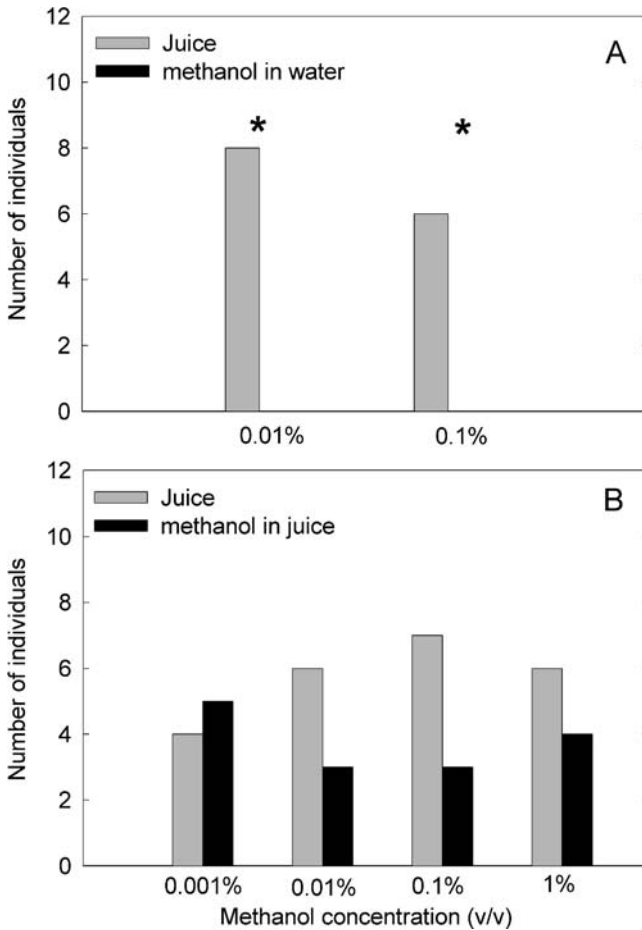


Fig. 3 Comparison of the responses of Egyptian fruit bats in odor experiments to (A) methanol in water vs. mango juice and (B) methanol in mango juice vs. mango juice. Asterisks denote significant differences ($P < 0.05$) from binomial tests

tracted nor deterred by the smell of methanol at any of the concentrations used in the experiments.

Discussion

We analyzed the volatile products of fermentation emitted by dates and rusty figs to test the hypothesis that compounds changing in emission during ripening may be used as odor cues by fruit-eating bats. We identified three volatile compounds produced by fermentation in both dates and rusty figs: ethanol, acetaldehyde, and acetic acid. However, we certainly did not identify all gaseous fermentation products from these fruits. A wide variety of volatiles are known to be produced during microbial fermentation in fruit, including acids, alcohols, esters, aldehydes, ketones, and polyols (Mangas et al., 1994; Vidrih and Hribar, 1999; Fleet, 2003). Further experiments with

more fruit samples and for longer times under low $[O_2]$ might help to identify more compounds.

Emission rates of ethanol, acetaldehyde, and acetic acid in dates all increased from one stage of ripening to the next. The opposite occurred with ethanol and acetaldehyde in rusty figs, whereas no changes in the emission rate of acetic acid during maturation were detected. Given that ethanol and acetaldehyde are the main products of fermentation in both dates and rusty figs, and that their emission rates change during ripening, these molecular species appear to be good candidates with which to test our hypothesis.

In contrast to acetaldehyde, for which only toxic effects are known, ethanol is both a nutrient, at low concentrations, and a toxin, at high concentrations (Fadda and Rossetti, 1998; Lieber, 2000). Ethanol may have both short- and long-term positive effects on a frugivorous animal (Dudley, 2000, 2002). In addition, ethanol concentration in fruit is correlated with its sugar content (Dominy, 2004; Dudley, 2004; Senesi et al., 2005), and it increases during ripening in several species consumed in the wild by Egyptian fruit bats (Sánchez et al., 2004).

Based on the above, we hypothesized that ethanol emitted by food serves as an odor cue to Egyptian fruit bats. The bats were not attracted by the smell of ethanol in water or in mango juice at concentrations similar to those in ripe fruit, compared to mixtures without ethanol. Sánchez et al. (2004) suggested that the presence of yeasts in fruit, via the ethanol they produce, could increase the probability of visits by potential seed dispersers; therefore, over evolutionary time the fostering of yeast growth may have been incorporated into the reproductive strategy of fleshy fruited plants. We did not find evidence, however, to support this hypothesis.

Janzen (1977) suggested that microorganisms and frugivorous vertebrates compete for sugars present in fruit. Consequently, decay-causing microorganisms produce compounds toxic to vertebrates, and vertebrates may have developed the means to detect those compounds. Our data support Janzen's hypothesis in that ethanol at concentrations greater than 1%, which often characterizes overripe unpalatable fruit, elicited avoidance in Egyptian fruit bats. Thus, our results suggest that ethanol at high concentrations is used as an odor cue aiding fruit bats to identify low-quality fruit.

In addition, the emission rate of methanol changed during maturation, both in dates and rusty figs. The increase in methanol is related to fruit softening because of the action of the enzyme pectin methylesterase (PME; Lefever et al., 2004). PME catalyzes the demethoxylation of cell-wall pectins, and its synthesis increases during fruit ripening (Frenkel et al., 1998; Supriyadi et al., 2003). Given that methanol emission rate is related to changes in fruit quality, we suspected that methanol might be used as an odor cue by fruit bats, particularly as an indicator of unpalatability because of its toxic effects. However, in contrast to our prediction, fruit bats were neither attracted nor deterred by the smell of methanol in water or mango juice at any concentration.

Cutaneous exposure to methanol, its inhalation, or ingestion can be deleterious because methanol is oxidized into formaldehyde and then to formic acid. The metabolic acidosis that characterizes methanol poisoning may cause reduced level of alertness, visual impairment, and even death (Shusterman et al., 1993). Nevertheless, not all mammal species are equally sensitive to methanol. Primates appear to be highly sensitive to it because of their limited ability to catalyze formic acid to carbon dioxide (Black et al., 1985). In contrast, rodents are far less sensitive because they are competent at metabolizing formic acid (Makar and Tephly, 1976; Black et al., 1985). It

seems that Egyptian fruit bats are not sensitive to methanol at the levels presented in our experiments.

We isolated the effect of the smell of two common aliphatic alcohols present in fruit. Our results partially support the hypothesis that volatile compounds that change in emission during fruit ripening may be used as odor cues by fruit bats. So far, we have no evidence that either ethanol or methanol attracts fruit bats, but rather our results suggest that the smell of ethanol at high concentrations may help them to recognize unpalatable fruit. The possibility still exists that either ethanol or methanol acts as attractant to fruit bats in synergy with other volatile compounds. Indeed, mixtures of different products of fermentation are known to attract fruit-eating moths (Cosse et al., 1994) and beetles (Rochat et al., 2000). In addition, ethanol and methanol might also act as long-distance cues to fruit bats for finding patches of fruiting plants, rather than as short-distance cues for assessing fruit quality.

Finally, although both ethanol and methanol at high concentrations are likely toxic to Egyptian fruit bats, these animals may behaviorally respond more to the possible consequences of ethanol ingestion than to the toxic effects of methanol. Thus, future studies on the physiology of ethanol and methanol pharmacokinetics in Egyptian fruit bats may help us to understand their behavior while selecting food.

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Sexual Dimorphism in Territorial Scent Marking by Adult Eurasian Beavers (*Castor fiber*)

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Abstract Mammalian scent marking is often associated with territorial defense. However, males and females may demonstrate different activity patterns and play different roles. Female mammals nurture the young during lactation, while males purportedly perform other tasks more frequently, such as territorial maintenance and defense. This paper investigates the contribution made by mated pairs of adult males and females to territorial scent-marking in the obligate monogamous Eurasian beavers (*Castor fiber*). We hypothesized that both sexes should show territorial behavior, and predicted that they deposit a higher proportion of scent marks at borders. We also hypothesized that a sexual dimorphism exists due to reproductive constraints on the females, and predicted that males should invest significantly more in scent-marking behavior than females during summer. We obtained behavioral data by radio tracking six mated pairs of Eurasian beavers during spring and summer 2000–2001 on two rivers in southeastern Norway. Our results showed that both males and females clustered their scent marks near territorial borders, but males deposited a larger number of scent marks than females and spent more time at borders. Males were also found to have a higher scent marking rate and scent marks per night than females during summer, but not during spring. Overall, scent marks per night were higher in males than females. We conclude that both males and females Eurasian beavers carry out territorial behavior by scent marking, but males carry a larger part of the territorial defense during summer when females lactate. Our results are discussed in the light of the codefense hypothesis.

Keywords *Castor fiber* · Monogamy · Parental care · Codefense · Territoriality · Mammals · Beavers · Olfactory signaling · Scent marking

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Introduction

Several nonexclusive hypotheses have been proposed to explain why animals scent-mark (see Back et al., 2002). Scent marks might serve several functions, which may change or vary with time of year or location of the mark. However, it is widely accepted that mammals scent mark their territories to advertise their occupancy and ownership of the territory (e.g., Gosling, 1982; Sillero-Zubiri and Macdonald, 1998), but it is still under debate how scent marks actually function in terms of territory maintenance (Gorman, 1990; Gosling, 1990; Richardson, 1991, 1993).

The “scent-matching” hypothesis posits that scent marks provide an olfactory link between a resident owner and his territory, and that this enables intruding animals to recognize the chance of escalated conflicts (Gosling, 1982; Gosling and Roberts, 2001). By matching the scent of a territory owner with those of nearby scent marks, an intruder employs the unique property of olfactory signaling that includes the provision of both a historical and a spatial record of a territorial individual’s behavior. Territory owners can thus signal their status to intruders in a way that cannot be mimicked and that is to their advantage in subsequent encounters (Gosling, 1982). Intruding animals (independent of gender), upon entering a foreign territory, quickly discover that the area is already occupied, as scent marks are placed at the border (Gorman and Mills, 1984; Richardson, 1993). The threat of being detected and possibly becoming involved in a fight should keep intruders to the border region, when it does not completely deter them from intruding (Sliwa and Richardson, 1998). According to the hypothesis, territory owners should also remove or replace marks of others (Gosling, 1986). It is, thus, crucial that both sexes maintain their scent marks in such a way that maximizes the success of matching (see Gosling, 1986; Gosling and Roberts, 2001; Roberts and Lowen, 1997). This is achieved both by replenishing their own scent marks on a regular basis and by removing or replacing marks of competitors within their territory (Roberts, 1998; Rich and Hurst, 1999).

Both sexes usually scent mark in mammals (e.g., Gosling and Roberts, 2001). However, males and females may demonstrate different activity patterns and play different roles. Female mammals nurture the young during lactation, while males purportedly perform other tasks more frequently, such as territorial maintenance and defense. If one sex ranged further than the other did, the former may encounter more intruders or unmarked regions and respond by marking at higher rates. Selection could then favor individuals of this sex with relatively larger or more active scent structures (Rosell and Schulte, 2004). Also, a seasonal difference in scent marking activity may have been developed due to energy constraints on females during summer.

The Eurasian beaver (*Castor fiber*) is a typical obligate monogamous species (Wilsson, 1971; Kleiman, 1977). They defend territories, invest in their territories by the construction of dams and lodges, have young with an extended period of maturation, and the adult male exhibits a relatively high degree of both direct and indirect parental care (Wilsson, 1971; Rosell, 2002). They live in family units consisting of an adult pair and their offspring (Wilsson, 1971; Nolet and Rosell, 1994). Breeding pairs maintain long-term pair-bonds and copulate during January–March (Wilsson, 1971). Kits are born on average on June 1, and the adult male as well as subadults of both sexes help rearing them (Wilsson, 1971). Some behavioral differences in adult males and females have been observed, especially during spring and summer when young are reared (Wilsson, 1971). Two recent studies on mated male and female Eurasian beavers suggest that males have significantly larger kernel

home ranges during the whole tracking period (March–August) and after parturition (Herr and Rosell, 2004), and that males allocate significantly more time to travel (Sharpe and Rosell, 2003).

In beavers, there are no external differences between the sexes except for visible nipples in lactating females (Wilsson, 1971). Both sexes possess two anal glands and two castor sacs where retention of urine creates the fluid castoreum (Rosell, 2002). During scent marking of their territories, castoreum is deposited on scent mounds or on the ground (Rosell and Sundsdal, 2001). The number of scent marks is significantly higher in spring when subadults disperse (Rosell et al., 1998). The anal gland secretion is occasionally deposited; however, its role in territorial scent marking is not yet fully understood (Rosell and Bergan, 1998; Rosell and Sundsdal, 2001). Both sexes and all age classes (>5 mo) participate in scent marking (Wilsson, 1971).

A variety of hypotheses have been assumed for scent marking in the two beaver species, i.e., the Eurasian beaver and the North American beaver (*C. canadensis*) (see review in Rosell, 2002). However, by testing alternative hypotheses, Houlihan (1989) confirmed the territorial function of North American beaver scent marks and rejected other interpretations (see also Schulte, 1998). Scent marking in the Eurasian beavers likewise serves a territorial function (Rosell, 2002). Scent marks are concentrated near territorial borders, apparently to maximize the signal effect to potential trespassers on or before entering the territory (Rosell et al., 1998; Rosell, 2002).

More information is needed about frequency of marking by different group members (age, social status, and sex) and behavioral context in which the signal is deposited. In this scenario and given the obligate monogamous nature of the Eurasian beaver, it is interesting to investigate whether scent marking behavior is similar between the sexes and if a seasonal difference exists. Previous studies on sex differences in scent marking in North American beavers have either focused on the reaction to experimentally constructed scent marks (Butler and Butler, 1979; Svendsen and Huntsman, 1988; Müller-Schwarze and Houlihan, 1991), or when based on direct observation of scent marking, included unequal numbers of males and females (Buech, 1995). Only anecdotal information exists on Eurasian beavers, based on animals in captivity (Wilsson, 1971). None of the studies above used observations of mated pairs.

This paper investigates the contribution made by mated pairs of adult males and females to scent marking in Eurasian beavers. We hypothesized that both sexes should show territorial behavior and predicted that they deposit a higher proportion of scent marks at borders. We also hypothesized that a sexual dimorphism exists due to reproductive constraints on the females, and predicted that males should invest significantly more in scent-marking behavior than females during summer. We examined the sexual dimorphism issue by presenting the rate of marking, activity patterns, and the marking context.

Methods and Materials

Study Area and Study Animals

We collected data in Eurasian beaver territories along the Gvarv and Saua Rivers in Telemark, southeastern Norway, during spring and summer of 2000 and 2001. Both rivers meander through a semiagricultural and mixed forest landscape. The Saua River forms part of the Telemark Canal, and at either end of the area studied are

canal locks and weirs. The Gvarv River follows a more natural flow regime and discharges into Lake Nordsjø, part of which forms a section of one of the territories. Both rivers are either too wide for beavers to build dams or too deep to make it necessary. Beavers have inhabited the area since the 1920s (Olstad, 1937). Hunting pressure within the study area is either very low or nonexistent, and the beavers are used to human presence.

Subjects consisted of 12 Eurasian beavers ($\bar{X} \pm SD$ weights: males = 21.3 ± 1.4 kg; females = 22.8 ± 2.6 kg), which were the reproducing pairs in six territories. Assessment of pairs was based on previous trapping records of weights, incidence of lactation, and behavioral observations (F. Rosell, unpublished data). We caught animals from a boat, by using landing nets, during September, March, and April 1999–2001 (Rosell and Hovde, 2001). Permission for capture, handling, and surgical procedures was provided by the Norwegian Experimental Animal Board and the Norwegian Directorate for Nature Management. Licensed veterinarians carried out all surgical procedures. To facilitate handling and tagging, each beaver was placed head first into a cloth sack and restrained while another researcher applied ear tags by maneuvering the beaver's ear out through a small hole cut in the sack. No beavers responded aggressively while confined in the sack; they tended to remain comparatively docile, and none was visibly injured by the procedure. Individuals were tagged with numbered plastic ear tags (3.5 cm; Dalton Continental, Lichtenvoorde, The Netherlands) or Monel metal ear tags (1.5 cm; National Band and Tag Co., Newport, KY, USA) or both. Each beaver was weighed, implanted with a microchip measuring 1 cm (Destron 490, St. Paul, MN, USA) in the neck, and sexed by the color of the anal gland secretion (Rosell and Sun, 1999; Rosell and Bjørkøyli, 2002). We assumed that all females gave birth during both 2000 and 2001 based on observations of nipple size, presence of kits, sound of kits inside the lodge, and transport of branches into the lodge during July (Wilsson, 1971).

During September, March, and April, prior to the 2000 field season, beavers from five of the six pairs studied were implanted with an Alterra TX30.3A1 intraperitoneal 30 MHz radio transmitter (63 g) equipped with temperature and movement sensor [Alterra (IBN/DLO), AA Wageningen, the Netherlands] (Ranheim et al., 2004). To recover completely, all animals were allowed a minimum of 1 wk before tracking began. However, the behavior and movement of the beavers did not appear to be affected by the implanted transmitters, except for the first 2 d when more time was spent inside the lodges. The transmitter signal was detected by using a one-element loop antenna (Alterra) and an Icom IC-R10 receiver (Icom Inc., Osaka, Japan). Signal reception range varied between 300 and 600 m from a boat (Nolet and Rosell 1994). In April 2001, the last two beavers (plus one with an failed intraperitoneal transmitter) were fitted with external tail transmitters (Advanced Telemetry Systems Inc., Isanti, MN, USA; Model 16 M ear tag for beaver tail, weight = 38 g) operating at 142 MHz (F. Rosell, unpublished data). Belt pliers were used to perforate the tail approximately 10 cm from the base of the tail and 3.1–2.4 cm from the lateral edge. The transmitter was secured with a bolt, washer, and nut. A plastic holder was inserted to facilitate the withdrawal of the bolt when the transmitter was recovered. The beavers were released near the capture site less than 10 min after capture. Animals showed normal behavior less than 15 min after their release. Signals were detected with a handheld RX-98 H tracking receiver with built-in foldable antenna (TVP Positioning AB, Lindsberg, Sweden). Mean ($\pm SD$) signal reception range from a boat was 353 ± 112 m (F. Rosell, unpublished data).

Ethical Considerations

Please refer to Sharpe and Rosell (2003) and Herr and Rosell (2004) for detailed information on animal welfare issues and Ranheim et al. (2004) for descriptions of surgical procedures.

Data Collection

The study was conducted between March 7 and August 8, 2000, and between April 8 and July 31, 2001. Three beaver pairs were followed during both years, two additional pairs only in 2000, and one extra pair only in 2001. On average, each beaver was followed 6.8 nights (range 4–10), adding to a total of 82 nights, equally divided between males and females. Data were collected by a two-person team (one observer and one recorder). A boat with outboard motor was used to follow the beavers. During dark periods, a spotlight was used to illuminate the animals. Noise from the engine and the light did not seem to affect normal behavior in the beavers as long as they were approached with care (Nolet and Rosell, 1994; Rosell and Hovde, 2001, personal observation). Binoculars were used when needed. Focal animal sampling (Altmann, 1974; Martin and Bateson, 1999) was used, and a single beaver was followed throughout each observation night. The predetermined focal animal was initially located in its daily resting place by the signal from its radio transmitter. After the animal emerged, the observation team attempted to stay in visual contact with it throughout the night. Whenever an animal disappeared from view, the radio signal was used to locate it again. Both members of a pair were followed on consecutive nights in order to obtain “paired observations” and to reduce the effects of seasonal changes in weather conditions and behavior. Which member of a pair was to be followed first was determined at random by tossing a coin.

Beavers are nocturnal and usually have one activity period during each night (Wilsson, 1971; Nolet and Rosell, 1994). Thus, an observation session began when the focal animal emerged from its daily resting place in the evening and was terminated after the break of dawn when the animal had spent ≥ 30 min inside a lodge or burrow. This period of activity was defined as the “principal activity period” (Corp et al., 1997). If a beaver left its daily resting place undetected, the activity period was set to begin midway between the last time the animal was known to be in the resting place and the time it was discovered to have left. The same procedure was used to determine the end of the period. Behavior was continuously recorded throughout observation sessions and classified into eight categories (Altmann, 1974; Martin and Bateson, 1999) along with time and position (Table 1). We assigned the last behavioral pattern prior to scent marking and the first after scent marking as the “context prior” and “context after,” respectively. Positions were recorded on a 1:5000-scale map of the study area. During each observation night, seven environmental variables were recorded every 2 hr: air temperature ($^{\circ}\text{C}$), water temperature ($^{\circ}\text{C}$), precipitation (presence/absence), moon (presence/absence), mist (presence/absence), cloud cover (pct.), and wind (5 categories).

Scent Marking Rate and Scent Marks per Night

We used scent marking rate (SMR) and scent marks per night (SM/night) to compare scent marking activity between male and female beavers. To obtain

Table 1 Categories of Eurasian beaver behavior and description of each category

Behavior category	Description of behavior
Swimming	Swimming for more than 2 min in one direction or more than 25 m
Scent marking	Depositing castoreum, including behavior initiating scent marking
Foraging	On land foraging, eating food, or diving for aquatics
Grooming	Grooming own fur
Amicable interaction	Nosing, social grooming, “play wrestling,” ^a and other social interactions between family members
Agonistic interaction	Chasing, biting, “stick display,” ^b or other agonistic behavior towards nonfamily or family members
On bank	Entering bank for a shorter or longer period, sitting, walking, or sniffing the ground, but without performing any other characteristic behavior
Other	Any behavior not mentioned above

^a Moving of the head in a circle in an exaggerated manner, signaling to a group member (Wilsson, 1971).

^b A display behavior involving the use of tools where the beaver holds an object, usually a stick, in its mouth while moving its upper body rapidly up and down (Thomsen et al., unpublished data).

standardized scent marking rates, we divided the number of scent marks by the length (in hours) of the principal activity of the beaver, subtracted by the time the animal was either out of sight, or its behavior could not be deduced. Nightly visits to lodges or burrows were included, as scent marking has not been reported to occur here. Males had longer activity periods than females ($\bar{X} \pm \text{SD}$ min/night: males = 570.0 ± 97.5 ; females = 493.0 ± 144.7 ; Wilcoxon signed ranks test: $W = 725.5$, $P < 0.001$). Therefore, a rate like SMR will not reflect the actual contribution of a beaver to the total number of scent marks deposited each night. Thus, we also compared scent marks per night (SM/night). Before doing so, we checked whether we had managed to keep track of males and females equally well, i.e., whether the percentage of time we had the beaver “in sight” (see above) during the principal activity period was the same for both sexes. Not surprisingly, we had more minutes of “in sight” data on males due to the longer principal activity periods ($\bar{X} \pm \text{SD}$ min/night: males = 495.0 ± 112.1 ; females = 442.0 ± 131.3 ; $W = 683.5$, $P = 0.001$). However, the percentage of time we had the beaver in sight was equal between the sexes albeit marginal ($\bar{X} \pm \text{SD}$ percent: males = 86.2 ± 0.083 ; females = 89.8 ± 0.068 ; $W = 280.0$, $P = 0.051$). Thus, we chose to use the visible counts of scent marking instead of adjusting them for time not in sight.

We divided our data into two periods: (1) spring (before parturition) and (2) summer (from parturition onwards). Parturition usually takes place around June 1, and was chosen as the division date (Wilsson, 1971; F. Rosell, unpublished data).

Spatial Distribution

Beaver territories can be considered as one-dimensional, as only a narrow strip of land is used along riversides (Nolet et al., 1994). Locations of territory borders used in this study were obtained from Campbell (2000) and Herr and Rosell (2004), who established borders based on minimum convex polygons (MCP) by fixes found during radio tracking performed concurrently with this study. We divided each beaver territory into two zones: (1) “border”—100 m bank length on both riversides

at shared borders, (2) “core”—the central part of the territory. A territory with two neighboring territories would have 400 m of border length. Border length did not vary considerably with territory size and a fixed length of 100 m bank length was used in all cases. Overlap between territories is minimal (Herr and Rosell, 2004), and 100 m bank length is, therefore, sufficient to encompass overlapping sections. Furthermore, the territories of pair members overlapped on average by $81.6 \pm 14.0\%$ SD (Herr and Rosell, 2004).

Data Analysis

Normality was tested by using Shapiro-Wilk test (SPSS Inc., 1999) and homogeneity of variance by using Levene’s test (Dytham, 1999). If assumptions about continuity, normality, and homogeneity were met, parametric tests were used. When assumptions of normality could not be met even after transformation of data, the equivalent nonparametric tests were used. SMR and SM/night were normality-distributed for overall year data and thus one-way ANOVA could be used to compare scent marking between pairs and Pearson correlation to investigate correlations within pairs (Sokal and Rohlf, 1995). We choose to use the nonparametric Wilcoxon signed ranks tests to compare SMR and SM/night between males and females due to the low sample size and problems of obtaining normality during seasons (Sokal and Rohlf, 1995). The sampling unit for paired statistics was the individual beaver in order to avoid the “pooling fallacy” (Martin and Bateson, 1999), which implies not to treat repeated measures of the same subject as independent samples. Chi-square tests were used to analyze spatial distribution of scent marks (Sokal and Rohlf, 1995). Before statistical testing, expected frequencies of scent marks in each zone were adjusted for bank length, as core bank lengths were much larger than border bank lengths. Spearman rank correlation was used to compare the context of male and female scent marking (Sokal and Rohlf, 1995). Data from males and females were pooled to obtain enough data in each behavioral category. Spearman rank correlation was used to control for environmental influences. Bonferroni adjustment for multiple comparisons was applied because of the high number of correlations (Sokal and Rohlf, 1995). Two-tailed tests were used in all analyses and $P < 0.05$ was used as the level of significance. Statistical tests were run in SPSS version 10.0 (SPSS Inc., 1999) and Analyze-it (version 1.71).

Results

Territorial Function

A total of 806 scent marks (SM) were registered (males = 480, females = 326; Table 2). Three animals (two males and one female) were the main contributors to the total number of scent marks deposited, with 20.5, 26.8, and 28.7%, respectively (Table 2). Those three animals came from two pairs (L2a and L2b), that accounted for 55.5% and 29.4% of all scent marks deposited by the six pairs (Table 2). The remaining four pairs each contributed 6.1% or less. Both SMR and SM/night varied significantly between pairs (one-way ANOVA; SMR: $F_{5,6} = 11.17$, $P = 0.005$; SM/night: $F_{5,6} = 12.81$, $P = 0.004$). Within pairs, male and female SMR and SM/night were correlated (Pearson correlation; Spring: $R^2 = 0.88$, $N = 6$, $P = 0.019$; Summer: $R^2 = 0.92$, $N = 6$, $P = 0.010$).

Table 2 Number of observation nights in six Eurasian beaver territories, number of scent marks observed, and relative contribution (in percent) to total number of scent marks within pairs, between pairs and between pairs with the sexes combined

Territory	Nights	No. scent marks		Contribution within pairs (%)		Contribution between pairs (%)		Contribution between pairs—sexes combined (%)
		Male	Female	Male	Female	Male	Female	
L1	14	27	2	93.1	6.9	3.3	0.2	3.6
L2a	20	231	216	51.7	48.3	28.7	26.8	55.5
L2b	18	166	72	69.6	30.4	20.5	8.9	29.4
L5	8	27	21	57.1	42.9	3.5	2.6	6.1
L6	14	18	11	62.1	37.9	2.2	1.4	3.6
N2	8	11	4	73.3	26.7	1.4	0.5	1.9
SUM	82	480	326					

All but four scents marks (three made by two females, one by a male) were deposited within the marker's own territory (border zone included). Males and females scent marked significantly more in the border zones than expected when compared to the time spent in border and core zones (Table 3, males: $\chi^2 = 1101.429$, $df = 1$, $P < 0.001$; females: $\chi^2 = 1142.360$, $df = 1$, $P < 0.001$). Both sexes also scent marked significantly more in the border zones than would be expected if the deposition had been according to bank length (Table 3, males: $\chi^2 = 935.348$, $df = 1$, $P < 0.001$; females: $\chi^2 = 696.193$, $df = 1$, $P < 0.001$).

Sexual Dimorphism

Within each pair, most scent markings observed were made by males, with the percentage ranging from about 52% to 75% (except for pair L1 where the male

Table 3 Scent marking by male and female Eurasian beavers in the border and core zones of their territories

	Border	Core
Males		
Obs ^a	311	168
Exp ^b	64/72	415/407
% ^c	64.9	35.1
Females		
Obs	231	92
Exp	39/53	294/270
%	70.9	29.1

^a Observed number of scent marks.

^b Expected number if scent marks had been randomly distributed according to time in zone/length of bank in zone.

^c Observed number of scent marks in percent.

Number of scent marks observed and number of scent marks expected according to time spent in each zone and expected according to bank length of the border and core zones, respectively.

made more than 90% of all scent marks; Table 2). There was no difference in SMR between the sexes when looking at the overall study period [Fig. 1a(I)]. However, this was attributable to only one female having a higher rate of marking than her mate. SM/night was significantly higher in males than females [Fig. 1b(I)]. From spring to summer, five males increased their marking rates and, of these, four males had a higher SM/night during summer. Among the females, a similar pattern was observed with four females having a higher SMR and five a higher SM/night in summer than in spring. In the couple from territory L2a, both SMR and SM/night dropped considerably between seasons [Fig. 1a(I–II) and b(I–II)]. During summer, both SMR and SM/night was significantly higher in males than in females. During spring there was no difference in SMR with two females having a higher rate than their mates. The same trend was observed for SM/night; however, only one female caused the difference [Fig. 1a(I–II) and b(I–II)].

Males and females deposited the same proportion of their scent marks in border and core zones (Table 3, $\chi^2 = 0.818$, $df = 1$, $P = 0.366$). However, males spent significantly more time in the border zones than females and spent a significantly higher proportion of their total activity time there (Table 4). This was also true when looking at the spring season. In the summer season, only absolute time was significantly different between the sexes, whereas proportion of time spent at borders was marginally nonsignificant (Table 4). Males would deposit more scent marks and females less scent marks than would be expected if the sexes had contributed equally to scent marking in each zone (Table 3, border: $\chi^2 = 9.993$, $df = 1$, $P < 0.001$; core: $\chi^2 = 19.167$, $df = 1$, $P < 0.001$).

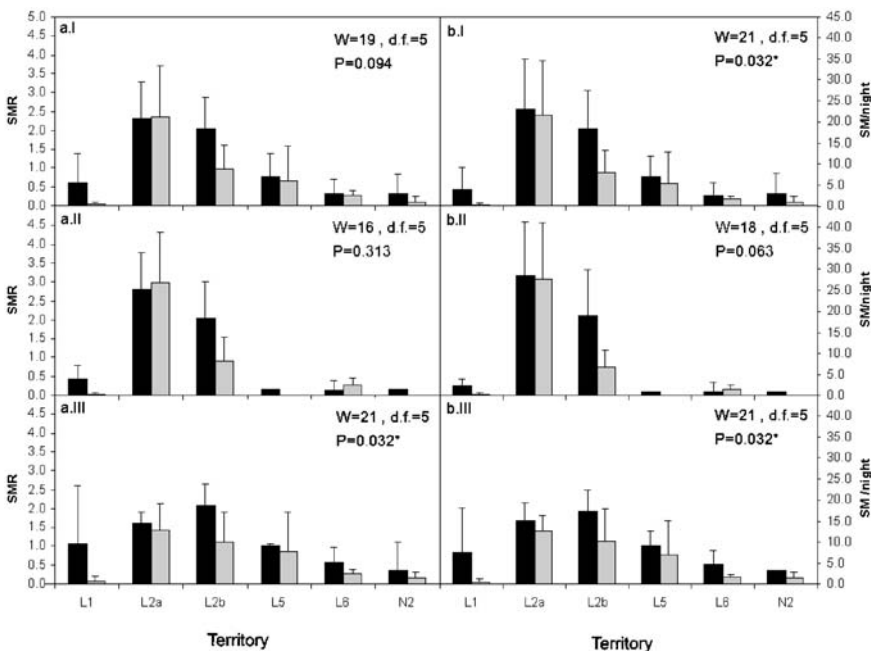


Fig. 1 Mean (SD) scent marking rates (SMR) (a) and scent marks per night (SM/night) (b) by the dominant male (black bars) and female (gray bars) Eurasian beaver. Results of Wilcoxon signed ranks test shown. (I) Overall (March 7–August 8); (II) spring (March 7–June 1); and (III) summer (June 1–August 8). Significant results ($P < 0.05$) are marked with an asterisk

Table 4 Mean (\pm SD) results of minutes per night spent at borders and time spent at borders in percentage of total activity time (*italics*) by male (M) and female (F) Eurasian beaver during spring, summer and overall

Season	Sex	Mean (\pm SD)	Paired <i>t</i> -test	<i>df</i>	<i>P</i>
Spring ^a	M	45.86 (51.27) <i>7.82 (7.66)</i>	5.11 -5.08	4	0.007* 0.007*
	F	24.14 (36.44) <i>5.34 (6.46)</i>			
Summer ^b	M	36.06 (45.88) <i>5.96 (7.30)</i>	3.05 -2.13	5	0.028* 0.086
	F	19.58 (22.68) <i>3.43 (3.91)</i>			
Overall	M	38.74 (46.98) <i>6.53 (7.24)</i>	6.70 -4.11	5	0.001* 0.009*
	F	23.04 (30.34) <i>4.18 (5.21)</i>			

^a March 7–June 1 (before parturition).

^b June 1–August 8 (after parturition).

Significant results ($P < 0.05$) are marked with an asterisk.

All eight behavioral contexts of scent marking (Table 5), both before and after, were correlated between males and females (before: $r_s = 0.82$, $N = 8$, $P = 0.012$; after: $r_s = 0.86$, $N = 8$, $P = 0.006$). There was a correlation between behavior before and after scent marking in both sexes (males: $r_s = 0.72$, $N = 8$, $P = 0.045$; females: $r_s = 0.90$, $N = 8$, $P = 0.002$). The most common context of scent marking in both sexes, before and after scent marking, was swimming (32.8–37.9%). The second most common context was scent marking (30.0–34.3%), i.e., about one third of scent markings would be succeeded by renewed scent marking (multiple marking). All but 1 of 122 (0.82%) and 1 of 91 (1.01%) multiple markings made by males and females, respectively, occurred at borders. Foraging came third (14.8–21.2%). All other events contributed with less than 6%.

Table 5 Behavioral context of scent marking by Eurasian beavers

Behavior ^a	Males	Males	Females	Females
	Prior	After	Prior	After
Swim	33.0	37.9	32.8	37.2
SM	30.0	30.0	34.3	32.9
Forage	21.2	15.3	18.9	14.8
Agonistic	4.7	3.9	1.9	1.8
Amicable	3.0	3.0	3.4	2.2
On bank	3.0	3.0	4.9	5.8
Groom	2.7	2.5	1.9	1.1
Other	2.5	4.4	1.9	4.3
Number of events	406	406	265	277

^a See Table 1 for description of behaviors.

Percentage distribution of behaviors prior to and after scent marking events and number of events recorded in each category, data from each sex pooled. SM: scent marking.

SMR was not correlated with any of the weather variables nor with date in either sex (Spearman correlation with Bonferroni method for multiple comparisons: r_s , all $P > 0.05$). This was also true for each season separately.

Discussion

Territorial Function

The “scent-matching” hypothesis has received support from studies of scent marking in both the North American beaver (Sun and Müller-Schwarze, 1998) and Eurasian beaver (Rosell, 2002; Rosell and Bjørkøyli, 2002; Rosell and Steifetten, 2004). By scent matching, competitors or mates match the odor from scent marks with the odor of conspecifics they encounter (Gosling, 1982; Gosling and McKay, 1990). Thus, it is crucial that both sexes maintain their scent marks in such a way that maximizes the success of matching (see Gosling, 1986; Roberts and Lowen, 1997; Gosling and Roberts, 2001). The high proportion of scent marks deposited at borders by both sexes confirms that scent marking serves a major function in territorial defense in Eurasian beavers (e.g., Nolet and Rosell, 1994; Rosell, 2002). Also, all (except one) multiple makings occurred at borders. This is in accordance with our first hypothesis and prediction and in agreement with earlier studies on the Eurasian beaver (Rosell et al., 1998; Rosell, 2002). However, in these studies, no information about sex was provided. Both sexes of beavers frequently replenish their own scent marks on a regular basis (this study) and remove or replace marks of competitors within their territory (e.g., Rosell, 2002), further supporting the hypothesis.

A high degree of individual variation in scent marking was found in this study, varying more between pairs than within. Moreover, scent marking behavior was correlated between mated pairs. Thus, apparently the sex of the beaver has less influence on scent marking than scent marking by their mate. This suggests that marking rates in beavers are regulated by the individual beaver in response to outside stimuli, and that both members of a pair respond in a similar way to these stimuli. Number of scent marks varies spatially and temporally in populations of most species of mammals and can be correlated with food availability, breeding activity, levels of dominance, and individual density (Gosling, 1990). It is possible that differences in these factors between the territories in the present study may explain the differences in scent marking. Furthermore, the two territories with the highest degrees of marking (L2a and L2b) were assumed to have an ongoing dispute over position of territory borders, possibly due to a split into two separate territories.

Sexual Dimorphism

Our second hypothesis that a sexual dimorphism exists as a result of reproductive constraints on females was only partly supported. The low sample size may have resulted in low statistical power and an inability to detect any further differences. Also, a wide application of our findings to other habitats should be carried out with some caution. Nevertheless, the data provide no convincing evidence of any biological effects that were undetected by statistical analysis (i.e., means, median, and percentages for males and females were similar).

Overall (spring and summer), SM/night was significantly higher in males than in females. Males were also found to have a higher SMR and SM/nights than females during summer, but not during spring. This is in accordance with our prediction. Scent marking involves investment both in terms of time and energy (Gosling, 1986). For instance, scent marking at higher intensity has been shown to reduce growth rate and body size in mice (Gosling et al., 2000). Patrolling the territory in connection with scent marking is time-consuming and could be physically costly for beavers. It seems likely that males invest more time in territorial defense than females during the breeding period, because males do not need extra energy to accommodate a developing a fetus and cannot initially provide direct care to newly born offspring. Kits are born during summer and are reliant on milk from the female (all females were assumed to give birth in our study). Thus, females are more constrained by parental care than males, and have less time and energy to spend on territorial activities. Lactation and infant care impose heavy burdens and constraints on female mammals (e.g., Campbell and Reece, 2002).

This coincides with our observation that males deposited a significantly larger number of scent marks and spent more time at borders. This finding is also in accordance with that of Sharpe and Rosell (2003), who—in a study concordant with ours—showed that males spend more time traveling, and with Rosell and Schulte (2004), who suggested that a difference in territorial behavior between male and female has led to differential investment in scent structures. They suggested that females have larger castor sacs (low flushing rates) and smaller anal glands (infrequent use) because of reduced employment of both glands for territorial purposes, whereas males have the opposite configuration because of enhanced use of both glands in territorial demarcation. Hence, behavioral dimorphism might select for morphological dimorphism in signal producing structures, even in an obligate monogamous species.

Codefense

Our results coincide with the general picture of obligate monogamous mammals where both sexes participate in the defense of a joint territory (Clutton-Brock, 1989). Most mammals are intrasexually territorial, but several findings suggest that beavers are both intra- and intersexually territorial. Adult beavers of both sexes countermark where other conspecifics (of both sexes) have marked (Rosell, 2002; Rosell and Steifetten, 2004). A display (“stick display”) used in territorial context is used both intra- and intersexually (Thomsen et al., unpublished data). Beavers are able to recognize the concept of “relatedness” by a pheromone in the anal gland secretion. This would deter extrapair copulations (EPCs) by females, since the mate would be able to detect young unrelated to him and could treat them unfavorably or potentially kill them, and consequently favor monogamy (Sun, 2003). Furthermore, it would make mate change during the breeding season unlikely because the kits would be exposed to risk of infanticide if a stranger of either sex enters the territory, although definite proof of infanticide in beavers is lacking (however, see Haines, 1955). Since beavers evolved in northern latitudes and mating occur in late winter, when mobility is restricted by environmental conditions, male roving costs would also be high (Nolet and Rosell, 1994) and therefore the potential for EPCs is low.

For a territorial species like the beaver, there are benefits in codefending the territory (e.g., Mathews, 2002). No other socially monogamous rodent shows the

same degree of construction activities or foraging and food storage behavior as the beaver (Baker and Hill, 2003). The benefits of defending a large territory rather than only the immediate vicinity of the lodge likely arise from the long-term payoff of reducing the rate of resource depletion within the site and thus increasing the long-term viability of the territory (Campbell et al., 2005). These authors argue that the settlement pattern and reproductive history have a lasting impact on the territorial system of beavers due to a combination of low adult mortality, high dispersal costs, and avoidance of resource depletion. All these factors lower the individual energy investment in defense and reduce the risk of being evicted from the territory. Male beavers exhibit a relatively high degree of both direct and indirect parental care (Wilsson, 1971; Rosell, 2002)—for instance, males will bring food to offspring. Hence, defending a territory could be seen as an effort to secure a breeding space with adequate food resources to support a pregnant female and later offspring, ultimately increasing the males' reproductive success (Clutton-Brock, 1989). Likewise, territory defense by females is likely to be resource defense (Clutton-Brock, 1989).

Thus, cooperation between male and female should raise the odds of maintaining control over a large resource-rich territory in which their young can be successfully raised. Male parental care, combined with the benefits of defending a larger territory, could have led to the evolution of codefense and consequently a high degree of monomorphism in scent marking. Further studies comparing allocation by either sex to territorial behavior (e.g., scent marking) outside the breeding season could confirm the hypothesis that females contribute less to the defense during certain times of the year due to reproductive constraints.

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The Frequency of Occurrence of Acyclic Monoterpene Alcohols in the Chemical Environment does not Determine Olfactory Sensitivity in Nonhuman Primates

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Abstract Using a conditioning paradigm, the olfactory sensitivity of five spider monkeys, three squirrel monkeys, and three pigtail macaques for six acyclic monoterpene alcohols that differ markedly in their frequency of occurrence in plant odors was assessed. The results showed that: (1) all three primate species have a well-developed olfactory sensitivity for acyclic monoterpene alcohols; (2) squirrel monkeys are significantly more sensitive for members of this class of odorants than the other two species and are able to detect all six odorants at concentrations below 0.1 ppm; and (3) there is a lack of positive correlations between olfactory sensitivity and the abundance of the acyclic monoterpene alcohols in flower odors and etheric oils. The results lend support to the growing body of evidence that suggests between-species comparisons of the number of functional olfactory receptor genes or of neuroanatomical features are poor predictors of olfactory performance. The findings do not support the hypothesis that olfactory sensitivity for members of a chemical class may be related to the frequency of occurrence of such odorants in a species' chemical environment.

Keywords Olfactory sensitivity · Detection thresholds · Acyclic monoterpene alcohols · Nonhuman primates · Chemical environment · Squirrel monkey · Spider monkey · Pigtail macaque

Introduction

Plasticity is one of the hallmarks of the olfactory system of mammals, and can be considered as an evolutionary adaptation to the ever-changing composition of an

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animal's chemical environment (Hudson, 1999). Several studies both in human subjects (Möller et al., 1999; Dalton et al., 2002) and in animal models (Wang et al., 1993; Voznessenskaya et al., 1994; Yee and Wysocki, 2001), for example, have demonstrated that repeated exposure to a particular odorant selectively increases an organism's sensitivity to that odorant. This raises the possibility that differences in the frequency of occurrence of odorants in the chemical environment of a species might at least in part account for the observed differences in olfactory sensitivity both within and between species. This hypothesis is supported by reports that show that spider monkeys, squirrel monkeys, and pigtail macaques are markedly more sensitive to acetic esters (Laska and Seibt, 2002a; Hernandez Salazar et al., 2003) and aliphatic aldehydes (Laska et al., 2003a,b, 2006) than to ketones (Laska et al., 2005c) and carboxylic acids (Laska et al., 2000, 2004). Whereas the former two classes of odorants are found in a large variety of fruit odors, the latter two classes are only rarely found as components of potential primate food odors (Maarse, 1991). Unfortunately, there is only limited knowledge as to differences in the frequency of occurrence of individual members of the aforementioned substance classes in the chemical environment of the three primate species.

Terpenes comprise by far the largest class of volatile chemicals in plant odors, and at least in human subjects, they often determine the characteristic odor quality of flowers, fruits, and etheric oils emanating from the vegetative parts of plants (Burdock, 2001). Gas chromatographic analyses of a large number of flower odors (Knudsen et al., 1993) and etheric oils (Kubeczka, 2002) have demonstrated that certain members of this class of volatiles differ markedly in their frequency of occurrence in plant odors, despite their similarity in chemical structure.

It was the aim of the present study to test the hypothesis that olfactory sensitivity for members of a chemical class may be related to the frequency of occurrence of such odorants in a species' chemical environment. To this end, we tested three species of nonhuman primates for their ability to detect six acyclic monoterpene alcohols that differ markedly in their abundance in flower odors and etheric oils. All three primate species include a considerable proportion of plant material in their diets (Caldecott, 1986; Cant, 1990; Lima and Ferrari, 2003). Employing odorants that share a number of molecular features, such as sum formula and oxygen-containing functional group, and differ from each other in others, such as stereoisomery, position of a functional group, or presence/absence of a double bond, allowed us to additionally address the question of the impact of these features on detectability of odorants.

Methods and Materials

Animals

Testing was carried out by using five adult female spider monkeys (*Ateles geoffroyi*), three adult male squirrel monkeys (*Saimiri sciureus*), and two adult males and one adult female pigtail macaques (*Macaca nemestrina*). All animals had served as subjects in previous olfactory experiments and were familiar with the basic test procedure. The animals of a given species were not genetically closely related to each other. Conditions of the animals' maintenance have been described in detail elsewhere (Laska and Seibt, 2002a; Hernandez Salazar et al., 2003).

Behavioral Tests

The experimental procedures for assessing olfactory detection thresholds in the three primate species have been described in detail elsewhere (Laska and Hudson, 1993; Hübener and Laska, 2001; Laska et al., 2003a,b). Briefly, the animals were tested by using a food-rewarded instrumental conditioning paradigm. Olfactory detection thresholds were determined by testing the animals' ability to discriminate between increasing dilutions of an odorant used as the rewarded stimulus (S+) and the odorless solvent diethyl phthalate alone used as the unrewarded stimulus (S-). In each test trial, each monkey sniffed at both options, and then decided for one of the alternatives by performing an operant response that, in the case of a correct decision, was food-rewarded. Ten such trials were conducted per animal and session, and at least three sessions per experimental condition were performed.

Odorants

A set of six odorants belonging to the class of acyclic monoterpene alcohols was used (Table 1). The rationale for choosing these substances was to assess the monkeys' sensitivity for odorants that have been shown to differ markedly in their frequency of occurrence in flower odors (Knudsen et al., 1993) and etheric oils (Kubeczka, 2002), thus allowing us to assess whether olfactory sensitivity correlates with abundance of these odorants in the chemical environment of the three species tested here. At the same time, the six odorants are structurally similar to each other, i.e., they share certain molecular properties, such as sum formula and type of functional group, and differ in other properties, such as stereoisomery, position of a functional group, or presence/absence of a double bond, thus allowing us to assess the impact of these structural features on detectability (Fig. 1). Acyclic monoterpene alcohols act as attractants for pollinators (Pichersky and Gershenzon, 2002; Raguso, 2004). All chemicals were obtained from Fluka (Taufkirchen, Germany) and had a nominal purity of at least 99%. They were diluted by using odorless diethyl phthalate as the solvent.

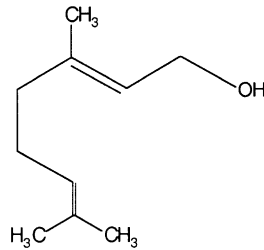
Table 1 Odorants used and their percentage of occurrence in flower odors and etheric oils

Veridical name	Chemical name	Percentage of flower odors ^a	Occurrence in etheric oils ^b
Linalool	3,7-Dimethyl- <i>trans</i> -1,6-octadien-3-ol	15.0	85.4
Geraniol	3,7-Dimethyl- <i>trans</i> -2,6-octadien-1-ol	4.8	48.8
Nerol	3,7-Dimethyl- <i>cis</i> -2,6-octadien-1-ol	3.9	31.7
Citronellol	3,7-Dimethyl-6-octen-1-ol	2.0	17.1
Lavandulol	2-Isopropenyl-5-methyl-4-hexen-1-ol	0.2	7.3
Myrcenol	2-Methyl-6-methylene-7-octen-2-ol	0.0	0.0

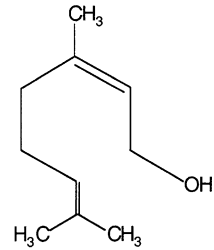
^aKnudsen et al. (1993): data from 441 taxa in 174 genera in 60 families of plants.

^bKubeczka (2002): data from 142 taxa in 98 genera in 47 families of plants.

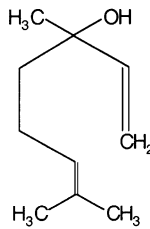
Fig. 1 Chemical structure of the six acyclic monoterpene alcohols used as odor stimuli



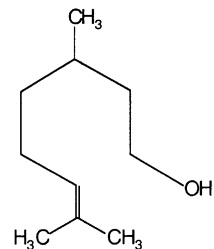
geraniol



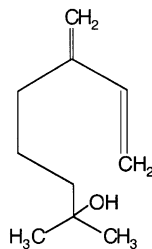
nerol



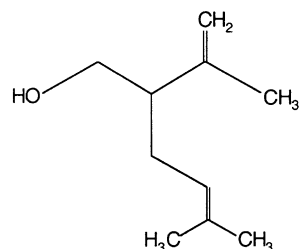
linalool



citronellol



myrcenol



lavandulol

Data Analysis

In the method described here, the animal had two options: (1) to correctly open the container that carries the positive stimulus (hit) and (2) to open the container that carries the negative stimulus (false alarm). For each individual animal, the percentage of hits from the best three consecutive sessions per dilution step, comprising a total of 30 decisions, was calculated and taken as the measure of performance. Significance levels were determined by calculating binomial z -scores corrected for continuity from the number of correct and false responses for each individual and condition.

Correlations between olfactory threshold values of a given species (individual scores per stimulus were used), and the frequency of occurrence of the odor stimuli in plant material was calculated by using the Spearman rank-correlation test. Across-species comparisons of performance were conducted by using Mann–Whitney U tests for independent samples. All tests were two-tailed, and, if not otherwise mentioned, the alpha level was set at 0.05.

Results

Spider Monkeys

Figure 2 shows the performance of the spider monkeys in discriminating between various dilutions of a given acyclic monoterpene alcohol and the odorless solvent. All five animals distinguished dilutions as low as 1:10,000 geraniol, 1:3000 nerol, 1:1000 linalool, 1:30,000 citronellol, 1:30,000 myrcenol, and 1:100,000 lavandulol from the solvent (binomial test, $P < 0.05$), with single individuals even scoring better.

The individual spider monkeys demonstrated similar threshold values, and with three of the six odorants (nerol, myrcenol, and lavandulol), they differed only by a dilution factor of 3 between the highest- and the lowest-scoring animal. In the case of citronellol, they even showed identical threshold values.

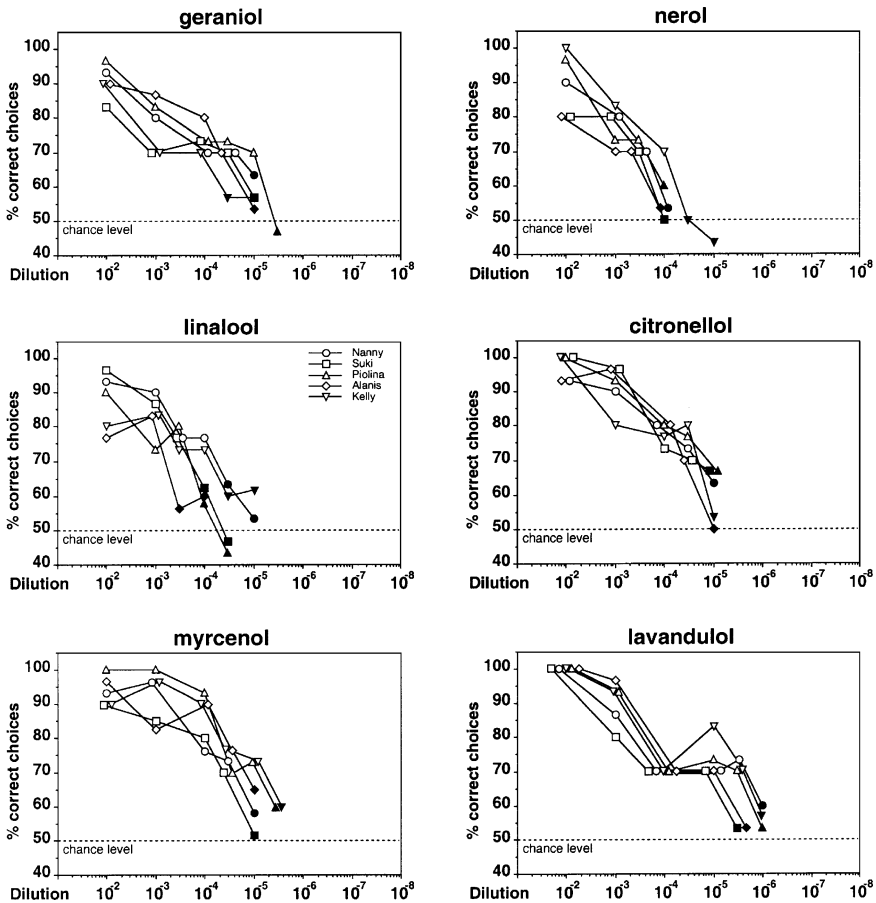


Fig. 2 Performance of five spider monkeys in discriminating between various dilutions of a given acyclic monoterpene alcohol and the odorless solvent. Each data point represents the percentage of correct choices from 30 decisions. The three different symbols represent data from each of the five individual animals tested. Filled symbols indicate dilutions that were not discriminated significantly above chance level (binomial test, $P > 0.05$)

sensitivity for a given odorant between individuals comprised a dilution factor of 10, and was found with geraniol and linalool.

Table 2 summarizes the threshold dilutions for the five spider monkeys and shows various measures of corresponding vapor phase concentrations (Weast, 1987), to easily compare the data obtained in the present study to those reported by other authors that used one of these convertible measures. With only one exception, threshold dilutions correspond to vapor phase concentrations below 1 ppm.

A significant negative correlation between the olfactory sensitivity of the spider monkeys for the six acyclic monoterpene alcohols tested and their frequency of occurrence in plant odors was found (Spearman, $r_s = -0.62$, $P < 0.001$). This means that the animals were more sensitive to monoterpene alcohols that presumably occur less frequently in their chemical environment compared to monoterpene alcohols that occur more frequently.

Squirrel Monkeys

Figure 3 shows the performance of the squirrel monkeys in discriminating between various dilutions of a given acyclic monoterpene alcohol and the odorless solvent. All three animals distinguished dilutions as low as 1:30,000 geraniol, 1:300,000 nerol, 1:30,000 linalool, 1:3 million citronellol, 1:300,000 myrcenol, and 1:10 million lavandulol from the solvent (binomial test, $P < 0.05$), with single individuals scoring even better.

Individual squirrel monkeys generally demonstrated similar threshold values, and with three of the six odorants, they differed only by a dilution factor of 3 (lavandulol), 10 (citronellol), or 33 (myrcenol) between the highest- and the lowest-scoring animal. The largest difference in sensitivity for a given odorant between individuals comprised a dilution factor of 1000 and was found with geraniol.

Table 3 summarizes the threshold dilutions for the three squirrel monkeys and shows various measures of corresponding vapor phase concentrations (Weast, 1987). In all cases, threshold dilutions correspond to vapor phase concentrations below 0.1

Table 2 Olfactory detection threshold values in spider monkeys expressed in various measures of vapor phase concentrations

	<i>N</i>	Dilution	Molecules/cm ³	ppm	log ppm	Mol/l	log Mol/l
Geraniol	1	1:10,000	1.1×10^{12}	0.041	-1.39	1.8×10^{-9}	-8.74
	3	1:30,000	3.7×10^{11}	0.014	-1.86	6.1×10^{-10}	-9.21
	1	1:100,000	1.1×10^{11}	0.0041	-2.39	1.8×10^{-10}	-9.74
Nerol	4	1:3000	4.0×10^{12}	0.15	-0.83	6.6×10^{-9}	-8.18
	1	1:10,000	1.2×10^{12}	0.044	-1.35	2.0×10^{-9}	-8.70
Linalool	1	1:1000	3.9×10^{13}	1.44	0.16	6.5×10^{-8}	-7.19
	2	1:3000	1.2×10^{13}	0.44	-0.35	2.0×10^{-8}	-7.70
	2	1:10,000	3.9×10^{12}	0.14	-0.84	6.5×10^{-9}	-8.19
Citronellol	5	1:30,000	5.0×10^{11}	0.019	-1.73	8.3×10^{-10}	-9.08
Myrcenol	3	1:30,000	4.3×10^{11}	0.016	-1.80	7.1×10^{-10}	-9.15
	2	1:100,000	1.3×10^{11}	0.0048	-2.32	2.2×10^{-10}	-9.67
Lavandulol	2	1:100,000	3.1×10^{11}	0.012	-1.94	5.1×10^{-10}	-9.29
	3	1:300,000	1.0×10^{11}	0.0037	-2.43	1.7×10^{-10}	-9.78

N indicates the number of animals.

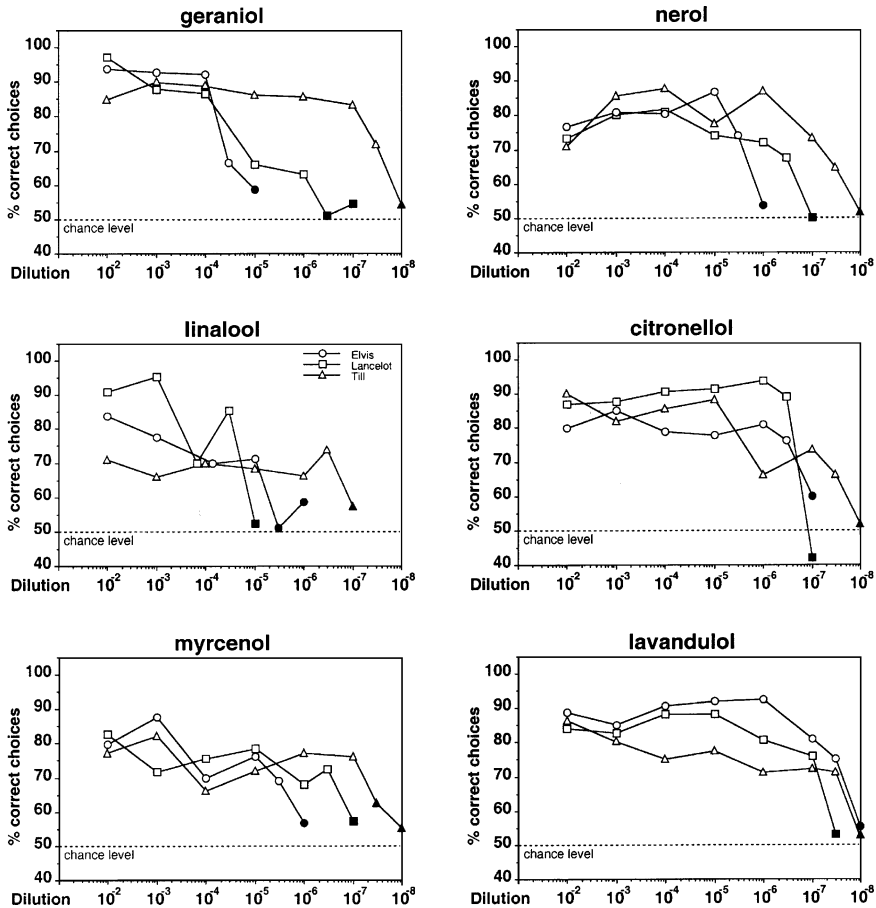


Fig. 3 Performance of three squirrel monkeys in discriminating between various dilutions of a given acyclic monoterpene alcohol and the odorless solvent. Each data point represents the percentage of correct choices from at least 30 decisions. The three different symbols represent data from each of the three individual animals tested. Filled symbols indicate dilutions that were not discriminated significantly above chance level (binomial test, $P > 0.05$)

ppm, and with citronellol and lavandulol, the animals were even able to detect concentrations below 1 ppb.

A negative correlation between the olfactory sensitivity of the squirrel monkeys for the six acyclic monoterpene alcohols tested and their frequency of occurrence in plant odors was found that fell short of statistical significance (Spearman, $r_s = -0.40$, $P = 0.09$).

Pigtail Macaques

Figure 4 shows the performance of the pigtail macaques in discriminating between various dilutions of a given acyclic monoterpene alcohol and the odorless solvent. All three animals distinguished dilutions as low as 1:100,000 geraniol, 1:30,000 nerol, 1:10,000 linalool, 1:10,000 citronellol, 1:1000 myrcenol, and 1:1000 lavandulol from the solvent (binomial test, $P < 0.05$), with single individuals scoring even better.

Table 3 Olfactory detection threshold values in squirrel monkeys expressed in various measures of vapor phase concentrations

	<i>N</i>	Dilution	Molecules/cm ³	ppm	log ppm	Mol/l	log Mol/l
Geraniol	1	1:30,000	3.7×10^{11}	0.014	-1.86	6.1×10^{-10}	-9.21
	1	1:1 million	1.1×10^{10}	0.00041	-3.39	1.8×10^{-11}	-10.74
	1	1:30 million	3.7×10^8	0.000014	-4.86	6.1×10^{-13}	-12.21
Nerol	1	1:300,000	4.0×10^{10}	0.0015	-2.83	6.6×10^{-11}	-10.18
	1	1:3 million	4.0×10^{19}	0.00015	-3.83	6.6×10^{-12}	-11.18
	1	1:30 million	4.0×10^8	0.000015	-4.83	6.6×10^{-13}	-12.18
Linalool	1	1:30,000	1.3×10^{12}	0.048	-1.32	2.2×10^{-9}	-8.67
	1	1:100,000	3.9×10^{11}	0.014	-1.84	6.5×10^{-10}	-9.19
	1	1:3 million	1.3×10^{10}	0.00048	-3.32	2.2×10^{-11}	-10.67
Citronellol	2	1:3 million	5.0×10^9	0.00019	-3.73	8.3×10^{-12}	-11.08
	1	1:30 million	5.0×10^8	0.000019	-4.73	8.3×10^{-13}	-12.08
Myrcenol	1	1:300,000	4.3×10^{10}	0.0016	-2.80	7.1×10^{-11}	-10.15
	1	1:3 million	4.3×10^9	0.00016	-3.80	7.1×10^{-12}	-11.15
	1	1:10 million	1.3×10^9	0.000048	-4.32	2.2×10^{-12}	-11.67
Lavandulol	1	1:10 million	3.1×10^9	0.00012	-3.94	5.1×10^{-12}	-11.29
	2	1:30 million	1.0×10^9	0.000037	-4.43	1.7×10^{-12}	-11.78

N indicates the number of animals.

The individual pigtail macaques generally demonstrated similar threshold values, and with three of the six odorants, they differed only by a dilution factor of 10 (myrcenol and lavandulol) or 30 (linalool) between the highest- and the lowest-scoring animal. The largest difference in sensitivity for a given odorant between individuals comprised a dilution factor of 300 and was found with citronellol.

Table 4 summarizes the threshold dilutions for the three pigtail macaques and shows various measures of corresponding vapor phase concentrations (Weast, 1987). With only one exception, threshold dilutions correspond to vapor phase concentrations below 1 ppm.

A positive correlation between the olfactory sensitivity of the pigtail macaques for the six acyclic monoterpene alcohols tested and their frequency of occurrence in plant odors was found that fell short of statistical significance (Spearman, $r_s = +0.39$, $P = 0.10$).

Across-Species Comparison of Performance

Considering all six odorants combined, the squirrel monkeys performed significantly better, i.e., showed lower detection thresholds than both the spider monkeys and the pigtail macaques (Mann–Whitney, $P < 0.001$). Furthermore, with all six odorants, the squirrel monkeys displayed the lowest individual detection threshold among the three primate species. No significant difference was found between the averaged performance of the spider monkeys and the pigtail macaques with the six acyclic monoterpene alcohols (Mann–Whitney, $P > 0.05$).

Discussion

The results of this study demonstrate, for the first time, that spider monkeys, squirrel monkeys, and pigtail macaques have a well-developed sensitivity for monomo-

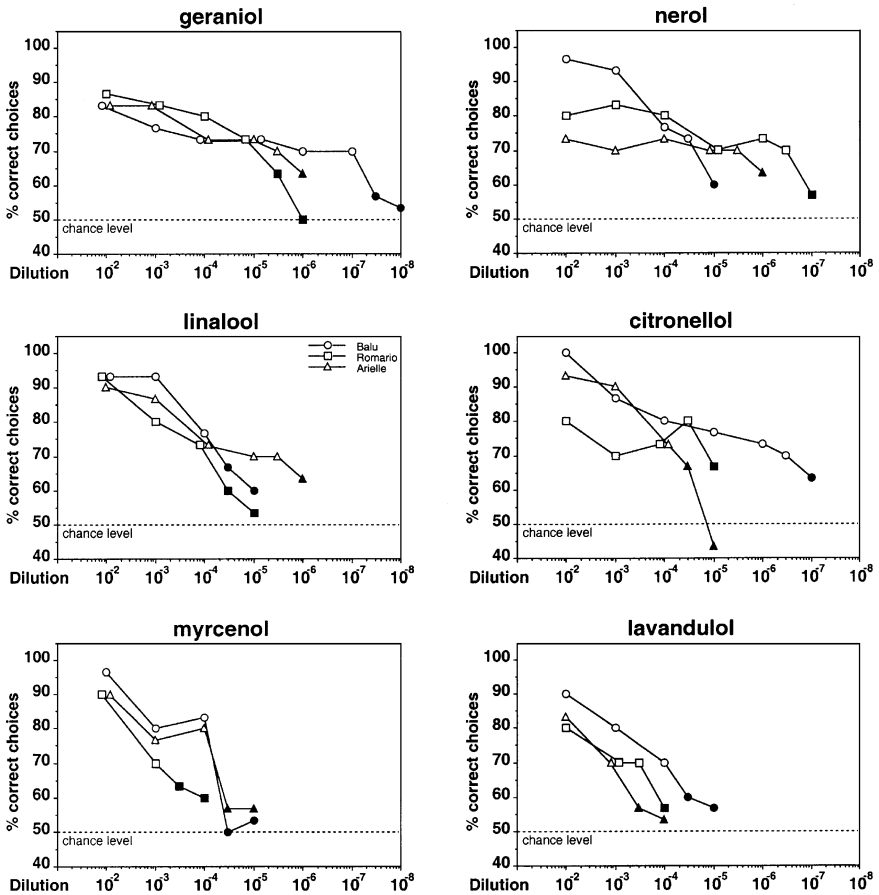


Fig. 4 Performance of three pigtail macaques in discriminating between various dilutions of a given acyclic monoterpene alcohol and the odorless solvent. Each data point represents the percentage of correct choices from 30 decisions. The three different symbols represent data from each of the three individual animals tested. Filled symbols indicate dilutions that were not discriminated significantly above chance level (binomial test, $P > 0.05$)

lecular odorants belonging to the class of acyclic monoterpene alcohols. These findings are in line with earlier studies that used the same methods and animals that showed that these species have a well-developed olfactory sensitivity for carboxylic acids (Laska et al., 2000, 2004), acetic esters (Laska and Seibt, 2002a; Hernandez Salazar et al., 2003), aliphatic alcohols (Laska and Seibt, 2002b; Laska et al., 2006), aliphatic aldehydes (Laska et al., 2003a,b, 2006), and ketones (Laska et al., 2005c), and that squirrel monkeys have excellent olfactory discrimination capabilities (Laska and Freyer, 1997; Laska and Hudson, 1995; Laska and Teubner, 1998; Laska et al., 1999a,b, 2005b). Thus, the present results lend further support to the idea that olfaction may play a significant role in the regulation of behavior in these primate species.

Although only between three and five animals per species were tested, the results appear robust, as interindividual variability was remarkably low and generally smaller than the range reported in studies on human olfactory sensitivity, i.e., within three

Table 4 Olfactory detection threshold values in pigtail macaques expressed in various measures of vapor phase concentrations

	<i>N</i>	Dilution	Molecules/cm ³	ppm	log ppm	Mol/l	log Mol/l
Geraniol	1	1:100,000	1.1×10^{11}	0.0041	-2.39	1.8×10^{-10}	-9.74
	1	1:300,000	3.7×10^{10}	0.0014	-2.86	6.1×10^{-11}	-10.21
	1	1:10 million	1.1×10^9	0.000041	-4.39	1.8×10^{-12}	-11.74
Nerol	1	1:30,000	4.0×10^{11}	0.015	-1.83	6.6×10^{-10}	-9.18
	1	1:300,000	4.0×10^{10}	0.0015	-2.83	6.6×10^{-11}	-10.18
	1	1:3 million	4.0×10^{19}	0.00015	-3.83	6.6×10^{-10}	-11.18
Linalool	2	1:10,000	3.9×10^{12}	0.14	-0.84	6.5×10^{-9}	-8.19
	1	1:300,000	1.3×10^{11}	0.0048	-2.32	2.2×10^{-10}	-9.67
Citronellol	1	1:10,000	1.5×10^{12}	0.056	-1.26	2.5×10^{-9}	-8.60
	1	1:30,000	5.0×10^{11}	0.019	-1.73	8.3×10^{-10}	-9.08
	1	1:3 million	5.0×10^9	0.00019	-3.73	8.3×10^{-12}	-11.08
Myrcenol	1	1:1000	1.3×10^{13}	0.48	-0.32	2.2×10^{-8}	-7.67
	2	1:10,000	1.3×10^{12}	0.048	-1.32	2.2×10^{-9}	-8.67
Lavandulol	1	1:1000	3.1×10^{13}	1.15	0.06	5.1×10^{-8}	-7.29
	1	1:3000	1.0×10^{13}	0.37	-0.43	1.7×10^{-8}	-7.78
	1	1:10,000	3.1×10^{12}	0.12	-0.94	5.1×10^{-9}	-8.29

N indicates the number of animals.

orders of magnitude (Stevens et al., 1988). In fact, for the majority of substances tested, there was only a factor of 3 or 10 between the threshold values of the highest- and the lowest-scoring animal of a species. Furthermore, with all substances tested, the animals' performance with the lowest concentrations presented dropped to chance level, suggesting that the statistically significant discrimination between higher concentrations of an odorant and the odorless diluent was indeed based on olfactory perception and not on other cues.

Figure 5 compares the olfactory detection threshold values obtained with the spider monkeys, squirrel monkeys, and pigtail macaques for the substances tested to those from human subjects. Although such across-species comparisons should be considered with caution, as different methods may lead to widely differing results (Hastings, 2003), it seems admissible to state that human subjects do not generally perform poorer than the nonhuman primates tested—despite the fact that the relative size of the human olfactory brain structures devoted to processing olfactory information is markedly smaller than that of the squirrel monkey, spider monkey, or the pigtail macaque (Stephan et al., 1988), and despite the fact that the number of functional olfactory receptor (*OR*) genes in *Homo sapiens* (≈ 350) is considerably smaller than that of *M. nemestrina* (≈ 700) and of *S. sciureus* and *A. Geoffroyi* (≈ 1000) (Rouquier et al., 2000; Glusman et al., 2001; Gilad et al., 2004). It should be mentioned that the threshold values of the human subjects for the acyclic monoterpene alcohols as depicted in Fig. 5 represent mean values (van Gemert, 2003), whereas the data points of the nonhuman primates represent individual threshold values found in the present study. Nevertheless, with four of the six substances tested, the human subjects demonstrated mean threshold values that were lower than the lowest individual threshold values of the spider monkeys.

Similarly, pigtail macaques generally did not perform poorer than spider monkeys, again despite the fact that the relative size of the olfactory bulbs and the number of functional *OR* genes in Old World primates are smaller than in New

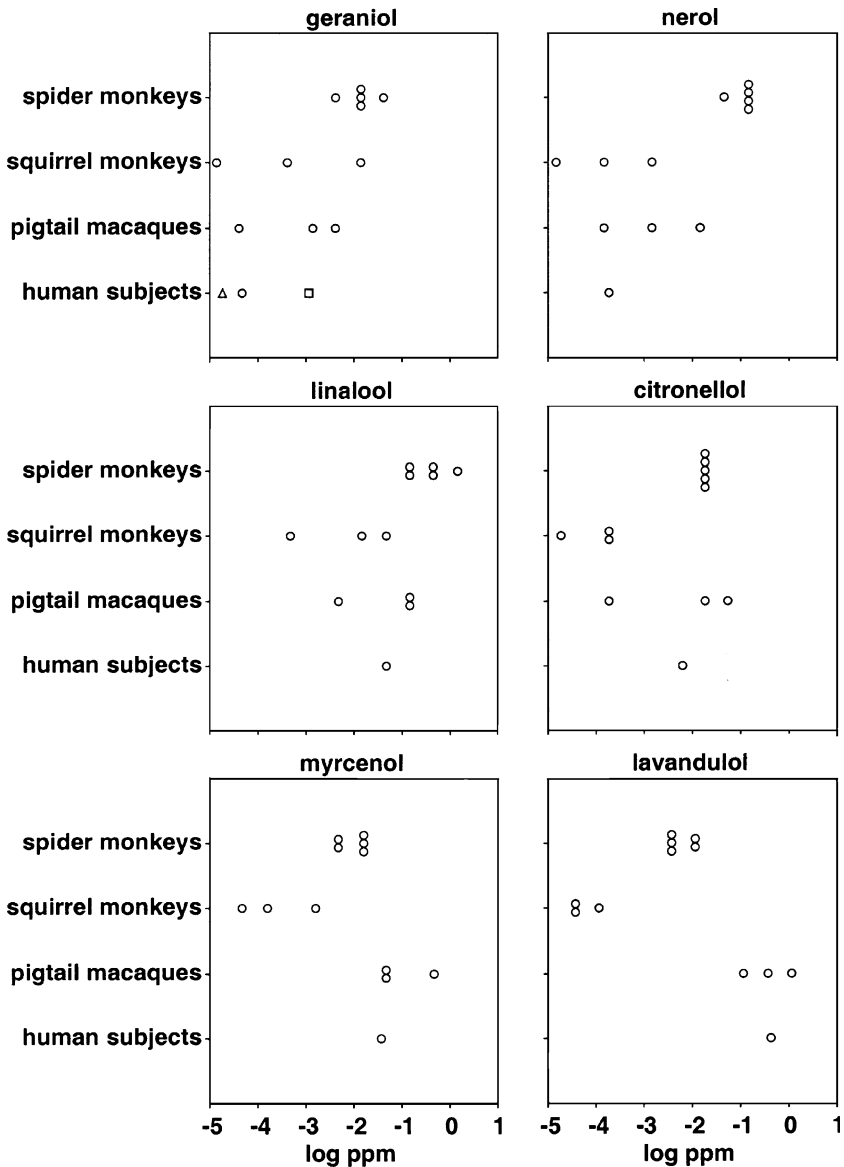


Fig. 5 Comparison of the olfactory detection threshold values (expressed as vapor phase concentrations) of the squirrel monkeys, the spider monkeys, and the pigtail macaques for the acyclic monoterpene alcohols tested to those of human subjects (van Gemert, 2003). Data points of the three monkey species represent individual threshold values, and data points of the human subjects represent mean values. Data points of the mouse (empty triangle; Schmidt, 1982) and the pig (empty square; Dorries et al., 1995) for geraniol are depicted in the same line as the human data and represent the lowest individual threshold values per species

World primates (Rouquier et al., 2000). These findings are in line with earlier studies that showed human subjects not to generally perform poorer in detecting aliphatic alcohols (Laska and Seibt 2002b) and carboxylic acids (Laska et al., 2000,

2004) compared to nonhuman primates, and Old World primates not to generally have a poorer sensitivity for aliphatic alcohols (Laska and Seibt, 2002b) and aldehydes (Laska et al., 2003a,b) than New World primates.

Thus, the present findings lend additional support to the notion that, at least within the order of primates, allometric comparisons of olfactory brain structures or differences in the number of functional *OR* genes do not allow us to draw generalizable conclusions as to olfactory sensitivity of any two species.

Unfortunately, only few data on olfactory sensitivity for acyclic monoterpene alcohols in nonprimate mammals are available. Figure 5 shows that squirrel monkeys, pigtail macaques, and human subjects are at least as sensitive for geraniol as the pig and the mouse, both species being regarded as having a particularly well-developed sense of smell. The findings are in line with earlier studies that reported all three primate species tested here to be at least as sensitive as, and with some substances even considerably more sensitive than, species such as the dog or the rat in detecting acetic esters (Laska and Seibt, 2002a; Hernandez Salazar et al., 2003) and aliphatic alcohols (Laska and Seibt, 2002b), although these nonprimate mammals possess relatively larger olfactory bulbs and do not show a reduction in their number of functional *OR* genes (Olender et al., 2004). This suggests that, here too, no reliable predictions as to species differences in olfactory performance between primate and nonprimate mammals are possible.

Our finding of a lack of significant positive correlations between olfactory sensitivity in spider monkeys, squirrel monkeys, and pigtail macaques and the frequency of occurrence of the acyclic monoterpene alcohols in flower odors and etheric oils is in contrast to our hypothesis, and the significant negative correlation found with the spider monkeys is difficult to understand. However, several possible explanations should be considered. First, we cannot exclude the possibility that the flower odors and etheric oils that have been analyzed for their contents of acyclic monoterpene alcohols (Knudsen et al., 1993; Kubezcka, 2002), although they include a large variety of plant species, may not be representative for the chemical environment of the primate species tested here. Thus, future studies should aim at assessing the specific composition of the volatile substances present in the habitat of the animal species under investigation.

Second, it might be that differences in ecological relevance rather than differences in frequency of occurrence of odorants may determine differences in olfactory sensitivity both within and between species. Pheromones, for example, have repeatedly been shown to be perceived (either by the main or the accessory olfactory system) at dramatically lower concentrations by species for which they serve a specific behavioral function compared to species for which this is not the case (Wyatt, 2003). Similarly, prey species have been shown to be much more sensitive to the odor of a predator compared to nonprey species (Laska et al., 2005a). Thus, future studies should aim at assessing the potential relevance of particular odorants in different behavioral contexts with a given species of animal.

Finally, it might be that the odorants tested here may be too similar to each other in their molecular structure to interact with sufficiently different subsets of olfactory receptor types to cause marked differences in sensitivity within a given species. This idea finds some support from a within-species comparison of performance with the different odorants. Geraniol and nerol, for example, represent stereoisomers; that is, they are identical in structure except for *cis-trans* isomery (see Fig. 1), and none of the three species tested here differed markedly in respective thresholds for these

two odorants. Similarly, spider monkeys, squirrel monkeys, and pigtail macaques showed similar detection thresholds for nerol and citronellol, suggesting that the presence or absence of a double bond (see Fig. 1) had little influence on detectability of these odorants. However, there were several cases in which the position of the functional alcohol group (see Fig. 1) clearly affected olfactory sensitivity (e.g., lavandulol and geraniol with the pigtail macaques, nerol and myrcenol with the spider monkeys, and lavandulol and linalool with the squirrel monkeys). Future studies should aim at assessing whether structural similarity of stimuli is restricted to the class of odorants tested here or whether this may represent a more general phenomenon.

In summary, the results of this study provide further evidence of a well-developed olfactory sensitivity in three species of nonhuman primates. The findings lend additional support to the suggestion that between-species comparisons of neuroanatomical features or of the number of functional *OR* genes are poor predictors of olfactory performance. Furthermore, the findings do not support the hypothesis that olfactory sensitivity for members of a chemical class may be related to the frequency of occurrence of such odorants in a species' chemical environment. We suggest that differences in the behavioral relevance of odorants and/or differences in the degree of structural similarity may account for both within- and between-species differences in olfactory sensitivity.

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Comparative Analysis of Volatile Constituents from Mice and their Urine

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Abstract We report the volatile composition of the body scent of male C57BL/6J mice in comparison to the volatile composition of their urine. From a total of 67 components, nitromethane, propanoic acid, dimethyldisulfide, 1-octene, 1-hexanol, hexanoic acid, indole, α - and β -farnesene, and one unidentified component were observed only in the volatiles from the body of mice. On the other hand, 3-penten-2-one, 3-methyl-2-buten-1-ol, 3-methyl-cyclopentanone, *p*-xylene, 3-hepten-2-one, 2,3-dehydro-exo-brevicommin, benzylmethylketone, and 13 unidentified components were only found in urine volatiles. All other substances were present in the volatiles of both mice and their urine. Aliphatic aldehydes from pentanal to decanal were prominent mouse odor components. Because receptors for these aldehydes have been extensively characterized in the main olfactory organ, these components may be important for mice in recognizing their conspecifics.

Keywords Aliphatic aldehydes · Odorant · Odorant receptor · Urine volatiles · Mouse body scent · Odor composition

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Introduction

Social signaling between mice involves volatile and nonvolatile components present in secretions from the skin, the reproductive tract, and in urine. Volatile cues recognized from a distance interact with the main olfactory system. In contrast, the vomeronasal system requires direct contact and sniffing for activation (Luo et al., 2003) and it can therefore process chemical cues independent of their volatility. A number of low molecular mass volatile compounds have been characterized that elicit specific behavioral responses. These compounds are perceived either through the main or the accessory olfactory organ (reviewed in Brennan and Keverne, 2004; Stowers and Marton, 2005). However, compared to the explosive increase in knowledge on the molecular and cell biology of the olfactory system following the cloning of the receptor genes (summarized by Julius and Katz, 2004), considerably less effort has been directed toward the analysis of the complex mixtures of volatile and nonvolatile chemicals that are used by mice for recognition of kin or territory, in reproduction, or for behavioral responses such as fear and aggression. This situation motivated us to compare the volatiles in the mouse body scent vs. urine-derived volatiles.

Body scent, which may be important for recognition of mice from a distance, has so far not been analyzed. In contrast, there are several reports of the composition of urine odor. Miyashita and Robinson (1980) identified 94 volatiles collected by a cryogenic trapping procedure from urine of male Swiss Webster mice. In the study of Schwende et al. (1986), volatiles were flushed from urine of male BALB/c mice by helium gas and collected on Tenax-filled tubes. Subsequent gas chromatography/mass spectroscopy (GC/MS) led to the characterization of 61 components, among them several substances considered to be mouse pheromones (reviewed by Novotny, 2003). Singer et al. (1997) and Willse et al. (2005) extracted urine pools collected from male C57BL/6 mice with ether, and analyzed the concentrated extract by GC/MS, eventually finding 50 volatile components. Finally, Lin et al. (2005) combined solid phase microextraction (SPME) of urine volatiles from male BALB/c mice with GC/MS and reported 131 components. This variability in the number of detected volatiles reflects differences in odor collection procedures, as well as the choice of the mouse strains and the diet used for raising the animals. In addition, there is surprisingly limited agreement in the chemical identity of the components described in these reports.

In this study, we present the odor composition of the body scent of mice in comparison to the volatile composition of urine odor. This comparison revealed both odor-source-specific and common components, and raises questions regarding the formation and function of mouse volatiles.

Methods and Materials

Mice

Male C57BL/6J mice were raised under specific-pathogen-free conditions at the Max-Planck-Institut für Immunbiologie (Freiburg, Germany), or purchased from Charles River Wiga Deutschland GmbH (Sulzfeld, Germany). Germ-free male C57BL/6 mice and corresponding controls raised under specific-pathogen-free

conditions were provided by Dr. B. Jilge (Tierforschungszentrum, Universität Ulm, Ulm, Germany).

To standardize the volatile components with respect to diet, randomly picked animals were kept singly in cages for 3–4 wk in ventilated hoods and fed on “Diät C 1000” (Altromin, Gesellschaft für Tierernährung GmbH, Lage, Germany) composed of casein, corn starch, saccharose, cellulose, vitamins, mineral substances and trace elements. At the time of body scent and urine collection, the mice were 12–13 wk old. The germ-free and corresponding control mice (three animals each) were used for mouse volatile and urine collection directly upon arrival.

Collection of Volatiles

Sampling of volatiles was performed under conditions that minimized the introduction of contaminants, e.g., from plastic materials. Mouse odor was collected from a horizontal stainless steel cylinder (length 14 cm, ID 9.8 cm) closed at one end, and supplied with a copper-ring-sealed ultrahigh vacuum flange on the other end (Fig. 1). Synthetic air (hydrocarbon-free; Messer-Griesheim, Sulzbach, Germany) was blown via a Mass Flow Controller GFC171 (ANALYT-MTC, Müllheim, Germany) at a rate of 200 ml/min through a charcoal filter (Small Cartridge ACCOSORB, Messer-Griesheim) attached to an inlet at the closed end of the cylinder into a stainless steel pipe (length 12 cm, 5 mm diameter) running parallel to the inner wall of the cylinder. The mouse volatiles were collected from the circulating air into freshly conditioned adsorption tubes (Tenax TA stainless steel prepacked sample tubes, Cat. No. N930-7005, Perkin-Elmer Instruments, Rodgau, Germany) directly connected to an outlet located opposite the inlet. Similarly, urine volatiles were collected at a flow rate of 100 ml air/min from custom-made, round-bottom glass dishes fitting into smaller stainless steel cylinders (length 11 cm, ID 3.8 cm) of otherwise similar design. By arranging a second Tenax or Carbotrap 300 tube (Cat. No. N930-7000) in tandem with the Tenax tube, it was shown that only minor amounts of highly volatile and polar compounds such as triethylamine (#1 in Table 1) were not completely adsorbed in the first tube.

Urine was collected at room temperature overnight from groups of three mice placed in metabolic cages (Tecniplast, Hohenpeißenberg, Germany) and then stored at -20°C . During urine collection, the animals were supplied with water but no food. The urine samples were normalized to a creatinine concentration of 0.12 mg/ml by the addition of water. Volatiles were collected from 1 ml adjusted urine for 4 hr; during this time, about half of the sample evaporated. Between collections from

Fig. 1 Steel vessel used for the collection of mouse volatiles. Purified air enters the vessel through a charcoal filter (lower right) and volatiles are collected in the absorption tube (upper right) directly connected to the steel cylinder

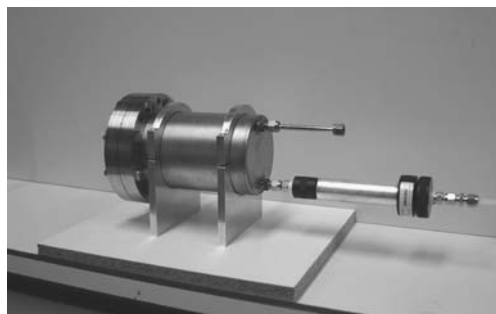


Table 1 Volatile components found by gas chromatography/mass spectroscopy

Peak no.	Retention time (min)	Substance/Characteristic fragments (relative intensity)	Frequency of occurrence		
			Mouse body odor (%)	Urine odor (%)	Source
1	5.40	Trimethylamine (b)	36	42	Merck
2	5.71	Acetone (a, b) ± Isopropylalcohol	100	100	Merck/Merck
3	6.31	Unknown [45 (7), 47 (10), 59 (2), 64 (3), 78 (4), 79 (100), 80 (6), 81 (5), 94 (4)]	36	50	
4	6.67	Nitromethane (b)	100	0	Merck
5	7.06	unknown [40 (2), 42 (11), 43 (100), 86 (13)]	9	100	
6	7.22	Acetic acid ± 2-Butanone (b; only in mouse odor)	100	96	Merck/Roth
7	7.59	2-Methyl-3-buten-2-ol	0	100	Aldrich
8	9.04	3-Methyl-2-butanone (b)	9	65	Aldrich
9	9.14	Benzene (a, b), contaminant	100	100	Merck
10	9.58	1-Methoxy-2-propanol	73	58	Sigma
11	9.99	2-Pentanone (b, d)	41	100	Sigma
12	10.39	Pentanal (b)	95	46	Merck
13	10.70	Propanoic acid	100	0	Merck
14	10.82	3-Hydroxy-2-butanone	95	100	Sigma
15	11.44	unknown [39 (11), 41 (14), 51 (5), 52 (8), 54 (53), 55 (100), 56 (5)]	18	96	
16	11.58	unknown [39 (33), 40 (24), 41 (47), 43 (100), 55 (18), 59 (22), 69 (37), 97 (39), 112 (42)]	9	100	
17	11.91	3-Methyl-3-buten-1-ol	14	15	Aldrich
18	12.19	3-Penten-2-one (b)	0	100	Sigma
19	12.51	Dimethyldisulfide (a, b)	32	0	Merck
20	12.94	Unknown [39 (26), 40 (11), 41 (54), 42 (14), 43 (100), 45 (31), 55 (9), 73 (31), 88 (9)]	55	15	
21	13.53	Pentanol (only in mouse odor) ± Toluol (a, b)	59	8	Sigma/Merck
22	13.98	3-Methyl-2-buten-1-ol	0	92	Aldrich
23	14.31	Unknown [38 (6), 39 (76), 40 (58), 41 (60), 44 (22), 53 (24), 55 (61), 83 (46), 84 (100)]	0	50	
24	14.36	Butanoic acid	86	4	Merck
25	14.77	1-Octene	91	0	Sigma
26	15.10	Hexanal	100	62	Aldrich

27	15.76	6-Hydroxy-6-methyl-3-heptanone (a)	100	100	Synthesized
28	15.97	Unknown [39 (16), 41 (20), 42 (19), 43 (100), 44 (9), 45 (20), 58 (16), 69 (21), 84 (18)]	50	100	
29	16.37	Hexamethyl-cyclotrisiloxan, contaminant	100	100	
30	17.19	3-Methyl-butanoic acid (c)	45	23	Sigma
31	17.46	3-Methyl-cyclopentanone (a, b)	0	81	Sigma
32	17.75	2-Methyl-butanoic acid	5	8	Merck
33	17.95	2-Furamethanol	0	0	Merck
34	18.13	Unknown [39 (51), 41 (61), 54 (63), 55 (100), 56 (41), 57 (29), 59 (30), 73 (43) 86 (16)]	0	85	
35	18.49	Unknown [39 (43), 41 (58), 43 (23), 54 (60), 55 (100), 56 (37), 57 (33), 59 (27), 73 (38)]	0	50	
36	18.75	<i>p</i> -Xylene (a)	0	4	Merck
37	18.78	1-Hexanol	14	0	Merck
38	19.83	2-Heptanone (a, b) + Bis-(methylthio)methane	5	100	Sigma/Alfa Aesar
39	20.06	Unknown [41 (26), 43 (100), 53 (5), 55 (6), 67 (4), 69 (6), 94 (4), 97 (4), 112 (4)]	0	100	
40	20.37	Heptanal	100	42	Sigma
41	21.26	Unknown [39 (32), 41 (44), 43 (22), 55 (100), 57 (44), 85 (42), 97 (26), 111 (43), 126 (37)]	36	100	
42	21.43	Unknown [39 (17), 40 (7), 41 (52), 42 (25), 43 (100), 68 (13), 69 (12), 83 (31), 111 (13)]	45	100	
43	22.17	3-Hepten-2-one (a, b)	0	100	Alfa Aesar
44	22.95	Unknown [39 (28), 41 (26), 43 (45), 55 (30), 57 (100), 67 (31), 68 (25), 97 (28), 112 (17)]	36	100	
45	23.40	Unknown [39 (35), 40 (9), 41 (24), 42 (91), 43 (3), 54 (22), 55 (100), 83 (30), 111 (28)]	36	65	
46	23.49	Benzaldehyde (a, b)	95	100	Merck
47	23.90	Unknown [39 (30), 40 (6), 41 (45), 43 (13), 53 (12), 55 (22), 57 (100), 69 (51), 97 (13)]	0	100	
48	24.25	Hexanoic acid	91	0	Sigma
49	24.40	Phenol (a, c)	50	100	Merck
50	24.49	Unknown [39 (34), 41 (25), 43 (100), 44 (23), 55 (65), 56 (24), 70 (39), 71 (22), 99 (58)]	0	100	
51	24.73	6-Methyl-5-hepten-2-one (a)	14	58	Merck
52	25.07	Unknown [39 (19), 40 (9), 41 (48), 53 (9), 57 (100), 67 (9), 69 (33), 97 (7), 126 (9)]	0	100	
53	25.28	Unknown [45 (100), 46 (62), 47 (58), 48 (33), 61 (85), 94 (41), 281 (94), 282 (27), 283 (20)]	32	100	
54	25.48	Octanal	100	46	Sigma
55	26.36	Unknown [39 (18), 41 (26), 43 (18), 45 (24), 58 (11), 59 (27), 60 (100), 114 (13), 129 (26)]	0	77	
56	26.43	Unknown [40 (9), 43 (100), 44 (28), 57 (82), 99 (76)]	0	88	
57	27.94	2,3-Dehydro-exo-brevicomin (a)	0	100	K. Mori, Tokyo
58	28.59	Unknown [39 (16), 41 (31), 43 (100), 68 (33), 69 (18), 71 (17), 86 (22), 95 (20), 97 (18)]	0	50	
59	28.95	4-Methylphenol	45	100	Sigma

Table 1 (continued)

Peak no.	Retention time (min)	Substance/Characteristic fragments (relative intensity)	Frequency of occurrence		
			Mouse body odor (%)	Urine odor (%)	Source
60	30.11	Unknown [39 (63), 41 (96), 43 (46), 53 (42), 69 (66), 71 (52), 79 (58), 91 (40), 93 (100)]	0	54	
61	30.37	Nonanal	100	50	Sigma
62	30.65	2-sec-Butyl-4,5-dihydrothiazole (a, b)	68	100	C. Mucignat, Padova
63	31.56	Benzylmethylketone	0	50	Aldrich
64	32.52	Unknown [73 (100), 193 (7), 267 (38), 268 (16), 269 (8)]	32	0	
65	34.85	Decanal	86	50	Sigma
66	35.43	Unknown [39 (24), 44 (2), 65 (51), 66 (42), 92 (37), 93 (100), 121 (86)]	0	46	
67	38.82	Indole	5	0	Aldrich
68	44.86	<i>E</i> - β -Farnesene (a)	23	0	M. Kumert, Jena
69	46.73	<i>(E,E)</i> - α -Farnesene (a)	9	0	P. Landolt, Wapato, WA
70	49.71	Diethylphthalate, contaminant	82	100	

Serial nos. 1 to 70 were assigned to peaks with average retention times indicated. Volatiles were identified by comparison with entries in the MS data bank and by comparison with standards. Components of unknown structure are defined by the mass and relative abundance (in brackets) of characteristic fragments. Three components (peak nos. 9, 29, and 70) were consistently observed contaminants. The occurrence of volatiles in both mouse and urine odor, or either in mouse or urine odor is indicated as a percentage of all samples analyzed [22 chromatograms derived from nine different groups of mice for body odor and 26 chromatograms for urine odor involving 11 urine samples from 11 different groups of C57BL/6J mice, cf. [Supplementary Material](http://dx.doi.org/10.1007/s10886-006-9091-2) (available for this article at <http://dx.doi.org/10.1007/s10886-006-9091-2> and is accessible for authorized users)]. Components previously identified are marked: (a) Schwende et al. (1986), Novotny et al. (1999a,b); (b) Miyashita and Robinson (1980); (c) Singer et al. (1997); (d) Montag et al. (2001).

different urine samples, the glass dishes and steel cylinders were rinsed with water and heated to 120°C for at least 1 hr.

Three mice were stimulated to urinate on paper tissue and the mice were then placed into the steel cylinder. Volatiles were collected for 1 hr. The vessel was then opened, inspected for the presence of urine, and the mice were again stimulated to urinate. Volatile body odor components were then collected for another hour in a fresh sample tube. Between collections with the next group of mice, the cylinder was rinsed with water and heated as described above. We had to eliminate some early mouse odor measurements due to urine contamination, which was subsequently prevented by the optimized procedure.

Analysis of Volatiles by Gas Chromatography/Mass Spectrometry

The volatiles were desorbed from the sample tubes in a Thermal Desorption System (Perkin-Elmer ATD400) and then separated by gas chromatography on an Agilent J&W DB-5 column (60 m × 0.25 mm, 0.25 μm; temperature program: 35°C for 5 min; 35–220°C at 3.75°C/min; 220–265°C at 7.5°C/min; 265°C for 3 min) in a Hewlett-Packard GC interfaced to an HP 5973 Mass Selective Detector.

Chromatograms were evaluated for 72 peaks in the following way. A data bank was set up from the mass spectra of all detectable peaks in two urine and three mouse odor chromatograms, which was then used to evaluate all other chromatograms for corresponding components. This procedure allowed reliable sorting of major components but was less successful for minor components. Because the retention times varied by 0.1–0.2 min for different chromatograms, assignments of the respective components required the individual comparison of retention time and mass spectra for all peaks in all chromatograms. Components were tentatively identified by comparison with the NIST 98 Mass Spectral Library (National Institute of Standards and Technology, Gaithersburg, MD, USA). Identities of some components were confirmed by comparisons of retention times and mass spectrum with those of commercially available or donated standards as detailed in Table 1. 6-Hydroxy-6-methyl-3-heptanone was synthesized according to the procedure described by Novotny et al. (1999a), using pyridinium chlorochromate (18 hr at room temperature in CH₂Cl₂) instead of CrO₃/aq. H₂SO₄ as the oxidant.

Results

Collection and Analysis of Volatiles

Using stainless steel vessels and highly purified air, we were able to eliminate most contaminants during volatile analysis (compare spectra in the upper panels of Fig. 2). Three remaining contaminants derived from the absorption tubes (peaks #9, 29, and 70, cf. Table 1) were useful landmarks in the chromatograms, and their abundance showed little variation. Typical chromatograms for mouse body and urine volatiles are shown in the lower panels of Fig. 2. Because the composition of urine volatiles may depend on the chow used for feeding (Brown et al., 1996), mice were kept individually on a synthetic diet for several weeks before the experiments. To obtain sufficient amounts of material for analysis in an adequate time, we always collected the body scent or the urine from groups of three randomly picked mice. We did not

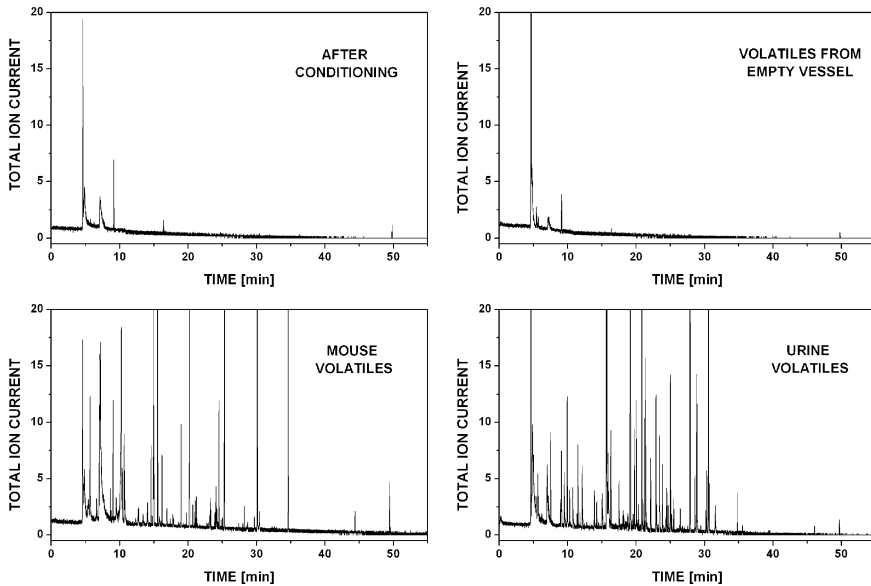


Fig. 2 Representative GC/MS spectra. (A) Spectrum obtained from an absorption tube directly after conditioning. (B) Spectrum obtained after volatile collection from the empty steel cylinder for 4 hr. (C) Volatiles collected for 1 hr from three mice. (D) Volatiles collected in 4 hr from urine

observe aggressive acts between these mice in the metabolic cage during urine collection. Although we cannot exclude aggressive behavior during mouse scent collection in the closed steel cylinder, we consider this unlikely because hair release caused by biting was not observed.

Our major concern was whether the collected mouse body volatiles were contaminated by urine components. We observed that the typical urine component 2,3-dehydro-exo-brevicommin (peak #57, cf. Table 1), which binds to the major urinary proteins (MUPs, Bacchini et al., 1992; Novotny et al., 1999b), was only detected in those chromatograms where the mice had urinated in the stainless steel vessel. When we stimulated the mice to urinate before insertion into the vessel, we consistently prohibited obvious urination in the vessel and did not detect 2,3-dehydro-exo-brevicommin in the spectra of mouse volatiles. However, small amounts of urine may be visually undetectable because marks may quickly dry in the air flux in the cylinder. Assuming that two abundant MUP ligands, 6-hydroxy-6-methyl-3-heptanone (peak #27) and 2-*sec*-butyl-4,5-dihydrothiazole (peak #62), are released solely in the urine and not excreted by MUP-producing glands (e.g., the submaxillary gland), a very rough estimate for the contribution of urine volatiles to the mouse body scent can be obtained from the ratio of the relative peak areas in the GC/MS spectra. The ratio of the medians for the mouse body and urine scents is 0.025 and 0.024 for peaks #27 and 62, respectively (Fig. 4 and Supplementary Material), suggesting that the mouse body volatiles may be contaminated by 2.5% of the urine volatiles collected from 1 ml urine (corresponding to a volume of 25 μ l). This limitation must be taken into account when comparing the mouse and urine scent data. However, for less abundant urine volatiles such as 2,3-dehydro-exo-brevicommin, this contamination falls below the detection level in the mouse body scent.

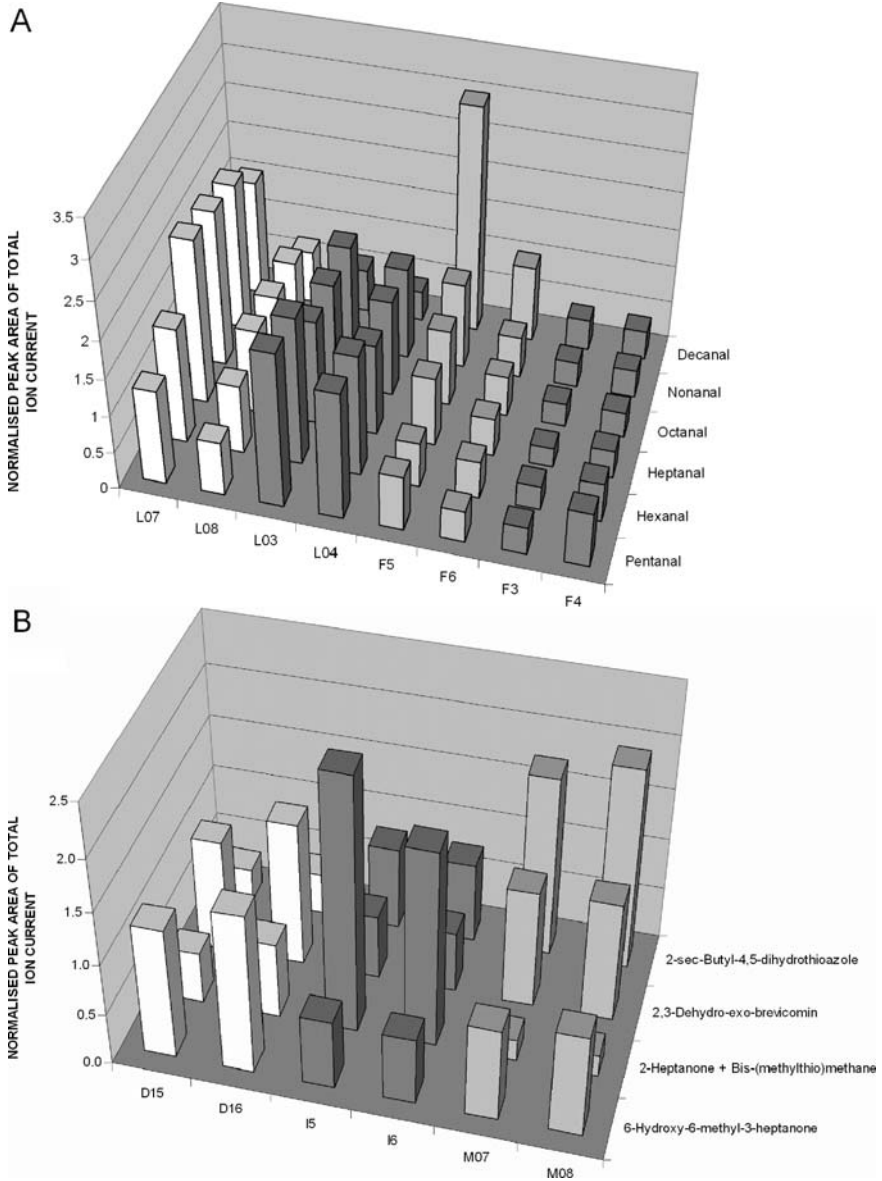


Fig. 3 Representative examples for demonstrating the reproducibility of the odor collection experiments. (A) Normalized peak areas of the total ion current for C₅–C₁₀ aldehydes. Odor was collected from three C57BL/6J mice for 1 hr (F3) and directly thereafter for another hour (F4) or from another three C57BL/6J mice (F5 and F6). A third group of this strain was used for collecting the samples L03 and L04, and 3 d later for samples L07 and L08. Average values of the peak area for the total ion current of the eight measurements were 0.16 for pentanal, 1.12 for hexanal, 0.59 for heptanal, 0.78 for octanal, 2.98 for nonanal, and 0.17 for decanal. (B) Normalized peak areas of the total ion current for pheromones. Note that bis-(methylthio)methane is not considered a pheromone, but overlaps with the 2-heptanone peak in the chromatogram. Double determinations (D15/D16, I5/I6, and M07/M08) from three urine samples collected from three animals each are shown. Average values of the peak areas for the total ion current of the eight measurements were 0.57 for heptanone + bis-(methylthio)methane, 3.87 for 2-sec-butyl-4,5-dihydrothiazole, 1.02 for 2,3-dehydro-exo-brevicomin, and 112.19 for 6-hydroxy-6-methyl-3-heptanone

In view of the large variability in the abundance of many components, it was important to document the reproducibility of successive mouse odor and duplicate urine odor measurements. As an example, Fig. 3A depicts the normalized abundance of *n*-aliphatic aldehydes from C₅ to C₁₀ in mouse body odor. Volatiles were collected from groups of three C57BL/6J mice for 1 hr (e.g., F3) and then for another hour (e.g., F4). With some exceptions, the agreement between successive samplings was reasonably good (compare equally marked pairs). However, for different groups of mice, aldehyde abundance varied by a factor of 5–10 (compare differentially marked pairs). Similar variability was observed in volatiles collected from urine samples normalized to a constant creatinine concentration (Fig. 3B). The four candidate pheromones chosen as examples differed in abundance by a factor of up to 10 when comparing urine samples from different groups of mice (compare differentially marked pairs). However, collection of volatiles from the same urine preparation was consistently reproducible (compare equally marked pairs). We concluded from these experiments that under our standardized conditions, variability in abundance was primarily attributable to the odor source rather than to the procedures used for collection and analysis of volatiles.

Composition of Volatiles

Taken together, we evaluated 22 chromatograms derived from 9 different groups of mice for body odor and 26 chromatograms for urine odor involving 11 urine samples from 11 different groups of C57BL/6J mice (Table 1 and [Supplementary Material](#)). A few measurements were performed with germ-free mice and controls raised under specific-pathogen-free conditions. All chromatograms were evaluated for the 67 components plus the three contaminants listed in Table 1. These 67 components were therefore characteristic of the total ensemble of samples analyzed in this study. The entire data set can be inspected in the [Supplementary Material](#). Table 1 also indicates the fraction from all measurements where a given volatile could be detected.

Prominent in the list were acids (#6, 13, 24, 30, 32, 48), ketones (#2, 6, 8, 11, 14, 18, 27, 31, 38, 43, 51, 63), aldehydes (#12, 26, 40, 46, 54, 61, 65), and alcohols (#2, 7, 10, 17, 21, 22, 33, 37, 49, 59), as well as components considered to be mouse pheromones (#27, 38, 57, 62, 68, 69; Novotny, 2003). The structures of about one third of the components remain to be identified. Nitromethane (#4), propanoic acid (#13), dimethyldisulfide (#19), 1-octene (#25), 1-hexanol (#37), hexanoic acid (#48), indol (#67), α - and β -farnesene (#68 and 69), and one unidentified component (#64) were only observed in the volatiles from mouse body scent. On the other hand, 3-penten-2-one (#18), 3-methyl-2-buten-1-ol (#22), 3-methyl-cyclopentanone (#31), *p*-xylene (#36), 3-hepten-2-one (#43), 2,3-dehydro-exo-brevicommin (#57), benzylmethylketone (#63), and 13 unidentified compounds (#7, 23, 34, 35, 39, 47, 50, 52, 55, 56, 58, 60, and 66) were only found in urine volatiles. All other substances were present in the volatiles of both mice and their urine.

Although we did only a few experiments with mice fed on a conventional chow under germ-free or, as a control, specific-pathogen-free conditions, some observations are noteworthy. Mouse odor components (#4, 6, 12, 13, 20, 24, 26, 27, 30, 32, 33, 36, 40, 46, 48, 51, 54, 61, and 65) and urine odor components (#2, 6, 11, 12, 14, 15, 16, 18, 26, 27, 28, 39, 40, 41, 42, 43, 44, 45, 46, 52, 53, 54, 57, 61, 62, and 65) were clearly present in germ-free animals. This set includes the aliphatic aldehydes, which

were therefore derived from the mice rather than their bacterial flora. One compound exclusively detected in germ-free mouse scent was 2-furanmethanol (#33). A more detailed comparison of germ-free and specific-pathogen-free mice would require a larger data set.

Figure 4 shows the medians and the quartile ranges on a logarithmic scale for all those components that were detected in more than 80% of the measurements (18 mouse body odor and 29 urine components). Small quartile ranges, e.g., for urine peaks #2, 11, 43, 44, and 46, indicated a good reproducibility in the detection of

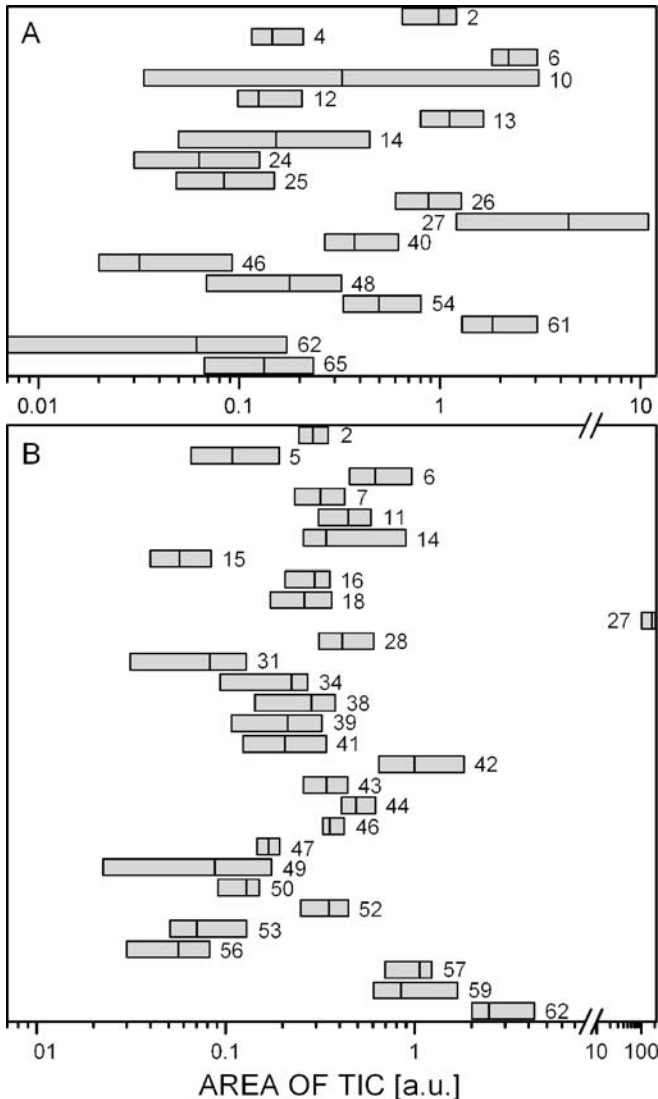


Fig. 4 Relative abundance of selected volatiles collected from the body of mice (A) or their urine (B). The peak area of the total ion current (TIC) is plotted on a logarithmic scale. The numbers refer to those listed in Table 1. Lengths of the horizontal bars represent the interquartile range of the measurements; median is indicated by a vertical bar within the horizontal bars

these volatiles. On the other hand, components such as mouse volatiles #10, 14, and 27 were highly variable in abundance. Inspection of the data after normalization to the medians suggested that the abundance of urine volatiles was less variable than the mouse volatiles (not shown).

Discussion

To our knowledge, the present study represents the first report on the composition of mouse body scent. Although most volatiles are present in both body and urine odors, several components were specific to one or the other scent. We find it intriguing that the mouse odor reproducibly contained aliphatic aldehydes from C₅ to C₁₀ as prominent components, because odorant receptors for these aldehydes are found in abundance in the main olfactory epithelium and they have been characterized in detail (Zhao et al., 1998; Krautwurst et al., 1998; Araneda et al., 2000, 2004; Kaluza and Breer, 2000). Behavioral experiments using these aldehydes may show that they are important to mice in recognizing their conspecifics. Interestingly, short-chain aldehydes have also been found in human odor (Bernier et al., 2000; Haze et al., 2001; Curran et al., 2005). These aldehydes can be produced through oxidative degradation of monounsaturated fatty acids such as palmitoleic acid and vaccenic acid (Haze et al., 2001), which are secreted by sebaceous glands. In the rat, these aldehydes have been detected in extracts from the preputial gland (Sioni et al., 2005).

Another remarkable observation is that the abundance of some components, e.g., 1-methoxy-2-propanol (#10), 6-hydroxy-6-methyl-3-heptanone (#27), phenol (#49), and methylphenol (#59) varied over a range of 10- to 100-fold in different experiments (Fig. 4 and [Supplementary Material](#)). This finding suggests the occasional discharge of these components, either spontaneously or in response to environmental or social cues. Triggers for the release of these components and their source remain to be defined.

It is puzzling that there is little overlap between the volatile components identified by different investigators in mouse urine. Therefore, reported volatile composition may strongly depend on the method used for odor collection and analysis as well as the type of mouse strain and diet used in the experiments. In Table 1, those compounds that have previously been identified are marked. However, many volatiles found in this study have not been described previously and *vice versa* (Miyashita and Robinson, 1980; Schwende et al., 1986; Singer et al., 1997; Montag et al., 2001; compare also Willse et al., 2005; Lin et al., 2005). We used a synthetic diet for feeding the mice, which should reduce the complexity of volatiles, and we have tried to rigorously exclude contaminants during odor collection. In addition, control experiments showed that the absorption tubes used for odor collection were efficient. These efforts toward a standardization of the methodology were successful, in that the set of observed components was generally reproducible for the body and urine scents of C57BL/6 mice, but there was a large variation in abundance such that minor components frequently were not detected (Table 1 and [Supplementary Material](#)).

We confirmed the presence of putative pheromones known to be present in male urine (2-*sec*-butyl-4,5-dihydrothiazole, 2-heptanone, 2,3-dehydro-*exo*-brevicomin, and 6-hydroxy-6-methyl-3-heptanone). Two further pheromones, α - and β -farne-

sene, which are secreted by the preputial gland (Novotny et al., 1990), were detected in some mouse samples but not in urine samples. We also did not detect the recently identified semiochemical (methylthio)methanethiol, a component of very low abundance (Lin et al., 2005).

In view of the importance of the mouse as a model for mammalian behavior and for the analysis of recognition and processing of olfactory signals, a better standardization for the collection and analysis of volatiles released by the animals and their urine appears desirable.

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Elemental Sulfur Identified in Urine of Cheetah, *Acinonyx jubatus*

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Abstract The urine of the cheetah, *Acinonyx jubatus*, is almost odorless, and probably for this reason, it has not attracted much attention from scientists. Using gas chromatography–mass spectrometry, we identified 27 and 37 constituents in the headspace vapor of the urine of male and female cheetah, respectively. These constituents, composed of hydrocarbons, short-chain ethers, aldehydes, saturated and unsaturated cyclic and acyclic ketones, 2-acetylfuran, dimethyl disulfide, dimethyl sulfone, phenol, myristic acid (tetradecanoic acid), urea, and elemental sulfur, are all present in the headspace vapor in very small quantities; dimethyl disulfide is present in such a low concentration that it cannot be detected by the human nose. This is only the second example of elemental sulfur being secreted or excreted by an animal. It is hypothesized that the conversion of sulfur-containing compounds in the cheetah's diet to elemental sulfur and to practically odorless dimethyl sulfone enables this carnivore to operate as if “invisible” to the olfactory world of its predators as well as its prey, which would increase its chances of survival.

Keywords Cheetah · *Acinonyx jubatus* · Urine · Territorial behavior ·
Mammalian semiochemicals · Elemental sulfur

Introduction

Although cheetah, *Acinonyx jubatus*, can reach a speed of more than 100 km/hr in seconds (Caro, 1994), they are exposed to severe competition for survival because they lack the physical strength to guard their territory and protect their young against stronger predators. In some nature reserves, predation accounts for more than 40% mortality in cheetah litters (Eaton, 1974). Cheetah are territorial, mostly solitary, animals with enormous home ranges. The average size of the home range of

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a female on the Serengeti plains is about 830 km². Male territories are about 4.5% of that of female territories (Caro, 1994). Male and female cheetah leave territorial marks by spraying objects with urine. Females in heat squirt urine on bushes, tree trunks, and rocks to attract males (Macdonald, 1984), but marking behavior is more common in males. Consensus has not been reached as to the function of this behavior in males. Both sexes as well as cubs take great interest in marks left by a strange male, and there is convincing evidence suggesting that territorial marking is a mechanism whereby contact and conflict between family groups, which could result in injury or death, is avoided (Eaton, 1974).

Probably because the urine is almost odorless, it has not elicited much interest from scientists. Continuing our research on the semiochemicals of mammals (Burger, 2005), we attempted a comprehensive chemical characterization of the volatile fraction of the urine of the cheetah.

Methods and Materials

Sample Collection

Urine was collected from four captive male cheetah (Cheetah Outreach Facility, Spier Wine Estate, Stellenbosch, South Africa) by catching sprayed urine in a glass beaker. Bladder urine was obtained from an anesthetized female by catheterization. These cheetah were kept in enclosures varying in size from about 100 × 50 m to 85 × 70 m with wire mesh fences and were fed a diet of chicken. On one occasion, urine was collected from two wild males at the research station of the Cheetah Conservation Fund, Otjiwarongo, Namibia. The urine samples were frozen as quickly as possible and kept at -25°C until used for analysis.

Analytical Techniques

Volatile constituents of the urine were sampled by sorptive sample enrichment in polydimethylsiloxane (PDMS) rubber by using a sample enrichment probe (SEP) (Burger et al., unpublished data) followed by thermal desorption in the injector of a gas chromatography–mass spectrometry (GC–MS) instrument (GC–MS, Carlo Erba QMD 1000). The volatile organic constituents were also extracted from the urine with dichloromethane (Merck, Residue Analysis Grade) for retention-time comparison (co-elution) with authentic synthetic compounds and quantification of the elemental sulfur and some of the less volatile compounds extracted from the urine. The concentration of the sulfur in cheetah urine was determined by GC–MS with sulfur as external reference.

Bioassays

Thick, green paper sheets (350 × 230 mm) were uniformly wetted on one side with 6 ml of a colloidal sulfur solution (3 µg S/ml) and air-dried to produce test papers coated on one side with 18 µg of sulfur, equivalent to approximately 18 ml of urine. Controls were prepared by wetting paper sheets with water, fresh cheetah urine, or with solutions of various odorants such as benzeneethanethiol and orange juice.

Bioassays were done at the Spier Cheetah Outreach Facility with four male and three female cheetah that were reasonably tame and accustomed to human visitors. Wet and dry sulfur-treated papers, wet and dry water-treated controls, and papers wetted with cheetah urine or with other odorants were presented 3 m apart along one of the fences of an enclosure where a cheetah was likely to spot and inspect it. The attention the cheetah paid to each test paper and control, as well as any peculiar behavior, was noted. Tests were also done at the Tygerberg Zoo with a breeding pair of wild cheetah, as well as a breeding pair each of the following species: lion (*Panthera leo*), Bengal tiger (*P. tigris*), caracal (*Felis caracal*), wild cat (*F. silvestris lybica*), and wild dog (*Lycaon pictus*).

Results and Discussion

Enrichment of the organic volatiles in the headspace of cheetah urine by using the SEP technique and GC–MS analysis of the enriched volatiles gave a total ion chromatogram (TIC) (Fig. 1) and mass spectra that could be used for the identification of most of the headspace constituents.

Urea (**35**) extracted from the headspace of the urine appears in the TIC as a broad peak at a retention time of 49.2 min, and cyanuric acid (2,4,6-trihydroxy-1,3,5-triazine) (**39**) as a broad peak at a retention time of 92.0 min. This compound is formed when urea is heated to temperatures above its melting point, and it was concluded that its presence in the TIC shown in Fig. 1 is due to the partial conversion of the urea to cyanuric acid in the injector of the GC–MS instrument. The saddlelike elution profile between these two peaks shows that this process continued to take place in the column during the chromatographic process. Some of the compounds listed in Table 1 were detected in the headspace vapor of the urine of only one of the sexes. The dimethyl disulfide (**9**) is present in the urine in such a small quantity that it could not be detected by the human nose.

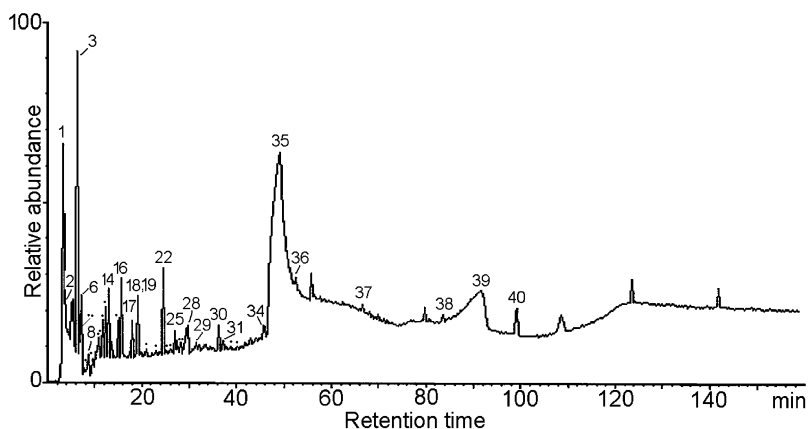


Fig. 1 TIC of volatile constituents extracted for 69 hr at 40°C from the headspace vapor of the urine of a female cheetah with a device in which 140 μ l of PDMS rubber was used, desorbed for 5 min at 220°C in the injector of the GC–MS system, and analyzed on a 40 m \times 0.3 mm glass capillary column coated with PS-089 (DB-5 equivalent) by using a temperature programming rate of 2°C/min from 40°C to 280°C

Table 1 Constituents identified in the headspace vapor of cheetah urine^a

No. in Fig. 1	Constituent	♂	♀
1	2-Butanone	✓	✓
2	Ethyl propyl ether		✓
3	2-Pentanone	✓	✓
4	Dipropyl ether		✓
5	3-Pentanone		✓
6	Butyl ethyl ether		✓
7	Heptane	✓	✓
8	1,2-Diethoxyethane		✓
9	Dimethyl disulfide	✓	✓
10	3-Hexanone	✓	✓
11	2-Hexanone	✓	✓
12	Butyl propyl ether		✓
13	Hexanal	✓	✓
14	Ethyl pentyl ether		✓
15	2-Acetylfuran	✓	
16	3-Methylcyclopentanone	✓	✓
17	4-Heptanone		✓
18	Cyclohexanone	✓	✓
19	2-Heptanone	✓	✓
20	Dimethyl sulfone	✓	✓
21	(<i>E</i>)-3-Hepten-2-one	✓	✓
22	Ethyl-3-methylcyclopentane	✓	✓
23	Benzaldehyde	✓	✓
24	2,4-Heptanedione		✓
25	Phenol	✓	✓
26	2-Octanone		✓
27	Octanal	✓	✓
28	Decane		✓
29	Acetophenone	✓	✓
30	2-Nonanone		✓
31	Nonanal	✓	✓
32	Phenylacetone	✓	✓
33	(<i>E</i>)-3-Nonen-2-one	✓	
34	Decanal	✓	✓
35	Urea	✓	✓
36	2-Undecanone	✓	✓
37	Sulfur S ₆	✓	✓
38	Myristic acid (tetradecanoic acid)	✓	✓
39	Cyanuric acid (2,4,6-Trihydroxy-1,3,5-triazine) ^b	✓	
40	Sulfur S ₈	✓	✓

^a Arranged in order of elution from an apolar column.

^b Formed from urea in the injector and on the column of the GC–MS instrument.

We were surprised to find elemental sulfur in all of the urine samples from cheetah males and from one female cheetah. Sulfur is not detected by GC when a flame ionization detector (FID) is employed. However, the cyclic S₆ (**37**) and S₈ (**40**) forms of the element can be separated and quantified by GC–MS analysis. In the present investigation, small quantities of S₆ and larger quantities of S₈ were found in

the headspace vapor and in dichloromethane extracts of the urine of both sexes. Employing sulfur as external standard, it was found that the concentration of sulfur in the urine of male cheetah with unlimited access to water is approximately 1 µg/ml. Sulfur is not an artifact formed from dimethyl disulfide because it was not detected when dimethyl disulfide was subjected to GC–MS analysis, either under the analytical conditions employed in the present investigation or under thermally harsher conditions.

It was not possible to determine the concentration of the highly volatile constituents of the urine because they were largely lost when the extract was concentrated and could not be detected in GC and GC–MS analyses. The less volatile constituents, dimethyl sulfone (**20**), benzaldehyde (**23**), phenol (**25**), acetophenone (**29**), and 2-nonanone (**30**) were present in concentrations of about 250, 1.5, 0.6, 1.4, and 1.5 ng/ml, respectively, in the extracted material. Although 2-piperidone (δ -valerolactam) was not detectable in the urine headspace vapor, it was identified as the major organic constituent of cheetah urine at a concentration of 1.7 µg/ml.

Several explanations can be offered for the presence of sulfur in cheetah urine. It is unlikely that this element could be a sex pheromone of the species because sulfur is present in the urine of both male and female cheetah. In view of the enormous home ranges of these animals, use of sulfur as a territorial-marking pheromone, or a component of such a pheromone, would have the advantage that it is much more persistent than the quite volatile organosulfur compounds present in the urine of other felids. It is also possible that the conversion of sulfur-containing proteins to elemental sulfur is just an alternative mechanism by which sulfur is excreted in its elemental form instead of being excreted as organosulfur compounds, as in many other felids. The conversion of organosulfur compounds to sulfur and dimethyl sulfone, also a practically odorless compound, could allow cheetah (especially females with small cubs that from a very early age accompany the female on hunts) to live in closer proximity to their prey than would otherwise be possible. In this regard, it has been found that organosulfur compounds derived from a protein-rich diet could be a cue by which prey can distinguish a potential predator (Epple et al., 1993; Nolte et al., 1994). If, as mentioned above, sulfur is at the same time used as a territorial-marking substance, it would imply that the cheetah must be equipped to detect sulfur much better than do their predators. With the limited information available from bioassays with cheetah in captivity, there is no proof that this could be the case.

Ideally, these possibilities should be tested by investigating the responses of male and female cheetah, other carnivores, and typical prey species to cheetah urine and sulfur in various combinations under completely natural conditions in the wild. This was impossible in the present investigation. It is not known which, and to what extent, urine constituents play a role in the sexual and territorial behavior of the cheetah, and there is no positive control that could have been used in experiments to determine the reaction of cheetah and other carnivores to cheetah urine and sulfur. In some exploratory experiments that were nevertheless done [with four male and three female cheetah at the Spier facility, as well as breeding pairs of wild cheetah, lion (*P. leo*), Bengal tigers (*P. tigris*), caracal (*F. caracal*), African wild cats (*F. silvestris lybica*), and wild dogs (*L. pictus*), at the Tygerberg Zoo], sulfur and cheetah urine elicited practically no response either in cheetah or in the other carnivores. Other odorants, especially benzeneethanethiol, elicited stronger

responses than male cheetah urine or sulfur. In an environment that must be saturated with semiochemical messages, no significance can be attached to the results of these bioassays as far as the possible role of sulfur as a component of a territorial-marking pheromone is concerned. On the other hand, our hypothesis—that the conversion of sulfur-containing compounds in their diet to elemental sulfur enables cheetah to operate as if “invisible” to the olfactory world of their predators as well as their prey—was not refuted by the results of these bioassays.

The secretion of elemental sulfur by a carabid beetle (Meinwald and Attygalle, personal communication) is the only other known example of the production of elemental sulfur by an animal. It is possible that sulfur is not so rare in animal excretions and secretions, but that it has been overlooked in the urine and/or feces of other species, as it is not detected by FID. It is hoped that biologists will be motivated by the discovery of sulfur in the urine of the cheetah to look for it in other biological material and to consider its possible role in other biological systems.

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Near-Infrared Reflectance Spectroscopy is a Rapid, Cost-Effective Predictor of Seagrass Nutrients

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Abstract Near-infrared reflectance spectroscopy was used to analyze nutrient composition of tropical and subtropical seagrasses in Queensland, Australia, as part of a broader study of impacts of grazing by dugongs on seagrass. Seagrass samples of 10 species were collected, transported to the laboratory, and separated into leaf and root/rhizome fractions. They were dried, ground, and near-infrared spectra (400–2500 nm) were collected. We used partial least-squares regression to develop calibration equations relating spectral data to standard compositional analyses performed in the laboratory. These compositional analyses focused on attributes believed to be important determinants of nutritional quality of marine vertebrate herbivores (nitrogen, organic matter, neutral detergent fiber, acid detergent fiber, lignin, neutral starch, water-soluble carbohydrates, and *in vitro* dry matter digestibility). Calibration equations for each attribute were developed separately

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for (1) roots/rhizomes and (2) leaves, irrespective of plant species. An equation that combined both plant parts was equally robust. These studies demonstrated the utility of near-infrared spectroscopy in providing rapid and cost-effective analysis of marine plants, which, in turn, permits a rigorous statistical approach to be applied to studies of foraging by marine herbivores.

Keywords Near-infrared spectroscopy · Seagrass · Grazing · Nutritional quality · Marine herbivores · Nutrient analyses · Ecological methods

Abbreviations

MPLS	modified partial least squares
NIRS	near-infrared reflectance spectroscopy
RMS	root mean square
SEC	standard error of calibration
SECV	standard error of cross validation
SEL	standard error of laboratory determination (=analytical precision)
r^2	simple coefficient of determination

Introduction

Analysis of nutrient content of vegetation is a key step in many ecological studies in marine environments. However, conventional methods of such analyses are frequently time consuming and expensive. In particular, replication and longitudinal experiments can be compromised because of the difficulty of conducting nutrient analyses over time. This constraint is particularly serious when studying the nutritional plant quality for those herbivorous species that may eat a variety of food plants growing under a variety of conditions.

Near-infrared reflectance spectroscopy (NIRS) offers an unrivalled potential to address these issues (Foley et al., 1998) and has become a widely used method for analyzing agricultural and food products. Advantages offered are analytical speed, no or minimal sample preparation (Osborne et al., 1993; Shenk and Westerhaus, 1993b), low cost, high precision, and suitability for longitudinal studies. Foley et al. (1998) reviewed the basics of NIRS, which essentially depends on establishing a statistical relationship between the spectrum of near-infrared light reflected by a sample and a set of standard laboratory analyses of components of interest. Defined chemical bonds absorb light at characteristic wavelengths. Once this relationship has been established, the concentration of constituents of interest in any new samples can be analyzed solely by collecting and processing spectra from the samples.

We were interested in several nutritional attributes of different species of seagrass available to herbivorous dugongs (*Dugong dugon* Müller; Mammalia, Sirenia) in tropical Australia as part of a broader study (Aragones, 1996) of the effect of grazing on seagrass community structure. That study generated 1165 samples for which we sought information on eight nutritional components—a total of 9320 determinations. This was beyond the capacity of our or any other laboratory to complete, and so we investigated whether NIRS would allow us to keep our preferred experimental designs at a much-reduced cost.

In this paper, we show that NIRS can be successfully used to analyze nutrient concentration and *in vitro* digestibility of many seagrass species sampled from diverse

habitats and localities in tropical and subtropical Australia. In addition, we evaluate implications of our results for further applications of this technique to seagrass—herbivore interactions and seagrass ecology. We conclude that NIRS can be used reliably to analyze seagrass nutrients and could be applied to many ecological studies.

Methods and Materials

Seagrass Collection

Samples of seagrasses were collected in tropical Australia including the Great Barrier Reef region and the Gulf of Carpentaria, and subtropical south Queensland (Moreton and Hervey Bays). The collection comprised 10 species (with two species having two morphs), representing the diversity of regional seagrasses: *Halophila ovalis* (R. Brown) Hooker, *H. minor* (Zollinger) den Hartog, *H. spinulosa* (R. Brown) Ascherson, *H. decipiens* Ostenfeld, *H. tricostata* Greenway, *Halodule uninervis* (Forsskal) Ascherson (narrow- and wide-leaf morphs), *Cymodocea rotundata* Ehrenberg and Hemprich ex Ascherson, *C. serrulata* (R. Brown) Ascherson and Magnus, *Syringodium isoetifolium* (Ascherson) Dandy, and *Zostera capricorni* Ascherson (narrow- and wide-leaf morphs). We included samples from several experimental treatments (grazing, cropping, and fertilization; Aragones, 1996) as well as material from unmanipulated plots established to measure variation in nutrients because of season, depth, and locality. Most samples came from intertidal beds, but a small subset was collected with SCUBA at Pipon Island (14°7'S, 144°32'E) down to 30 m. We collected a total of 1165 samples.

Sample Preparation

Samples were washed and cleaned of adherent substrate in clean seawater and sorted into species. All were divided into leaf and root/rhizome fractions, except for *H. tricostata* and *H. decipiens*, where leaves and roots/rhizomes were combined because their leaves are so small. Fractions were placed in labeled paper bags and dried to constant mass in a forced draft oven at 60°C. Each sample was ground through a 1-mm screen in a Cyclone mill (Udy Corporation, Fort Collins, CO, USA) and stored in a polyethylene jar. One week prior to collection of NIRS (see below), we opened and equilibrated samples in a chamber maintained at 11% r.h. and 12°C. This ensured that moisture content of all samples was similar (Shenk and Westerhaus, 1993b).

Routine NIRS Scanning and Analyses

Collection of Spectral Data

Near-infrared reflectance spectroscopy is commonly used in agriculture, and standard methods have been developed (Anon, 1995). In our work, we followed these standard procedures carefully and so only briefly detail our procedures.

We acquired spectra of all samples using a near-infrared reflectance spectrophotometer (Model 6500, NIR Systems, Silver Springs, MD, USA), equipped with a

spinning-cup module, housed in a room maintained at $22 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ r.h. We acquired the mean spectrum from two 25-mm standard cells collected to a set RMS error (using first-derivative spectral data), of 50μ absorbance units. Spectral data were collected from 400 to 2498 nm at an interpolated data gap of 2 nm. This ensured collection of spectral data of high precision (Shenk and Westerhaus, 1993b). Reflectance (R) readings were converted to absorbance (A) values: $A = \log(1/R)$. The instrument was maintained and operated, spectral data manipulated, and calibrations developed using the NIRS3 software package (Shenk and Westerhaus, 1992).

Many of our samples were too small (~ 0.5 – 2.0 g) for use of standard cells. We made three “micro” ring cups that allowed collection of spectra from these samples. These cups had the same external dimensions as standard ring cups supplied commercially (ISI, Port Matilda, PA, USA), but the internal dimensions were reduced by 70%. We made the cups from black anodized aluminum and spectrally matched, spectroscopic-grade quartz glass (Behmn, Dayton, OH, USA) in front of each cell. We routinely used “micro” cells for samples as small as 0.3 g throughout the project, once we verified spectral data for the same samples scanned in each cell type did not differ.

NIRS Analysis

Selection of the Calibration Set for Chemical Analyses

We chose a subset of samples for detailed chemical analyses (calibration set) using the algorithms CENTER and SELECT (Shenk and Westerhaus, 1991a,c). These algorithms use principal component analysis and Mahalanobis distances (Mahalanobis, 1936) to rank spectra relative to the population average. This procedure captures a subset of the full spectral (and hence chemical) variation present in the data set.

Samples for the calibration set were selected over several months because sample collection was designed to be cumulative through time due to the limitations of the study. Processing of samples was slow because each field sample had to be sorted first into different plant species, and then each into fractions (leaves vs. roots/rhizomes). The first 200 samples represented a diversity of seagrasses of various species and morphs, collected from various sites and depths. We selected 70 of these samples for initial detailed chemical analyses. Once we had acquired spectra from all 1165 samples that were collected for the studies described by Aragonés (1996), we again used the SELECT algorithm to select further 118 samples. We included 10 further samples identified as population outliers by their Mahalanobis distances, bringing the final calibration set to 198 samples. In several instances, there was insufficient sample for all chemical analyses, and so we selected the nearest neighbor, again based on Mahalanobis distances, for inclusion in the calibration set.

A Comparison between Leaf or Root/Rhizome and Whole Plant-Based Calibrations

Samples selected for the calibration set were divided into two groups: leaves and roots/rhizomes. Thus, two options were possible for calibration: a separate calibration for each of the fractions of leaves or roots/rhizomes, and a whole plant calibration, combining both fractions. Accordingly, we evaluated whether calibra-

tion equations developed for a single plant part were superior to a broad-based calibration developed with multiple plant parts. Plant part calibrations were developed separately for leaves or roots/rhizomes. A broad-based calibration was developed combining both plant fractions, as well as a few detrital matter samples. We assumed that leaves and roots/rhizomes samples selected in the broad-based calibration also would have been chosen in separate selections for each plant part because they were part of the same population. Thus, they would have still been representative of each group. Grouping by plant part rather than species was the simplest possible division, as our samples included 10 separate species. Predictions from single-product calibrations (i.e., roots/rhizomes or leaves) were compared to those of the broad-based calibration equations by comparing their standard error of cross validation (SECV), r^2 , regression slope, and “bias.” Bias is an estimate of the mean difference between laboratory-determined and NIRS-predicted values for calibration or monitoring sets (Smith and Flinn, 1991). Bias confidence limits were set to distinguish between no bias and a bias no greater than $1.0 \times$ standard error of calibration (SEC) with 90% confidence when using a two-tailed type 1 error probability of 0.10 (Shenk and Westerhaus, 1993a). Shenk and Westerhaus (1993a) suggested that if bias were greater than the confidence limit, the calibration set may be insufficient and should be expanded.

Chemical Analysis (Laboratory Reference Methods)

Samples in the calibration set were analyzed for eight constituents. We assayed organic matter (OM) by burning a sample in a muffle furnace at 550°C for 4 hr. Total nitrogen (N) was assayed using a semi-micro Kjeldahl method calibrated against ammonium sulfate standards. Cell-wall constituents of seagrass have been implicated as important determinants of feeding by several authors (Lanyon and Marsh, 1995a; Preen, 1995), and we measured them as neutral detergent fiber, acid detergent fiber, and acid lignin using an ANKOM plant fiber analyzer (ANKOM, Fairport, NY, USA; Komarek, 1994) following Van Soest et al. (1991). Total water-soluble carbohydrates were extracted using 80% aqueous ethanol and water (Radojevic et al., 1994) and quantified as fructose equivalents using the anthrone reaction (Jermyn, 1975). Insoluble material remaining from this extraction was analyzed for starch enzymatically using a commercial total starch assay kit (Megazyme Total Starch Kit; Megazyme, Australia). We solubilized any resistant starch with dimethyl sulfoxide prior to enzymatic treatments. Finally, we measured *in vitro* dry matter digestibility of each sample (Choo et al., 1981) in ANKOM filter bags. The *in vitro* digestibility method attempts to simulate digestive processes in herbivorous mammals using the enzymes pepsin and cellulase and is potentially useful as a way of integrating all individual assays of each sample.

Accuracy of NIRS analyses depends entirely on accuracy of analyses of the calibration set. Therefore, to ensure the quality of data in the calibration set, we analyzed all samples in duplicate and repeated those that differed by more than 2% for N and 5% for other measures. Additionally, a randomly chosen subset ($N = 20$) of laboratory samples (called the laboratory validation set) was reanalyzed in duplicate to estimate precision of analyses. All analyses were expressed on a percent dry weight basis. A correlation analysis between calibration and laboratory validation analyses (sets) was performed for each reference method.

Table 1 Composition of seagrass used to construct calibration equations

Species	Part	Nitrogen	Organic matter	Neutral detergent fiber	Acid detergent fiber	Acid lignin	Starch	Water-soluble carbohydrate	<i>In vitro</i> digestibility
<i>Zostera capricorni</i>	Leaf (12)	1.91 (0.05)	63.9 (0.5)	42.3 (0.7)	28.0 (0.5)	15.4 (0.4)	1.1 (0.1)	0.1 (0.0)	90.2 (0.3)
	Root/rhizome (14)	0.66 (0.03)	56.2 (0.8)	34.3 (0.7)	26.2 (0.6)	15.1 (0.6)	7.1 (0.5)	0.1 (0.0)	82.3 (0.8)
<i>Halodule uninervis</i>	Leaf (30)	2.9 (0.1)	68.6 (0.2)	48.2 (0.2)	32.6 (0.2)	18.9 (0.2)	1.2 (0.1)	0.1 (0.0)	90.1 (0.1)
	Root/rhizome (35)	0.84 (0.01)	65.3 (0.5)	28.3 (0.3)	20 (0.2)	8.4 (0.2)	13.7 (0.5)	0.2 (0.0)	83.7 (0.2)
<i>Halodule spinulosa</i>	Leaf (9)	1.10 (0.0)	64.8 (0.6)	35.1 (0.4)	27.0 (0.3)	11.2 (0.4)	1.4 (0.2)	0.1 (0.0)	89.0 (0.4)
	Root/rhizome (12)	0.63 (0.0)	68.0 (0.6)	36.4 (0.3)	26.7 (0.3)	8.8 (0.2)	0.6 (0.1)	0.2 (0.0)	86.1 (0.8)
<i>Halodule minor</i>	Leaf (5)	1.95 (0.07)	50.9 (0.8)	31.9 (0.6)	20.7 (0.5)	11.5 (0.5)	0.8 (0.1)	0.1 (0.0)	90.1 (0.4)
	Root/rhizome (7)	0.56 (0.03)	44.2 (0.9)	24.3 (0.5)	19.8 (0.8)	8.8 (0.6)	1.8 (0.3)	0.3 (0.0)	91.3 (0.4)
<i>Cymodocea serrulata</i>	Leaf (4)	1.67 (0.19)	71.2 (2.3)	46.2 (2.4)	29.2 (0.5)	15.3 (0.8)	0.2 (0.1)	0.1 (0.0)	89.7 (2)
	Root/rhizome (4)	0.75 (0.07)	73.4 (1.8)	39.5 (2.6)	30.2 (2.1)	15.8 (1.9)	1.4 (0.3)	0.6 (0.1)	81.0 (2.8)
<i>Cymodocea rotundata</i>	Leaf (6)	2.60 (0.1)	67.4 (1.3)	50.7 (0.7)	32.9 (0.7)	19.0 (0.8)	0.8 (0.2)	0.1 (0.0)	87.0 (1.0)
	Root/rhizome (8)	0.91 (0.0)	60.7 (1.8)	42.2 (1.4)	32.5 (1.3)	19.9 (1.0)	2.3 (1.2)	0.2 (0.1)	73.0 (1.5)
<i>Syringodium isoetifolium</i>	Leaf (5)	1.36 (0.11)	61.4 (1.5)	37.0 (1.1)	26.7 (1.0)	10.2 (1.0)	1.6 (0.3)	1.9 (0.5)	96.9 (0.7)
	Root/rhizome (5)	0.78 (0.05)	75.5 (4.3)	34.3 (1.2)	26.3 (1.3)	11.1 (1.4)	3.9 (0.9)	6.4 (1.5)	84.7 (0.5)
<i>Halophila ovalis</i>	Leaf (17)	1.73 (0.06)	56.7 (0.5)	32.3 (0.4)	21.9 (0.3)	10.8 (0.4)	0.9 (0.08)	0.1 (0.0)	93.0 (0.4)
	Root/rhizome (20)	0.62 (0.02)	49.0 (0.6)	26.5 (0.4)	21.4 (0.3)	9.0 (0.3)	1.0 (0.2)	0.2 (0.0)	92.3 (0.3)
<i>Halophila trichostata</i>	Whole plant (3)	1.03 (0.1)	65.9 (1.1)	33.1 (0.6)	24.7 (0.9)	8.1 (0.5)	5.6 (0.9)	0.1 (0.0)	93.5 (0.8)
<i>Halophila decipens</i>	Whole plant (2)	0.75 (0.0)	51.5 (0.9)	27.5 (1.3)	20.4 (1.0)	4.9 (1.0)	2.6 (0.4)	0.1 (0.0)	92.5 (0.8)

All values expressed as % dry matter: mean (SE).

NIRS Calibrations

A calibration model for each of the constituents of interest was developed by regression of spectral absorbances and chemical analyses of each sample. We assessed a number of different regression methods including multiple linear regression and partial least-squares regression, but in all instances, modified partial least-squares (MPLS) regression (Martens and Naes, 1989; Shenk and Westerhaus, 1991b,c) was superior. MPLS uses all spectral data in contrast to alternative approaches such as multiple linear regression that uses a small subset of spectral data. We applied several transformations [calculation of first derivative (Osborne et al., 1993) and detrending (Barnes et al., 1989)] to the spectral data to remove the effect of particle size differences and to minimize autocorrelations between spectral measurements. These are recommended standard procedures (Anon, 1995).

The MPLS approach requires cross validation to prevent overfitting (i.e., using too many terms in the equation) and to select the optimum number of terms for each calibration equation (Osborne et al., 1993). Cross validation involves dividing the sample set into N groups and performing calibration on $N - 1$ groups with the remaining group being used as an independent validation set. This exercise is repeated until all samples have been cross-validated and residuals of each prediction are pooled to provide an SECV. A final regression model is then fitted to the data using the number of factors determined by the cross-validation procedure. Cross validation is an efficient procedure because all samples are used for both calibration and validation and avoids the need to set aside samples for a validation set (Osborne et al., 1993; Shenk and Westerhaus, 1993a). Another advantage of cross validation is that outliers from prediction residuals are identified readily (Shenk and Westerhaus, 1991a, 1993a; Osborne et al., 1993).

Table 2 Performance of combined plant part calibration equations for estimating the nutritional composition of seagrass

Component	N	Mean	Range	SEL	SEC	SECV	SECV/ SEL	r^2	Bias	Bias limit
Nitrogen	198	1.15	0.38–3.60	0.06	0.07	0.08	1.33	0.99	−0.01	0.04
Organic matter	192	61.4	32.0–77.4	2.00	1.93	2.17	1.08	0.96	0.01	1.16
Neutral detergent fiber	198	34.2	19.2–59.8	2.54	2.14	2.79	1.10	0.94	0.00	1.28
Acid detergent fiber	198	25.0	12.9–41.2	2.02	1.89	2.15	1.06	0.91	−0.02	1.13
Acid lignin	195	13.0	2.1–30.2	3.18	3.11	3.35	1.05	0.73	0.31	1.86
Starch	175	5.6	0.2–30.6	0.90	1.08	1.25	1.40	0.98	−0.36	0.65
Water-soluble carbohydrate	184	1.7	0.01–7.95	0.55	0.58	0.60	1.09	0.90	0.02	0.35
<i>In vitro</i> dry matter digestibility	198	86.7	62.7–98.1	2.48	2.52	2.76	1.11	0.86	−0.19	1.45

All values expressed as % dry matter. Number of samples used in the calibration (N), standard errors of laboratory analysis (SEL), calibration set (SEC), cross validation (SECV), ratio of SECV to SEL, bias, and limits of the bias associated with NIRS equations developed using a combined population of plant fractions (leaf + root/rhizome).

Predictions from Calibration Equations

Calibration equations obtained from MPLS regressions with cross validation for each nutritional attribute were used to predict constituent values of the entire sample population using the PREDICT algorithm of the NIRS3 software (Shenk and Westerhaus, 1992). These calibrations were monitored statistically for performance. Linear regressions of predicted values (\hat{Y}) vs. laboratory reference values (X) were determined for each constituent. Other statistics (r^2 , SEC, SECV, bias and bias confidence limits calculated as percentage of means, and slope) were computed through NIRS3 (Shenk and Westerhaus, 1992).

Results

Details of composition of the 198 samples used in the calibration set are presented in Table 1. There was no significant difference (paired t test, range of $P = 0.856$ – 0.072 ; $N = 20$) in predicted values for any of the eight constituents for material from large samples (>2 g) scanned in standard cells or small amounts (0.3–0.5 g) of the same material scanned in micro cells. The lower P value (0.072) was for water-soluble carbohydrates, values for which tended to vary little, and thus there is a greater tendency for the comparisons to approach significance. We also examined correlations between the values from the large and small cells. In all cases, the correlations were very close to 1, so we are satisfied that our estimates of composition of small samples of seagrass leaf and rhizome are valid.

Table 3 Performance of plant part specific calibration equations for estimating the nutritional composition of seagrass

Component	Roots and rhizomes fraction					Leaf fraction				
	N	SECV	r^2	Bias	Bias limit	N	SECV	r^2	Bias	Bias limit
Nitrogen	102	0.07	0.92	0.00	0.03	85	0.099	0.99	0.01	0.04
Organic matter	94	1.92	0.98	0.14	0.90	70	1.92	0.96	0.09	0.88
Neutral detergent fiber	102	2.52	0.89	0.19	1.25	78	2.27	0.96	0.38	1.01
Acid detergent fiber	102	1.61	0.95	-0.20	0.75	78	2.82	0.90	0.00	1.11
Lignin	100	2.76	0.79	-0.23	1.48	74	3.97	0.72	0.44	1.67
Starch	89	1.43	0.98	-0.45	0.69	64	0.13	0.26	0.23 ^a	0.07
Water-soluble carbohydrate	95	0.08	0.88	0.05	0.40	70	0.05	0.92	0.04	0.23
<i>In vitro</i> dry matter digestibility	104	2.78	0.91	-0.07	1.34	74	2.03	0.78	-0.26	0.96

Standard errors of cross validation (SECV), simple coefficient of determination (r^2), bias of the prediction, and limits of the bias associated with NIRS analysis of nutrient composition and *in vitro* digestibility using single plant-fraction prediction equations for root and rhizome fractions of seagrass.

^a See results for explanation of high bias estimate.

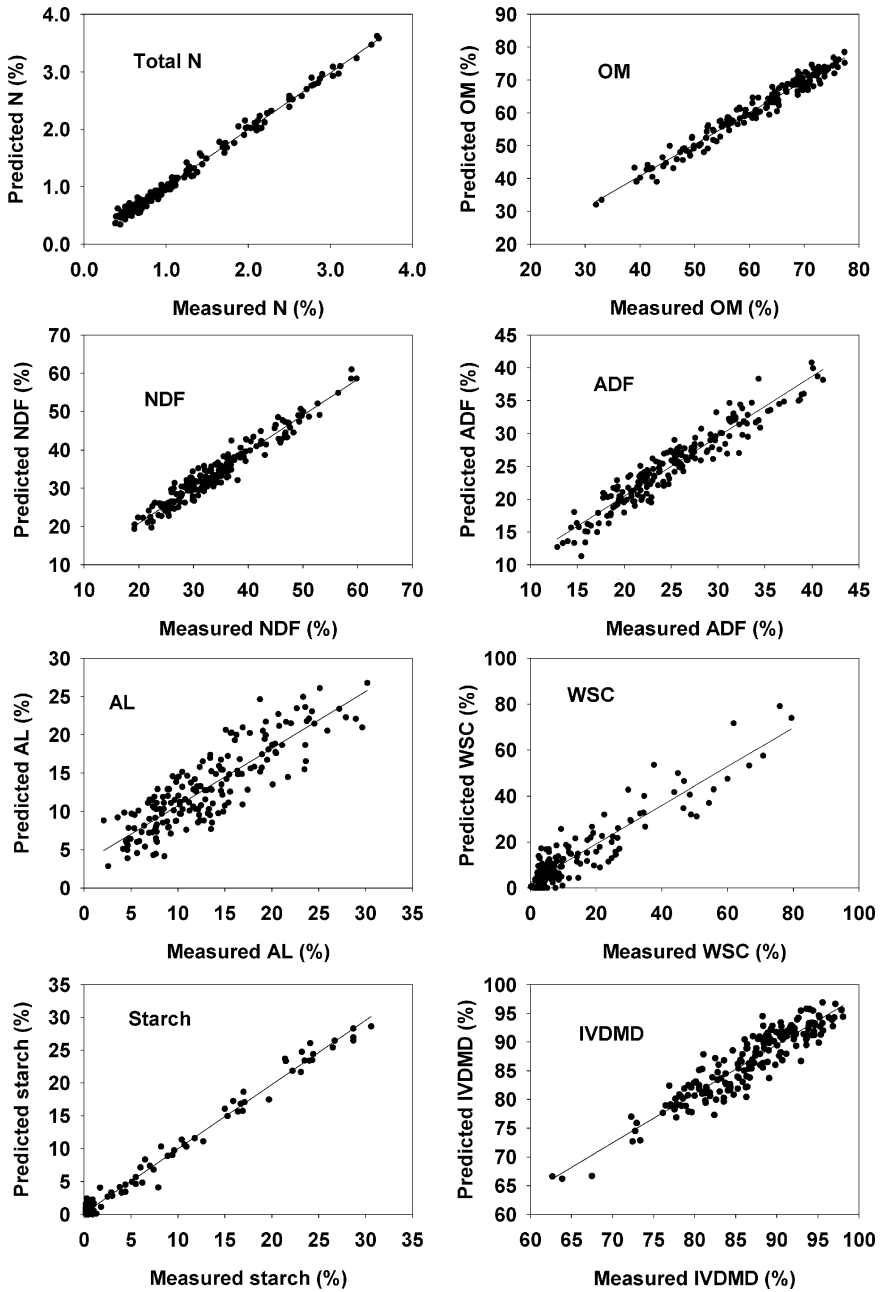


Fig. 1 Relationship between the nutritive value of seagrass measured in the laboratory and value predicted by near-infrared spectrometry. All values are expressed as % dry matter. For statistics, see Table 2

Laboratory precision [standard error of laboratory determination (SEL)] generally was excellent (Table 2) but was best for those constituents that were best characterized chemically. For example, acid lignin is a poorly defined, heterogeneous material that is difficult to analyze and had a relatively high SEL ($\bar{x} = 12.95$; SEL = 3.18). In contrast, total *N* is well defined and easy to analyze and had a relatively low SEL ($\bar{x} = 1.15$; SEL = 0.06).

Final or “global” calibration equations, developed by combining all samples irrespective of plant fraction or species, performed as well as those developed separately for roots and rhizomes (combined) and leaves (Tables 2 and 3). Only two constituents, organic matter and neutral detergent fiber, had consistently lower SECVs using calibrations based on single plant fractions than those determined from calibrations based on combining all samples. The calibration equation for starch in seagrass leaves was poor—reflected by an $r^2 = 0.26$. This was caused primarily by the small range of values for starch in leaves of seagrasses (0.2–0.8% starch of dry matter). In contrast, starch in roots and rhizomes was up to 30% of dry matter, and these data strongly influenced performance of the global equation.

The global set of MPLS-based calibration equations was superior to other regression methods in terms of SECV and coefficient of determination. Overall performance of calibration equations for the different constituents and *in vitro* digestibility of seagrasses was excellent (Table 2 and Fig. 1). This is best reflected by the closeness of the SECV/SEL ratio to 1.0. SEL values usually were low, reflecting precision of routine laboratory analyses. Proximity of values of the SECV/SEL ratio to unity (Table 2) confirms that NIRS analysis lost little precision relative to standard laboratory analytical techniques. Predicted values for all components except acid lignin agreed closely with the laboratory reference values and had r^2 values >0.90.

Discussion

This study has shown that NIRS is a suitable and powerful technique for measuring nutrient composition and *in vitro* digestibility of seagrass. The key advantage of using NIRS is that sampling regimes can be developed that satisfy statistical and biological criteria without being unduly constrained by the logistics of making traditional laboratory measurements of a large number of samples. In the context of the broader study for which these methods were developed (Aragones, 1996), only 17% of the total samples collected were subject to traditional laboratory analyses, and NIRS was used to predict the remaining 83%. This led to major savings of time and money.

Collecting duplicate spectra from a single sample takes at most 1–2 min, and, depending on how instrument software is configured, results for all calibrated constituents appear within seconds. Of course, developing calibration equations takes time, but selecting a suitable subset of samples to use in the calibration equation and validating the calibration equation take only a matter of hours in addition to standard laboratory work to chemically analyze the subset. Clearly, NIR analysis is suitable for larger data sets rather than those containing only a few samples.

A second advantage is that calibration equations are available for future studies, so that there will be no need to analyze an additional large sample set. We believe

that analysis of some samples from a future study is important to check that all new samples fall within the bounds of the existing calibration population. If not, then the calibration set can be expanded as we demonstrated here.

This study introduced several innovations that make NIRS particularly useful for ecological research in marine environments. First, development of a microsample cell demonstrated that multiple analyses could be made on small amounts of material. Sample cells for NIRS have been developed for a wide range of materials, and there is little constraint on the type and amount of material that is needed to collect spectra. In marine studies, there is often only a small amount of material available, especially from manipulative studies, and demonstration that NIRS can be used successfully for samples as low as 0.3 g of dry matter is significant. A current study (Bité and Lawler, unpublished data) is using even smaller samples with success using spinning cup inserts made by the manufacturers of the spectrophotometer.

Second, we have shown that broad-based or global calibration equations are just as good as those restricted to plant fractions. Traditionally, NIRS-based calibration equations have been narrow. For example, in analysis of protein in wheat grain, equations often are developed for wheat harvested in a single season at a single site (Batten, 1998). In contrast, especially ecological studies in marine environments usually focus on compositional changes across years, across sites, and among species (e.g., Lanyon and Marsh, 1995b). Our development of broad-based equations that incorporated leaves, roots, and detritus suggests that the method is particularly powerful for ecological studies.

Some studies (e.g., Meuret et al., 1993; Shenk and Westerhaus, 1993b) also have shown that whole plant-based calibrations differ only slightly from single-product calibrations. Smith and Flinn (1991) suggested that although development of broad-based calibrations is tedious, broad-based equations are more cost-effective than conventional techniques. This suggests that an initial broad-based calibration is more useful to develop, and expands with additional small extension populations, as required, than to gather another large set of samples for development of a specific new calibration each time this need arises. Shenk and Westerhaus (1993a) noted that development of global calibrations was made possible only by technological advances in desktop computing and software. This approach will be important for wildlife nutritional studies. The ability to limit calibrations to one equation per constituent, rather than one per constituent per plant part, will also increase the savings of adopting NIRS.

In NIRS, there always will be a trade-off between developing a robust equation of wide applicability and one that is tailored precisely to a particular set of conditions. For example, we were careful to ensure that all our samples were equilibrated to an equivalent dry matter content before scanning. This is because water has a strong absorbance in the near-infrared spectra, and this absorption can mask other absorbances that may be of interest. Similarly, we were careful to use a single, specified grinder and to keep that grinder in good order so that we produced a uniform particle size. Additional robustness of a calibration equation can be achieved by including variation for particle size and sample moisture. For example, if samples were to be prepared and scanned in several different laboratories, these issues might be accorded more weight. A small amount of precision may be sacrificed for a more robust equation, depending on the uses to which the equation is to be put. Potential users of this technique should not necessarily try to emulate the detail of the approaches that we used for equation development, but any laboratory

analyst has to think carefully about an equation's end use before deciding what analytical precision and accuracy are acceptable.

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A Modified Method for Determining Tannin–Protein Precipitation Capacity Using Accelerated Solvent Extraction (ASE) and Microplate Gel Filtration

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Abstract The protein precipitation assay used by Robbins et al., (1987) *Ecology* 68:98–107 has been shown to predict successfully the reduction in protein availability to some ruminants due to tannins. The procedure, however, is expensive and laborious, which limits its utility, especially for quantitative ecological or nutritional applications where large numbers of assays may be required. We have modified the method to decrease its cost and increase laboratory efficiency by: (1) automating the extraction by using Accelerated Solvent Extraction (ASE); and (2) by scaling and automating the precipitation reaction, chromatography, and spectrometry with microplate gel filtration and an automated UV–VIS microplate spectrometer. ASE extraction is shown to be as effective at extracting tannins as the hot methanol technique. Additionally, the microplate assay is sensitive and precise. We show that the results from the new technique correspond in a nearly 1:1 relationship to the results of the previous technique. Hence, this method could reliably replace the older method with no loss in relevance to herbivore protein digestion. Moreover, the ASE extraction technique should be applicable to other tannin–protein precipitation assays and possibly other phenolic assays.

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Keywords Tannins · Polyphenolics · Protein precipitation · Protein binding · Phenolic analysis · Ruminants · Nitrogen · Plant–herbivore interactions · Plant defense · Chemical ecological method

Introduction

Plant polyphenolics (tannins) are estimated to be the fourth most abundant biochemical in terrestrial biomass, following cellulose, hemicelluloses, and lignin (Hernes and Hedges, 2000). In rapidly cycling soft tissues such as leaves and needles, tannins can be more abundant than lignin (Hedges and Welikey, 1989; Benner et al., 1990). Because of their prevalence and biological importance, the determination of tannins and their impacts is important in many fields including ecology, nutrition, soil science, and human health (Haslam et al., 1999). With regards to plant–herbivore interactions, the effects of tannins can range from beneficial [e.g., as antioxidants (Hagerman et al., 1998a) and by increasing protein bypass in the foregut of ruminants (Waghorn and Shelton, 1997)] to toxic (Bryant et al., 1992; Reed, 1995; Provenza et al., 2000) or antinutritional (Feeny, 1976; Rhoades and Cates, 1976).

Due to the complexity of tannin interactions, there is currently no single analytical technique that adequately quantitates the suitability of tannin-containing feeds to herbivores. With regards to ruminants, Makkar (2005) recommends employing a suite of assays to assess the potential effects of tannins. Although such a battery of tests reveals much information, to date few tannin assays have been shown to directly correlate to a measurable biological effect in ruminants.

One type of assay that has been successful at predicting a biological effect of tannins in ruminants is protein precipitation. The reduction in protein availability due to tannins has been demonstrated *in vivo* and successfully developed as a laboratory assay (Martin and Martin, 1982; Robbins et al., 1987; Hanley et al., 1992). Subsequent studies have questioned whether the predictive equations conceived by Robbins et al. (1987) and validated by Hanley et al. (1992) for mule deer (*Odocoileus hemionus hemionus*) and black-tailed deer (*O. hemionus sitkensis*) are applicable to other ruminants. Awassi sheep (*Ovis aries*) and crossbred goats (*Capra hircus*) appear less adept at retaining protein when consuming tannin diets (Degen et al., 1995), and results from digestion trials with blue duikers (*Cephalophus monticola*) (Shipley and Felicetti, 2002) may also deviate from the predictions of Robbins et al. (1987). There is considerable observed variability in animal physiological responses to tannins (Martin and Martin, 1984; Robbins et al., 1991; Hagerman and Robbins, 1993), and structural differences are known to affect tannin function (Clausen et al., 1990; Ayres et al., 1997; Kraus et al., 2003). Therefore, such discrepancies to the equations of Robbins et al. (1987) are plausible, and reveal the need to examine carefully the utility of protein precipitation assays, when such assays are appropriate, and for which animals they apply.

The Degen et al. (1995) study investigated Awassi sheep and goats, which may produce few or no salivary tannin-binding proteins (Austin et al. 1989), and thus are understandably less capable of efficient protein digestion when consuming tannin-rich diets compared to deer. However, the Degen et al. (1995) study utilized the protein precipitation assay of Makkar et al. (1988) with “the conditions of Martin and Martin (1982).” The Makkar et al. (1988) assay was shown to correlate well with

the protein precipitation assay outlined in Hagerman and Butler (1978). However, the protein precipitation assay utilized by Robbins et al. (1987) (i.e., the Martin and Martin, 1982 assay) correlated poorly with the Hagerman and Butler (1978) assay at least for some oak species (Martin and Martin, 1982), discrepancies that Martin and Martin (1982) attributed to possible structural and protein-binding variation of the tannins between species. Considering the documented variation in protein precipitation due to tannin structural heterogeneity (Kraus et al., 2003) and the diversity of tannins found in plants, it is not surprising that the correlation between assays and protein digestion *in vivo* is not high. In fact, it emphasizes that the use of alternative assays to predict protein digestion must be carefully validated against the original assays or against *in vivo* data.

In addition, both the Degen et al. (1995) and Shipley and Felicetti (2002) studies include a small sample size of forages, which limits their comparison to the Robbins et al. (1987) predictions for protein digestion. Therefore, it is difficult to say whether the equations of Robbins et al. (1987) are useful for ruminants other than mule deer and black-tailed deer. Regardless, even if these equations are not inclusive of all ruminants, future comparative work on other herbivores with this assay system will lead to greater understanding of tannin chemistry and herbivore digestive efficiency and adaptation.

With regard to the role tannins play in reducing protein availability to ruminants, numerous assays are currently available (see Scalbert, 1992; Waterman and Mole, 1994; Hagerman et al., 1997; Hoffmann et al., 2002; Makkar, 2003; Henson et al., 2004). Makkar (2005) suggests that the radioisotope technique (Hagerman and Butler, 1980; Hagerman et al., 1998b) is particularly attractive for determining protein availability to ruminants because of its sensitivity and recent simplification (Henson et al., 2004). To the best of our knowledge, only the assay used by Robbins et al. (1987) has been directly and quantitatively linked to protein digestion in ruminants. Moreover, the available techniques rely on slow and potentially inefficient extraction methodologies, are not conducive to laboratory automation, and do not minimize waste production.

The goal of this work was to modify the methodology of Robbins et al. (1987) to accelerate and simplify the extraction process by using Accelerated Solvent Extraction (ASE), and to scale the protein-binding assay to a microplate system to improve its efficiency and cost-effectiveness. Our objectives were to optimize the system for time and cost while maintaining the protein-binding sensitivity, equivalence of results, and predictive capabilities of the original method employed by Robbins et al. (1987) for ruminant (e.g., Cervid) herbivores.

Methods and Materials

Reagents and Sample Preparation

Bovine serum albumin (BSA) (Albumin, Bovine, Globulin-free, Catalogue No. A-7638) and Sephadex G-25 (Medium grade, Catalogue No. G25150) were obtained from Sigma (St. Louis, MO, USA). Sephadex G-25 PD-10 columns were obtained from Amersham Biosciences (Piscataway, NJ, USA). Acetate buffer (0.2 M, pH 4.9) was prepared as described by Martin and Martin (1982). All methanol used was HPLC-grade and all water used was 18 Ω Mohm. Leaf and stem samples were

collected in the field, immediately frozen on dry ice, and subsequently lyophilized. The dried samples were ground in a Wiley mill (40-mesh) and stored in airtight containers at room temperature out of direct sunlight until analyzed.

Extraction of Phenolics

Hot aqueous methanol extraction was conducted with a sonicator and heat block as described by Robbins et al. (1987). Briefly, 1 g plant material was extracted $\times 3$ in 15 ml hot aqueous methanol, filtered, and combined. The final solution was brought to 50 ml volume.

ASE extraction was conducted on a Dionex Model ASE-200 solvent extractor using aqueous methanol (50:50, vol/vol) and 11 ml cells with three cellulose filter disks. The extraction consisted of two 5-min static (extraction) periods at 1500 psi N_2 and 100°C. One g plant material was extracted, and the subsequent supernatant from the extractions and washes totaled approximately 30 ml, which were then diluted to 50 ml volume.

For additional comparison, we extracted each sample by using a cold aqueous methanol technique. One g plant material was extracted $\times 4$ (15 min each, 0°C) in a sonicator with aqueous methanol (50:50, vol/vol). The four extracts were combined and brought to 50 ml volume.

Macro BSA Precipitation Assay

The technique of Martin and Martin (1982), as modified by Robbins et al. (1987), was followed. For each plant sample, 1.39 ml of a 1 mg BSA/ml acetate buffer stock solution were combined with four aliquots of plant extract, diluted such that a range between 0.1 and 0.7 mg of BSA was precipitated from the stock BSA solution. Precipitation reactions were allowed to proceed under refrigeration for 12 hr, after which samples were centrifuged. The supernatant was decanted and filtered through a Sephadex G-25 PD-10 column and collected in a clean test tube. The precipitate was washed with 0.51 ml acetate buffer, centrifuged, and the supernatant was decanted into the Sephadex column again. Finally, the column was washed with 3.5 ml buffer to optimize recovery of protein. The filtered supernatant was assayed by using a standard Bradford protein assay (Bradford, 1976). For extraction technique comparison, BSA precipitation capacity was estimated from an optimal dilution (i.e., where precipitation of protein was approximately 50%). This dilution level was determined by trial and error from at least 4 dilution trials per forage. Duplicate extractions and duplicate BSA precipitations for each extraction were conducted to assess the precision and accuracy of the extraction technique. For BSA precipitation assay comparison, precipitation capacity was estimated from a regression of at least three different dilutions of plant extract.

Microplate BSA Precipitation Assay

To conserve materials and automate the protein precipitation assay, we scaled the volumes and concentrations of BSA, plant extracts, and Bradford assay such that all reactions, chromatography, and spectrometry could be carried out in a microplate environment. To do so, aliquots of forage extract between 2.7 and 59.1 μg (dry weight basis) diluted to 100 μl were combined with 150 μl BSA (310 $\mu\text{g/ml}$) in

acetate buffer on a 2 ml well volume microplate. The entire plate was then vortexed, capped, and allowed to precipitate overnight at 4°C. Fourteen standards ranging in concentration from 5.0 to 37.4 µg/ml BSA and two blanks were prepared on each microplate by using aqueous methanol (50:50, vol/vol), 310 µg BSA/ml solution, and acetate buffer, such that volumes of each reagent were identical to that of the unknowns. The following day, a Sephadex filter plate was prepared by pipetting 300 µl Sephadex G-25 slurry (1.0 g/5 ml acetate buffer, allowed to swell for at least 3 hr) into each well of 350 µl cellulose acetate filter microplate (Whatman Unifilter). The Sephadex wells were packed by centrifuging the filter plate (750 g) for 5 min at 20°C, and discarding the filtrate. The sample preparation plate was then centrifuged (750 g, 5 min, 20°C) to sediment the precipitated pellet, and 40 µl supernatant from each well were added to the center of each Sephadex-packed well on the filter plate. The filter plate was then centrifuged (750 g, 5 min, 20°C), and the filtered supernatant was collected on an assay microplate (400 µl flat-bottomed polystyrene). One hundred sixty µl of diluted Coomassie Brilliant Blue G-250 dye reagent [(1 part dye, 4 parts distilled water; Bio-Rad Protein Dye Reagent, Bio-Rad Corporation)] were then added to each well, and the entire plate was carefully vortexed to mix each sample, and centrifuged (500 g, 40 sec, 20°C) to eliminate any bubbles formed during pipetting of the dye. After 6 min incubation (Bradford, 1976), the absorbance at 595 nm was read on a microplate spectrophotometer (Molecular Devices SpectraMax Plus³⁸⁴). Absorbance was transformed into µg of BSA by the use of blanks and a standard curve on each microplate. BSA concentrations of the unknowns were calculated from the standard curve, and precipitated BSA was calculated by difference.

Results

Tannin Extraction Method Comparison

Protein precipitation capacity of plant samples ranged between 0.06 ± 0.01 and 0.61 ± 0.02 mg BSA/mg forage dry matter for the hot methanol extraction method, $0.08 \pm$

Table 1 Bovine serum albumin (BSA) precipitation capacities of eight plants compared by extraction method^a

Plant sample	Extraction method		
	Hot	ASE	Cold
<i>Epilobium angustifolium</i> leaf	0.61 ± 0.02	0.66 ± 0.02	0.37 ± 0.01
<i>Betula papyrifera</i> leaf	0.49 ± 0.02	0.47 ± 0.05	0.27 ± 0.01
<i>Salix pulchra</i> leaf 1	0.33 ± 0.01	0.36 ± 0.01	0.25 ± 0.01
<i>Equisetum sylvaticum</i>	0.33 ± 0.01	0.27 ± 0.02	0.20 ± 0.10
<i>Salix alaxensis</i> stem	0.28 ± 0.01	0.23 ± 0.02	0.17 ± 0.01
<i>Salix pulchra</i> stem	0.22 ± 0.02	0.17 ± 0.02	0.11 ± 0.02
<i>Betula papyrifera</i> stem	0.12 ± 0.02	0.08 ± 0.01	0.07 ± 0.01
<i>Salix pulchra</i> leaf 2	0.06 ± 0.01	0.11 ± 0.00	0.04 ± 0.00

^a Hot extraction was performed as described by Robbins et al. (1987). ASE and cold extraction methods are outlined above. Precipitation capacities are expressed as mean \pm standard deviation. All values are expressed in mg BSA precipitated/mg forage dry matter. $N = 2$ for each plant sample.

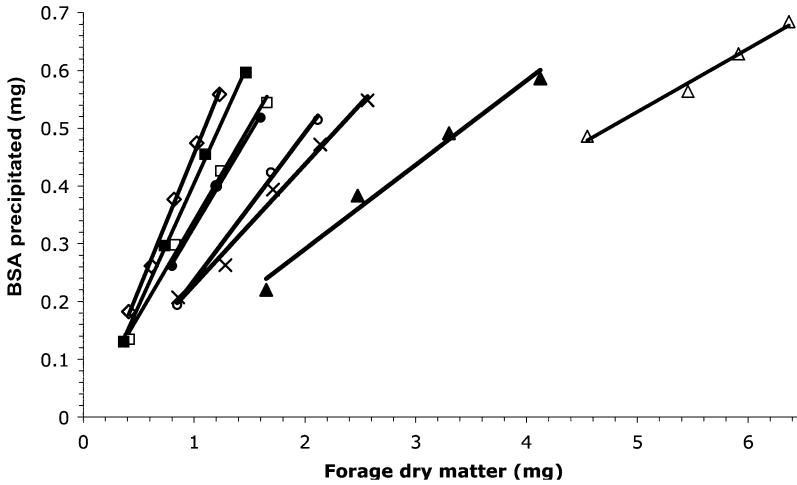


Fig. 1 The macro BSA precipitation capacities (Martin and Martin, 1982; Robbins et al., 1987) of eight plant extracts. Symbols and regression equations for each plant sample type: Δ *Betula papyrifera* stem, $Y = -0.0183 + 0.1093X$; \blacktriangle *Salix pulchra* stem 1, $Y = -0.0018 + 0.1461X$; \times *Salix alaxensis* stem, $Y = 0.0201 + 0.2085X$; \circ *Salix pulchra* stem 2, $Y = -0.0185 + 0.2553X$; \bullet *Salix pulchra* leaf 1, $Y = 0.008 + 0.3213X$; \square *Betula papyrifera* leaf, $Y = 0.011 + 0.3283X$; \blacksquare *Salix pulchra* leaf 2, $Y = -0.0195 + 0.4242X$; \diamond *Epilobium angustifolium* leaf, $Y = -0.0154 + 0.4716X$. Coefficients of determination for all regressions are 0.98 or higher

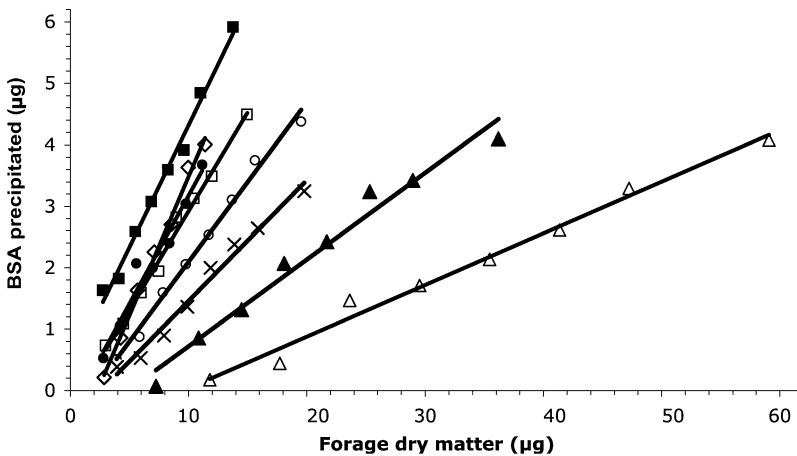


Fig. 2 Microplate BSA precipitation capacities of eight plant extracts. Symbols and regression equations for each plant sample type: Δ *Betula papyrifera* stem, $Y = -0.8065 + 0.0842X$; \blacktriangle *Salix pulchra* stem 1, $Y = -0.6844 + 0.1411X$; \times *Salix alaxensis* stem, $Y = -0.5096 + 0.1965X$; \circ *Salix pulchra* stem 2, $Y = -0.4995 + 0.2598X$; \square *Betula papyrifera* leaf, $Y = -0.293 + 0.3214X$; \bullet *Salix pulchra* leaf 1, $Y = 0.3488 + 0.3529X$; \blacksquare *Salix pulchra* leaf 2, $Y = 0.3427 + 0.3977X$; \diamond *Epilobium angustifolium* leaf, $Y = -1.0266 + 0.4516X$. Coefficients of determination for all regressions are 0.96 or higher

0.01 and 0.66 ± 0.02 mg/mg for ASE extraction, and 0.07 ± 0.01 and 0.37 ± 0.01 mg/mg for the cold methanol extraction (Table 1). Mean relative error was 7.1% for the hot methanol extraction technique, 7.4% for ASE extraction, and 11.9% for cold extraction.

There was a high correlation and a nearly 1:1 relationship between BSA precipitation capacities of the forages for the ASE extraction and the hot extraction techniques (Table 1, $r^2 = 0.95$, slope = 1.06). The homogeneity of the slope comparing techniques to the line $y = x$ revealed there was no significant difference in BSA precipitation capacity between ASE and hot methanol extraction techniques ($t = 0.46$, $P = 0.66$). The cold extraction technique correlated well with hot extraction ($r^2 = 0.95$) and ASE extraction ($r^2 = 0.94$) BSA precipitation capacities, but less protein was precipitated compared to the hot and ASE extraction techniques (slope = 0.58, $t = -6.53$, $P < 0.001$; and slope = 0.54, $t = -6.86$, $P < 0.001$, respectively).

BSA Precipitation Assay Method Comparison

For the comparison of the macro- and microplate BSA precipitation assays, we reextracted the plant samples by using the ASE extraction method. The macroassay of the eight plant samples revealed BSA precipitation capacities ranging between 0.11 and 0.47 mg BSA/mg forage dry matter, with coefficients of determination for

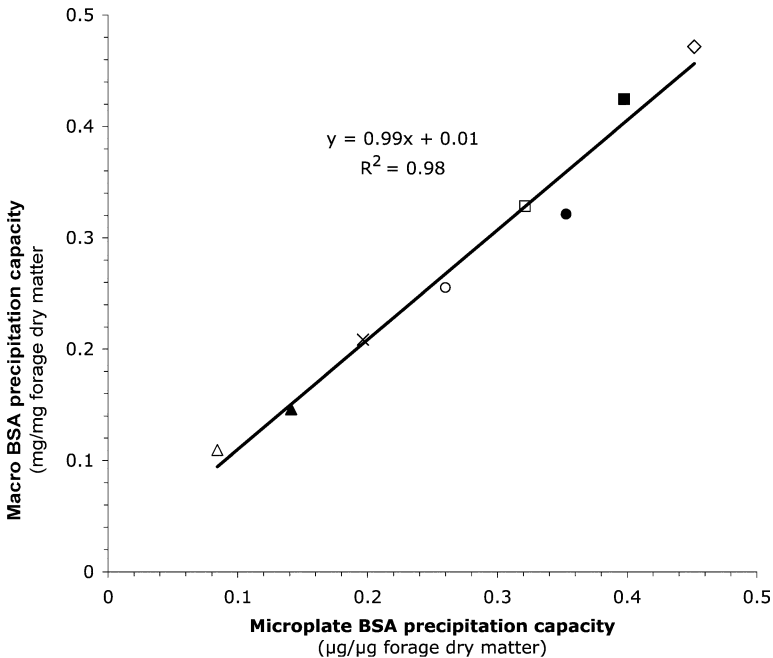


Fig. 3 BSA precipitation capacity comparison between the macro technique (Martin and Martin 1982; Robbins et al., 1987) and the new microplate technique. Symbols representing each plant sample type: ◇ *Epilobium angustifolium* leaf; ■ *Salix pulchra* leaf 2; ● *Salix pulchra* leaf 1; □ *Betula papyrifera* leaf; ○ *Salix pulchra* stem 2; × *Salix alaxensis* stem; ▲ *Salix pulchra* stem 1; △ *Betula papyrifera* stem

each sample ranging between 0.98 and 0.99 (Fig. 1). In comparison, the microplate method resulted in BSA precipitation capacities of the same plant extractions ranging between 0.08 and 0.45 $\mu\text{g BSA}/\mu\text{g}$ forage dry matter, with corresponding coefficients of determination ranging between 0.96 and 0.99 (Fig. 2).

The BSA precipitation methods were highly correlated, and produced a nearly 1:1 relationship (Fig. 3, $r^2 = 0.98$, slope = 0.99). The homogeneity of the slope comparing techniques to the line $y = x$ revealed there was no significant difference between BSA precipitation capacity techniques ($t = -0.22$, $P = 0.83$).

Discussion

Tannin Extraction Method Comparison

Numerous methods exist for extraction of plant phenolics, but the standardization of a particular technique is integral to preserving the specific phenolic compounds and their reactivities of interest to the analyst (Hagerman, 1988; Cork and Krockenberger, 1991; Waterman and Mole, 1994; Mueller-Harvey, 2001). We show here that ASE extraction of tannins appears to be a suitable alternative to the hot methanol extraction technique used by Robbins et al. (1987) for the BSA precipitation assay. The relationship between extraction techniques is nearly 1:1 and correlation is high (slope = 1.06, $r^2 = 0.95$), suggesting minimal change in the protein precipitation capabilities of the extracted tannins within and between samples when extracted under high pressure and temperature.

The repeatability of the ASE extraction technique compared to the hot methanol technique is nearly identical (7.4% and 7.1% mean relative error, respectively). However, we feel the ASE extraction technique is superior to the manual extraction technique because it is automated and thus minimizes potential human error, and it is rapid and, therefore, allows much higher throughput in the laboratory. The hot methanol extraction technique used by Robbins et al. (1987) is prone to sample spillage and loss due to the boiling aqueous methanol solutions. Moreover, although we used 11 ml cells in our ASE, which constrained our extractions to approximately 1 g of dried plant material, and resulted in approximately 50 ml final dilutions, smaller cells (1.5 ml volume) are available for the extraction process. The smaller cells could reduce sample size requirements, and reduce final extraction volumes to approximately 10 ml or less. Hence, there are potentially further savings in solvent costs and waste production, while at the same time allowing the investigator to use much smaller samples (i.e., individual leaves) to obtain equivalent information. As a precaution, however, we feel that analyzing small samples of potentially nonhomogeneous solids can be a major source of experimental error, so there may be advantages to using 1 g samples of the dry solid.

It is likely that ASE extraction of tannins is a useful extraction technique for other popular protein precipitation assays (i.e., Makkar et al., 1988; Hagerman et al., 1998b; Henson et al., 2004) and possibly other phenolic assays (see Scalbert, 1992; Waterman and Mole, 1994; Hagerman et al., 1997; Makkar, 2003). Further investigation into the usefulness of ASE for polyphenolic extraction and use in alternative assays is recommended.

BSA Precipitation Assay Method Comparison

As noted earlier, several assays are currently available for the investigation of protein–tannin binding interactions, and each assay has its advantages and disadvantages. Many binding assays appear to provide similar results (e.g., Martin and Martin, 1982; Hagerman, 1987). Nevertheless, to our knowledge, only the binding assay developed by Martin and Martin (1982) and used by Robbins et al. (1987) has been directly and quantitatively linked to protein digestion in ruminant herbivores. Protein binding capacities of our optimized microplate assay and the Robbins et al. assay were nearly identical (Fig. 3), strongly suggesting that the two techniques are equivalent, and that the microplate assay should be as reliable and accurate in predicting protein digestion in ruminants as the Robbins et al. (1987) assay.

The microplate protein–tannin binding assay was developed in large part to reduce the costs of the assay as well as to increase laboratory efficiency. The macrotechnique used by Robbins et al. (1987) and others requires the use of at least four PD-10 chromatographic columns as well as numerous other laboratory consumables for each plant sample (e.g., numerous pipette tips, several disposable UV–VIS spectroscopy cuvettes, test tubes, and substantial amounts of Bradford dye reagent). Alternatively, the microplate assay technique can accommodate at least 10 samples per 96-well plate, and requires substantially smaller amounts of reagents and Sephadex G-25 per sample. Although the microplate technique consumes 3 microplates per run (including a relatively expensive filter plate), it reduces overall laboratory consumable expenses 8-fold (e.g., \$25 per sample for the Robbins et al. assay vs. \$3 per sample for the microplate assay, at current prices), and generates approximately 6-fold less organic waste.

Likewise, the microplate assay reduces laboratory preparation time by nearly half, particularly because many operations can be performed by using a multichannel pipettor, and because the spectroscopy and its analysis is automated and rapid. Moreover, the microplate method lends itself to robotic automation, making possible further and substantial reductions in preparation time. Robotic plate preparation would likely increase precision of the assay as well.

Although we attempted to optimize and automate all aspects of the microplate assay, we are certain that further optimization and automation can be accomplished. We were least satisfied with the preparation and quality of the Sephadex column beds, and we expect that improvements in this process will lead to more precise results. A consistent Sephadex gel bed volume and density is critical to the cleanup and consistent recovery of the samples prior to protein assay, and their preparation by using the slurry method requires practice and care. We recommend the use of a column loader (such as that produced by Millipore) and powdered G-25 Sephadex beads for preparing the filter plate gel columns. With our current filter plate system, a column loader delivering approx. 60–65 μl of beads would be ideal, but unfortunately, loaders are currently only available for 45 or 80 μl deliveries.

In summary, we have shown that the combination of ASE extraction and the microplate BSA precipitation assay produces virtually equivalent results to the methods used by Martin and Martin (1982) and Robbins et al. (1987), and the new technique sacrifices minimal precision and accuracy. Consequently, the new technique preserves the predictive capabilities of the macrotechnique for estimating the protein availability of tanniferous plants to ruminant herbivores. The new technique reduces laboratory time more than 2-fold per sample, and decreases the

cost of the protein–tannin binding assay more than 8-fold. We feel that precision and efficiency can be further improved by using robotic automation, and sample dry weight requirements and chemical waste production may be reduced by using smaller ASE extraction cells. The reduction in cost and the increase in analytical capacity should provide ecologists and ruminant nutritionists greater opportunity at a broader scale than previously feasible for exploring the reduction of available protein to ruminant herbivores due to tannins.

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Early Herbivore Alert: Insect Eggs Induce Plant Defense

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Abstract Plants are able to “notice” insect egg deposition and to respond by activating direct and indirect defenses. An overview of these defenses and the underlying mechanisms is given from a tritrophic perspective. First, the interface between plant and eggs is addressed with respect to the mode of attachment of eggs on the plant surface. It is elucidated which plant cells might respond to components from insect eggs or the egg deposition. The scarce knowledge on the elicitors associated with the eggs or the egg-laying female is outlined. Since endosymbiotic microorganisms are often present on the eggs, and microorganisms are also abundant on the leaf surface, the role of these hidden players for eliciting oviposition-induced plant responses is considered. Furthermore, the question of which physiological and molecular processes are induced within the plant in response to egg deposition is addressed. Second, studies on the response of the herbivorous insect to oviposition-induced plant defenses are outlined. Third, the importance of oviposition-induced plant volatiles and contact cues for host and prey location of parasitoids and predators is discussed in the context of other informative chemicals used by carnivores when searching for food. Finally, physiological and ecological costs of oviposition-induced plant responses are addressed.

Keywords Plant defenses · Oviposition · Egg · Secretion · Endosymbiotic microorganisms · Elicitor · Terpenoids · Green leaf volatiles

Introduction

Most herbivorous insects start attacking a plant by laying eggs on it. For example, many Lepidoptera and folivorous Hymenoptera do not feed upon leaves during the adult stage, but females deposit their eggs on those plants or plant parts where

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Table 1 Oviposition-induced plant responses acting directly against the herbivore or addressing the third trophic level

Plant	Herbivore	Effect of egg deposition	Induction by	Elicitor	Induction time	Reference
Direct defenses						
Pea	Bruchids	Neoplasma growth (local response only)	Egg cement No (ovipositional) wounding	3-Hydroxy-propanoates	1 d	Doss et al., 1995, 2000; Oliver et al., 2000
Rice	Planthoppers	Ovicidal substance (local response)	Egg on watery oviposition lesion Ovipositional wounding	Unknown	2 d	Seino et al., 1996; Suzuki et al., 1996
Potato	Colorado potato leaf beetle	Hypersensitive response (local response only)	Egg shell or egg cement No (ovipositional) wounding	Unknown	1 d	Balbyshv and Lorenzen, 1997
Cabbage	Large/small white cabbage butterfly	Hypersensitive response (local response only)	Egg cement No (ovipositional) wounding (?)	Unknown	1 d	Shapiro and DeVay, 1987
	Large white cabbage butterfly	Oviposition deterrence (local response, systemic response indicated)	Egg on undamaged surface No (ovipositional) wounding (?)	Unknown	1 d	Blaakmeer et al., 1994

Indirect defenses						
Elm	Elm leaf beetle	Oviposition-induced volatiles attract parasitoids (local + systemic response)	Egg on surface-damaged leaf Female removes leaf epidermis at oviposition site	Oviduct secretion coating the egg	3 hr–5 d	Meiners and Hilker, 1997, 2000; Meiners, unpublished data
Pine	Pine sawfly	Oviposition-induced volatiles attract parasitoids (local + systemic response)	Egg in slit pine needle Female saws a needle tangentially to lay eggs inside	Oviduct secretion coating the eggs; a small protein or peptide	3 d	Hilker et al., 2002a, 2005
Bean	Bug	Oviposition-induced volatiles attract parasitoids (local + systemic response)	Egg on undamaged surface No (ovipositional) wounding But plus feeding damage	Unknown	3–4 d	Colazza et al. 2004a,b
Brussels sprouts	Large white cabbage butterfly	Oviposition-induced leaf contact cues arrest parasitoids (local response)	Egg on undamaged surface No (ovipositional) wounding	Secretion of accessory gland which glues the egg onto the surface	3–4 d	Fatouros et al., 2005a

hatching larvae will find suitable food. Also, in those herbivores where adults and larvae do not have separate feeding niches, such as, for example, in many herbivorous Coleoptera and Hemiptera, the choice of a plant for egg deposition is the initial step for starting a further generation damaging the plant by feeding activity.

Numerous studies address plant defenses that are induced by feeding herbivores (Tumlinson et al., 1993; Karban and Baldwin, 1997; Agrawal et al., 1999; Dicke and van Loon, 2000; Turlings and Wäckers, 2004). These plant responses start when feeding damage and loss of foliage have already begun. However, a plant is able to act “just in time” to prevent herbivores from feeding at all. Plants have evolved several mechanisms and respond to the very first step of insect attack. Induced direct and indirect plant defenses as responses to insect egg deposition are known. These oviposition-induced reactions aim to rid the plant of eggs and/or kill them, thus preventing feeding damage by the larvae that would hatch from these eggs (Hilker and Meiners, 2002; Hilker et al., 2002b).

Direct plant defenses induced by insect egg deposition may affect either the eggs themselves or the egg-laying female (Table 1). Egg deposition can induce the formation of a neoplasm that elevates the egg from the plant surface. Such exposed eggs easily drop off the plant (Doss et al., 1995, 2000). Rice plants even produce an ovicidal substance that kills the eggs in response to egg deposition by planthoppers (Seino et al., 1996; Suzuki et al., 1996; Yamasaki et al., 2003). Another mechanism to get rid of the eggs is to form necrotic tissue where eggs are laid. Such a hypersensitive response detaches the eggs from the plant surface and makes them fall to the ground. Larvae hatching on the ground suffer a rarely high mortality and rarely find their way back to their host plant (Shapiro and DeVay, 1987; Balbyshev and Lorenzen, 1997). A further direct defense mechanism induced by egg deposition addresses the egg-laying female by rendering those leaves deterrent that already carry eggs or that are adjacent to egg-laden leaves (Blaakmeer et al., 1994).

The known indirect plant defense mechanisms induced by egg deposition act by supporting egg parasitoids to locate their hosts (Table 1). For three tritrophic systems, it has been shown that insect egg deposition induces a plant volatile pattern that attracts egg parasitoids (elm: Meiners and Hilker, 1997, 2000; pine: Hilker et al., 2002a; beans: Colazza et al., 2004a), whereas the study of a further system consisting of *Brassica*, *Pieris*, and *Trichogramma* suggests that egg deposition induces the change of plant surface chemicals, thus arresting the egg parasitoids by contact cues in the vicinity of the eggs (Fatouros et al., 2005a).

Up to now, leaves especially are known as the plant organs responding to insect egg deposition by defensive mechanisms (see Table 1). The only exceptions are fruits, i.e., pea pods forming neoplasms in response to egg deposition (Doss et al., 1995, 2000), and stems of rice plants producing ovicidal benzyl benzoate after egg deposition (Seino et al., 1996). No seeds are known to “whisper for help” (Steidle et al., 2005) by emission of oviposition-induced volatiles, even though small seeds also can emit sufficient amounts of volatiles to attract herbivores, regardless of whether they carry eggs or not (Ignacimuthu et al., 2000). Although it is not known if egg deposition can induce defensive responses in, e.g., seeds, roots, or flowers, we suggest that these and plant organs other than leaves are capable of reacting to the presence of insect eggs and activating effective defensive mechanisms.

Almost all plant organs are well known to be able to form galls when attacked by gall insects (Shorthouse and Rohfritsch, 1992). Whereas numerous plant galls are induced by larval feeding activity in plant tissue, several plant galls are induced by

oviposition or by both ovipositional wounding and larval feeding activity (Rohfritsch, 1992; Hilker et al., 2002b). Gall formation of plants may be considered as a mechanism initially evolved for defensive purposes by isolating the galling insect, which in turn has counteradapted to this plant response and evolved the ability to exploit the growing plant tissue for shelter and nutrition (Zweigelt, 1931). Thus, studies on the response of plant organs other than leaves to insect egg deposition will be necessary to understand the plasticity of plant defenses against this first step of attack by herbivores.

When considering plants in the tritrophic systems studied so far for their response to insect egg deposition, numerous questions on both the mechanisms and ecological functions arise. How does the plant “notice” an egg deposition? Is there any evidence that the egg-laying herbivore female counteradapts or even exploits the plant’s response to egg deposition? How does the egg parasitoid recognize oviposition-induced volatile patterns within a jungle of other volatile cues? How important are these oviposition-induced plant cues for host location behavior of egg parasitoids in comparison to other informative chemicals? In the following, these and other questions will be addressed with respect to molecular, physiological, behavioral, and evolutionary aspects.

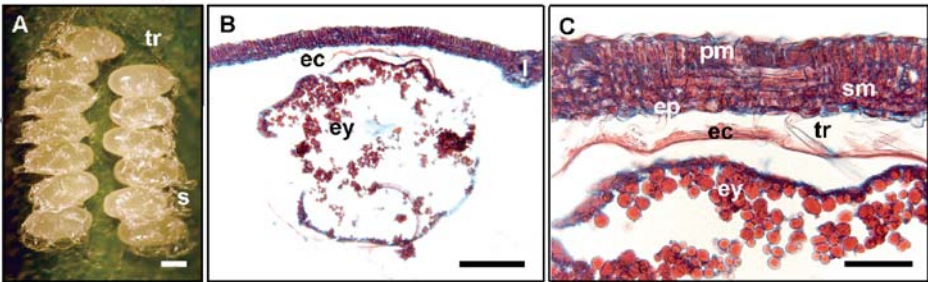
The Plant and its Response to Insect Egg Deposition

The Interface between Plant and Eggs

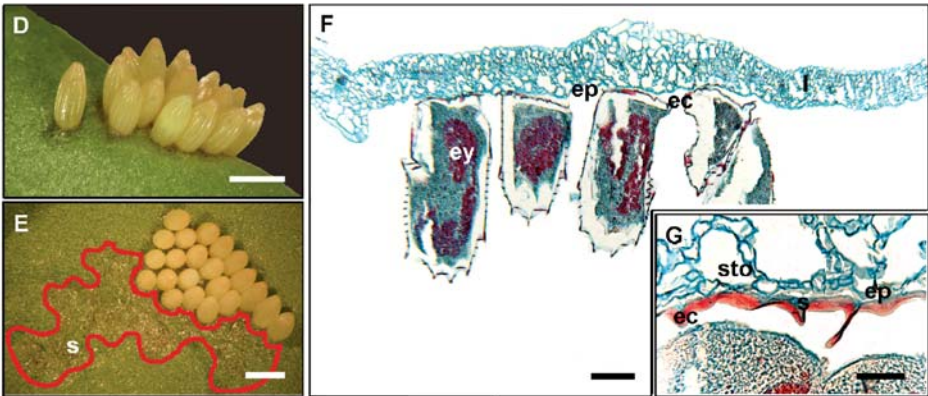
Since so far mostly leaves have been studied and found to display oviposition-induced defenses, a closer morphological view of the interface between leaves and eggs of herbivorous insects is presented here (Table 1). The degree of how tightly an egg is associated with the leaf varies greatly and may also depend on the physicochemical characteristics of the leaf surface (Müller and Riederer, 2005). Eggs may be attached just loosely to a leaf like the eggs of the willow leaf beetle, *Phratora vulgatissima*, which are laid on the lower side of a willow leaf (Fig. 1A, B). In this species, the secretion coating the eggs has only very loose contact with the plant surface. The egg adheres especially to the trichomes of the lower plant surface (Fig. 1C). The eggs of many lepidopteran species are tightly glued onto the lower leaf surface. In *Pieris brassicae*, sometimes surface scratches are visible on Brussels sprouts leaves with eggs (Nina Fatouros, personal communication). In the samples studied here, the surface of Chinese cabbage leaves with eggs has not been observed to be damaged before oviposition (Fig. 1D). The transparent shiny secretion of the accessory glands of *P. brassicae* females is not only found directly associated with the eggs, but also smeared on leaf tissue around the egg mass. Where secretion touches the leaf surface, the tissue becomes brownish (Fig. 1E). It is not yet known whether this is due to the closure of stomata by the secretion (Fig. 1F, G) and subsequent death of the cells, or whether it is a hypersensitive response. In contrast, some herbivorous insects damage the leaf at the site of oviposition. This damage may be limited to the epidermis and the adjacent spongy parenchyma, as has been observed for the elm leaf beetle (Fig. 1H–J). The elm leaf beetle prefers to lay eggs on leaf veins (Meiners and Hilker, 1997), thus getting close to the vascular bundles after removal of the epidermis (Fig. 1K, L). Other herbivorous insects severely damage leaf tissue, as has been described for the egg deposition of pine sawflies, which slit a needle tangentially with the ovipositor, remove parenchymatic tissue, and damage one vascular bundle (Hilker et al., 2002a).

The different degrees of egg attachment on or insertion into leaf tissue suggest that different cells of a leaf are able to perceive the information that an egg has been laid. When eggs of *P. brassicae* just touch the wax layer, egg components eliciting the plant's defensive response probably need to move across the wax layer to the epidermal cell wall (Fatouros et al., 2005a). In the elm leaf beetle, the elicitor inducing the oviposition-induced defense (Meiners and Hilker, 2000) is probably interacting with the parenchymatic tissue. Since sawflies deeply disrupt pine needle tissue, all types of damaged cells (e.g., epidermal cells, parenchymatic tissue, endodermal cells) need to be considered as being able to perceive the information of a recent egg deposition (Hilker et al., 2002a).

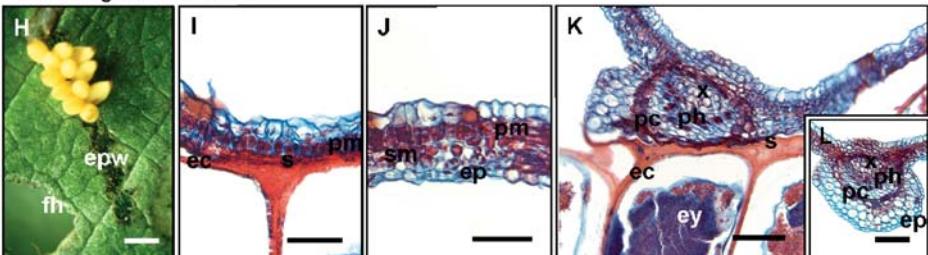
Phratora vulgatissima



Pieris brassicae



Xanthogaleruca luteola



Microorganisms in the Interface between Plants and Eggs

Are leaves, insect eggs, and/or their mothers the only interacting organisms for oviposition-induced plant responses, or are hidden players such as microorganisms involved (Dicke and Hilker, 2003)? When damage is inflicted to leaf tissue prior to oviposition (see Table 1), microorganisms might invade wounds and contribute to the oviposition-induced response. These microorganisms or their products might be transferred from the insect into the wound. Furthermore, microorganisms living on the plant surface where they may occur as symbionts, commensalists, or phytopathogens might be involved in the induction process (Dickinson and Preece, 1976; Morris et al., 1996; Kinkel, 1997; Varma et al., 2004). However, wounding is known to be insufficient to induce indirect defensive responses that attract egg parasitoids. In bean leaves, feeding damage *per se* does not induce the release of volatiles that attract the egg parasitoid *Trissolcus basalis* (Colazza et al., 2004a). Also, feeding damaged elms do not emit volatiles that attract egg parasitoids (Meiners and Hilker, 1997). Neither do the ovipositional wounds conducted by the elm leaf beetle (with the mandibles) or by the pine sawfly (with the ovipositor) induce a volatile pattern to which the respective egg parasitoid responds positively (Meiners and Hilker, 2000; Hilker et al., 2002a). Thus, induction of plant defensive responses by insect egg deposition obviously requires elicitors that are specific for the egg or egg-associated components rather than elicitors produced by microorganisms living on the plant surface.

Plant defensive responses to feeding activity have been discussed as being elicited by components produced by microorganisms in the insect gut and then released via

Fig. 1 Mode of attachment of eggs of herbivorous insects to leaf surface. (A) Egg mass of the willow leaf beetle *P. vulgatissima* on a hairy leaf of *Salix viminalis*. Leaf trichomes (tr). Transparent secretion (s) visible on the top of the eggs. Bar = 500 μ m. (B) Cross section (overview) through a leaf of *S. viminalis* with an egg of *P. vulgatissima* on the lower surface. Overview. ey, egg yolk; ec, egg chorion; l, leaf. Bar = 200 μ m. (C) Close view on cross section through a leaf of *S. viminalis* with an egg (eg) of *P. vulgatissima* on the lower surface. It is just adhered to the trichomes (tr) and hardly has any contact with the lower epidermis (ep). sm, spongy mesophyll; pm, parenchymatic mesophyll; ec, egg chorion; ey, egg yolk. Bar = 50 μ m. (D) Egg mass of *P. brassicae* on the lower surface of a *Brassica oleraceae* leaf. Bar = 1 mm. (E) Egg mass of *P. brassicae* partially removed from the leaf. The red lining shows where the eggs lay prior to removal. Leaf tissue turns brownish underneath the eggs and right next to them around the egg mass. s, shiny transparent secretion. Bar = 1 mm. (F) Cross section (overview) through a cabbage leaf (l) with eggs (ey, egg yolk; ec, egg chorion) of *P. brassicae*. The epidermis (ep) is not damaged. The eggs are closely attached to the epidermis. Bar = 200 μ m. (G) Close view on cross section through a cabbage leaf with eggs of *P. brassicae*. The egg chorion (ec) covered by secretion (s) is tightly attached to the epidermis (ep) and is even covering the stomata (sto). Bar = 50 μ m. (H) Egg mass of the elm leaf beetle *X. luteola* on the lower surface of an elm leaf, *Ulmus minor*. The lower part of the egg mass has been removed. At the place where eggs have been removed, the epidermal wounding (epw) is visible, which a female is conducting at the oviposition site with her mouth parts prior to egg laying. This epidermal wounding is clearly distinguishable from a feeding hole (fh) visible on the lower left side of the photo. Bar = 1 mm. (I) Cross section (close view) through an elm leaf with eggs of the elm leaf beetle. The epidermal cells and spongy mesophyll cells are removed so that the egg with chorion (ec) and secretion (s) adheres to the parenchymatic mesophyll cells (pm). Bar = 100 μ m. (J) Cross section (close view) through an elm leaf without eggs. ep, epidermis; sm, spongy mesophyll; pm, parenchymatic mesophyll cells. Bar = 507 μ m. (K) Cross section (close view) through an elm leaf vein with an egg mass of the elm leaf beetle on it. The epidermal cells and mesophyll cells are removed so that the eggs with their secretion (s) are closely attached to the parenchymatic cells surrounding the vascular bundle (ph, phloem; x, xylem; ec, egg chorion; ey, egg yolk). Bar = 100 μ m. (L) Cross section (close view) through an elm leaf vein without egg mass. The intact vascular bundle is visible with the xylem (x), phloem (ph), and the parenchymatic cells (pc) surrounding the vascular bundle. Bar = 100 μ m

the regurgitate into the plant wound during feeding (Spiteller et al., 2000). However, Tumlinson and Lait (2005) have argued that the bacterial biosynthesis rates of the analogs of eliciting components presented by Spiteller et al. (2000) are much too low to meet the requirements for a necessary rapid elicitor accumulation. Instead, they found evidence that fatty acid amide elicitors are biosynthesized in the membranes of the crop and anterior midgut tissues of lepidopteran larvae (Lait et al., 2003). Up to now, there are no hints that endosymbiotic microorganisms are involved in the production of egg-associated elicitors, even though symbiotic microorganisms are well known to be deposited with the eggs (e.g., Hilker, 1994; Kellner, 2002).

The Elicitor of Oviposition-Induced Plant Responses

If the plant is not wounded by oviposition and eggs just “touch” the plant surface, the components necessary for eliciting an oviposition-induced plant response might act either just via induction by touching or they may need to pass the waxy plant cuticula to reach the epidermal cells.

Plant responses to touch and wind have been summarized by Schaller and Weiler (2002). These responses range from changes of the cytosolic calcium concentration to an increase in ethylene production. Both changes of calcium concentrations (Arimura et al., 2000; Maffei et al., 2004) and accumulation of reactive oxygen species are also well known to occur in response to feeding herbivores (e.g., Felton and Eichenseer, 1999, and references therein).

If just the touch of the plant surface by insect eggs is not sufficient to elicit the defensive response and when no wounding occurs along with egg deposition, then the eliciting components associated with the eggs need to gain access to the plant epidermal cell. Thus, they need to be either highly lipophilic to pass the waxy plant surface, or need to possess the ability to “cut” their way enzymatically through the plant cuticula, or need to be accompanied by components smoothing their way (compare e.g., Rosetto et al., 2003). When the elicitor has reached the epidermal cell wall, it might act via receptors or form channels to enter the cell. Truitt et al. (2004) provide evidence that volicitin, an elicitor from the regurgitate of lepidopteran larvae that induces indirect plant defenses, is binding with a plasma membrane protein of the wounded plant (Alborn et al., 1997). When considering the ability of an elicitor to induce plant volatiles, Engelberth et al. (2000) suggest that formation of “pores” is also a possible mechanism. Several channel-forming components especially from phytopathogenic fungi are known to change permeability of biomembranes (Minardi, 1995; Zimmermann et al., 1999; Engelberth et al. 2000). However, they have not been described yet from insect eggs.

If plant cells are wounded by oviposition, an elicitor might enter them via the disrupted cell membrane. In addition, microwounding of the leaf surface that is visible only at high magnification will need closer consideration in future. For example, insect females might easily scratch the leaf surface by ovipositor hairs when laying eggs. Similarly, crawling of insect larvae also has been found to leave microwoundings on the leaf surface that are only visible by REM (Hall et al., 2004). These “larval footsteps” induce significant plant responses, such as increases of superoxide concentrations and GABA accumulation (Bown et al., 2002).

The chemistry of the elicitor of a direct plant defensive response to egg deposition is known only from bruchid beetles (Table 1). Their eggs laid onto pea pods induce the formation of neoplasms. The elicitors are long-chain α , γ -

monounsaturated C₂₂ diols and α , γ -mono- and diunsaturated C₂₄ diols, mono- or diesterified with 3-hydroxypropanoic acid.

The elicitors of indirect oviposition-induced plant defensive responses studied so far have been detected in the secretion coating the eggs and adhering them to plant tissue. In both the elm leaf beetle and in the pine sawfly, this secretion is produced in the *oviductus communis* (Meiners and Hilker, 2000; Hilker et al., 2002a). When applying these secretions onto artificial wounds of leaf tissue that mimic the wounding applied by the female prior to egg deposition (see above), the oviduct secretion *per se* is able to induce the indirect plant response, and no eggs are necessary for this eliciting process. For the pine sawfly, it has been shown that the oviduct secretion loses its activity when treated with a proteinase. The oviduct secretion only keeps its eliciting activity if kept in Ringer solution. Storage in just water results in a loss of activity. Hemolymph of the pine sawfly has no eliciting activity. A comparison of hemolymph and oviduct secretion by SDS-PAGE revealed that oviduct secretion contains a small protein that is not present in the hemolymph. Therefore, this protein is a candidate component that needs to be isolated and tested for its eliciting activity in future studies by applying it into wounded pine needle tissue like the sawfly females does during oviposition (Hilker et al., 2005).

A further candidate as elicitor of oviposition-induced plant responses might be jasmonic acid (JA), which has been detected inside the eggs of several lepidopteran species in much higher concentrations than in plant tissue or larval diet. “Chorions appear to contain small amounts of JA” (Tooker and De Moraes, 2005). It is not yet known whether JA is indeed present at the very outer surface of the eggs. The fact that the leaf surface is usually not visibly damaged when moths deposit eggs raises the question of how JA can invade the plant cell through a waxy egg chorion and a lipophilic plant cuticle.

Plant physiological and molecular processes that are initiated by an oviposition-associated elicitor are almost a black box. When considering the direct plant response to bruchid eggs, enhanced mitogenesis was observed. However, it is unknown how this is controlled and leads to a neoplasm with limited growth. In rice plants, a specific gene (*ovc*) is induced by egg deposition of a planthopper. This gene is involved in the production of the ovidical substance, benzyl benzoate (Yamasaki et al., 2003). Indirect plant responses to insect egg deposition require modification of the biosynthetic activity of the terpenoid pathways especially, since changes of the quantity and/or quality of the plant’s terpenoid volatiles have been detected for egg-laden bean and elm leaves as well as for pine needles with eggs (Wegener et al., 2001; Mumm et al., 2003; Colazza et al., 2004b). For example, pine needles with eggs emit the sesquiterpene (*E*)- β -farnesene in higher quantities than do egg-free pine needles (Mumm et al., 2003). Current studies address the question of how the increase of (*E*)- β -farnesene in the headspace of egg-laden pine needles is regulated at the molecular level.

Systemically Induced Plant Responses

In elm, pine, and beans (see Table 1), the defensive plant response is not strictly limited to the site of egg deposition, but egg-free leaves adjacent to the site of oviposition also release volatiles attractive for the egg parasitoids. Egg-free leaves adjacent to egg-laden ones might just adsorb the oviposition-induced volatiles produced by egg-carrying

leaves and release them again with some delay. However, it has been proved experimentally that no such adsorbance, but systemic induction, occurs. Egg-free leaves adjacent to egg-laden ones indeed show a systemically induced response (Meiners and Hilker, 2000; Hilker et al., 2002a; Colazza et al., 2004a).

How is the signal systemically transferred in the plant from the egg-laden part to the adjacent egg-free part? Application of jasmonic acid (JA) through the cut elm and pine twig, respectively, revealed that this treatment also leads to a volatile pattern attracting the respective egg parasitoids. Comparisons of the components of the headspace of JA-treated twigs and untreated controls show that the pattern is not identical to the one of oviposition-induced twigs, but very similar. For example, JA-treated pine twigs also show a significant enhancement of the emission of (*E*)- β -farnesene. These results indicate that JA might act as a hormone transferring the information from the oviposition-induced site to adjacent, egg-free parts of the plant.

The Egg-Laying Herbivore

The defensive responses of a plant may deliver not only information on the herbivore presence to the third trophic level, but also on the degree of infestation and the defense status to the herbivores themselves. Several studies are available showing that the emission of leaf volatiles in response to herbivore attack is exploited by the herbivores themselves. As outlined below, most of these studies address the herbivore's response to feeding-induced plant volatiles. We are just starting to analyze the herbivore's behavior in response to oviposition-induced volatiles.

The Herbivore's Response to Feeding-Induced Plant Volatiles

If herbivore-induced plant volatiles convey information on the presence of competitors or the risk of high predation and parasitization, herbivorous insects should avoid such plants. Indeed, herbivorous females about to oviposit can be strongly deterred by feeding-induced plant volatiles. For example, the cabbage looper, *Trichoplusia ni*, is less attracted to cabbage damaged by larval feeding and laid fewer eggs on feeding-damaged plants (Landolt, 1993). In addition, females of *Heliothis virescens* are significantly deterred from oviposition by the nocturnal volatiles of feeding-damaged tobacco plants (De Moraes et al., 2001). Similarly, females of *Manduca quinquemaculata* lay fewer eggs on feeding-damaged and JA-treated plants (Kessler and Baldwin, 2001).

If herbivore-induced plant volatiles inform herbivores about the presence of a suitable host plant or the presence of mates or gregarious partners, they are expected to be attracted to these plants (Loughrin et al., 1996; Ruther et al., 2000). Several studies are available showing herbivore attraction to feeding-induced plants (Landolt, 1993; Harari et al., 1994; Loughrin et al., 1996; Bolter et al., 1997; Dicke and van Loon, 2000; Kalberer et al., 2001; Prokopy and Roitberg, 2001; Horiuchi et al., 2003). For example, female *Plutella xylostella* show oviposition preferences for cabbage plants already damaged by conspecific larvae, even though these plants also attract larval parasitoids. Shiojiri and Takabayashi (2003) suggest that this oviposition behavior of *P. xylostella* may be beneficial for the herbivore since it might result in an encounter-dilution effect against the larval parasitoid.

The Herbivore's Response to Oviposition-Induced Plant Volatiles

As suggested by Kessler and Baldwin (2002), the exploitation of herbivore-induced plant volatiles by the herbivores themselves might favor selection for genetic variation in emission of induced volatile compounds in natural plant populations. In the systems studied so far, the phenotypic variation of induced plant odor is obviously not sufficient to prevent its exploitation by herbivores. For example, studies on the response of the elm leaf beetle to oviposition-induced elm volatiles indicate that this beetle is able to adjust its response to plant volatiles according to the infestation rate.

Females of the elm leaf beetle *Xanthogaleruca luteola* orientate to volatiles of both untreated elm leaves and differently treated ones (treatment by feeding, mechanical damage, oviduct secretion). However, when elm twigs are heavily infested by eggs and additionally damaged by feeding of conspecifics, female *X. luteola* clearly avoid such twigs. Odor from uninfested leaves is significantly preferred to odor from heavily infested leaves (feeding damage and eggs). In contrast, at low infestation levels, the beetle's response changes. Odor from uninfested leaves is no longer preferred, but instead volatiles from elm twigs poorly infested by some conspecifics (feeding damage and egg deposition) become highly attractive. The elm leaf beetle females also prefer to lay eggs upon uninfested leaves over heavily infested ones (Meiners et al., 2005).

The herbivore's response to elm leaf odor in dependence of the infestation level would be especially adaptive if the egg parasitoid's response to oviposition-induced elm leaf odor also follows these dependencies. The egg parasitoid *Oomyzus gallerucae* is attracted by odor from elm leaves with eggs and feeding damage (Table 1). Odor from feeding damaged leaves without eggs does not attract the egg parasitoid (Meiners and Hilker, 1997, 2000). However, we do not know yet whether the egg parasitoid prefers odor from leaves with a high egg load to odor from leaves with few eggs and little feeding damage. If so, the herbivore's risk of egg parasitization at low infestation levels is expected to be lower, thus rendering the herbivore's preference for low-infested leaves over heavily infested ones advantageous.

Elms might differ in their thresholds and lag times to produce and emit induced volatiles. Underwood et al. (2005) show in a model and in a field study that increasing lags and thresholds for the production of induced resistance traits might cause increasing aggregation of herbivores and their damage. Herbivores might have adapted to link the induced plant volatiles (kairomones) with the potential presence of suitable host plants. Local densities of elms, beetles, and parasitoids might determine the outcome of this tritrophic interaction (Meiners et al., 2005).

Egg Parasitoids and Predators

Complexity of Plant Odor and the Role for Host/Prey Location

Indirect oviposition-induced plant responses address egg parasitoids and predators of eggs. Plant volatiles or changes of leaf surface chemicals induced by egg deposition provide reliable information on the presence of hosts and prey. Furthermore, the large plant biomass when laden with eggs releases oviposition-

induced volatile information in quantities that can easily be detected. However, egg parasitoids and predators are also able to respond to volatiles from plants without host eggs (see review by Romeis et al., 2005) or with feeding damage only (Kessler and Baldwin, 2001).

Volatiles from feeding-induced plants do not provide carnivores with information on the presence of eggs, but just give a hint on the infestation of the plant. Carnivores responding to these volatiles are not expected to be specialized to prey upon eggs, but instead to be generalists. If they are specialists, they might be forced to take a chemical detour (Vet and Dicke, 1992) because other cues are not available. Indeed, the generalist carnivorous bug *Geocoris pallens* is attracted by feeding-induced plant volatiles and then preys upon the eggs found on such plants (Kessler and Baldwin, 2001). Among the feeding-induced plant volatiles, the release of the so-called general green leaf volatiles (i.e., C₆ aldehydes, C₆ alcohols and their esters) has been shown in numerous studies to increase after feeding damage (Karban and Baldwin, 1997; Dicke and Hilker, 2003). Also, carnivores specialized on insect eggs have been shown to be attracted by these volatiles. For example, *Trichogramma chilonis* shows a positive response to green leaf volatiles such as (Z)-3-hexenyl acetate and hexyl acetate (Reddy et al., 2002). These volatiles are also known to be emitted in small amounts by uninfested plants, and volatiles from uninfested plants often have been found to act as attractants or parasitization stimuli for *Trichogramma* spp. (for references: see Romeis et al., 2005). Also, surface extracts of leaves without eggs can stimulate parasitization (Nordlund, 1994; Dirie and Gabriel, 1998). However, volatiles from undamaged host plants may also act as repellents for egg parasitoids (Nordlund, 1994; Romeis et al., 1997, 1998). Thus, the response of egg parasitoids to plant volatiles is of high genotypic and phenotypic plasticity (De Jong and Pak, 1984; Bjorksten and Hoffmann, 1998; McGregor and Henderson, 1998). Among the factors influencing the egg parasitoid's response to chemical stimuli, the role of previous experience to these chemicals has been studied intensively (Vet and Dicke, 1992; Steidle and van Loon 2002a,b).

The chemical context in which plant volatiles are perceived is important for a parasitoid's response. During host location, the larval parasitoid *Microplitis croceipes* can learn compounds in a mixture (Meiners et al. 2003). Another braconid wasp, *Cotesia kariyai*, learns a blend of unspecific plant compounds better when it is embedded in a blend of specifically host-induced compounds (Fukushima et al., 2002). This context specificity of the response of parasitoids to plant volatiles seems to play a role when considering responses of egg parasitoids to oviposition-induced plant volatiles.

The olfactory background in which the stimuli are presented can affect recognition of individual compounds (Meiners et al., 2003). Studies on the response of *Chrysonotomyia ruforum* to pine volatiles induced by oviposition of the pine sawfly (Table 1) revealed that it is important for the egg parasitoid to perceive the oviposition-induced volatile together with the background of noninduced pine volatiles. (*E*)- β -Farnesene is the only component detected that occurs in significantly higher quantities in oviposition-induced pine than in egg-free controls (Mumm et al., 2003). However, even experienced egg parasitoids are not attracted to (*E*)- β -farnesene, at either concentration tested. When this sesquiterpene is offered in a mixture with odor from egg-free pine, however, it becomes attractive. Odor from egg-free pine does not attract the parasitoid (Mumm and Hilker, 2005). Thus, to attract this parasitoid it is necessary to contrast the oviposition-induced volatile with

other plant volatiles. Although a background contrast is well known to be important for the perception of color, the importance of background odor has been somewhat neglected so far for the detection of specific volatiles (Smith, 1998; Kelling et al., 2002). However, since induction of plant volatiles by egg deposition often involves small quantitative or qualitative changes of the plant odor, the background context at which these induced volatiles are perceived might be very important to detect them within the plethora of other volatiles.

Time Frame of Oviposition-Induced Plant Responses

Oomyzus gallerucae, the major egg parasitoid of the elm leaf beetle, was shown to respond to volatiles from elm leaves with eggs 3 hr after herbivore egg deposition (Table 1). The attractiveness of odor from elm leaves with eggs vanishes when eggs are being laid for more than 5 d on an elm leaf and are close to larval hatching (Meiners, unpublished data). In other systems, the time scale of indirect plant responses to oviposition is larger. *Chrysonotmyia ruforum*, a eulophid wasp parasitizing the eggs of the pine sawfly *Diprion pini*, is not attracted by odor from pine needles with 1-d-old eggs, but 3 d after egg deposition, odor from pine needles is attractive (Hilker, unpublished data; Hilker et al., 2002a). *T. basalis*, an egg parasitoid of the Southern green stink bug *Nezara viridula*, is attracted to volatiles from egg-laden bean leaves until the host eggs are about 3 to 4 d old. After 5 d, *Nezara* eggs become unsuitable for parasitization (Colazza et al., 2004a).

The studies by Fatouros et al. (2005a) indicate that a trichogrammatid egg parasitoid is arrested by oviposition-induced leaf surface chemicals at a time when eggs are most suitable for parasitization, i.e., 3 d after egg deposition. *Trichogramma brassicae* females most successfully parasitize 3-d-old eggs of the large white cabbage butterfly. About 80% of 3-d-old eggs are parasitized, whereas the rate of successful parasitization of freshly laid eggs and 1-d-old eggs is about 40% and 55%, respectively. The trichogrammatid wasp is also arrested on leaves with freshly laid eggs and 1-d-old eggs. However, this arrestment is due to scales and possibly other deposits left by the ovipositing female. When these host cues have lost their arresting activity 3 d after oviposition, the eggs seem to have induced a change of surface chemicals that act as arrestants at this time. Thus, in this system, the trichogrammatid wasp is first arrested by host cues, and only later, when host eggs have become most suitable, do oviposition-induced leaf contact cues seem to play a role.

All of these examples indicate a fine-tuned and cost-efficient time frame for the release of volatile or contact synomones by egg-infested plants. This is characterized by providing notice of the presence of suitable eggs and by ceasing the emission of synomones when the eggs are unsuitable. This guarantees that parasitoids will respond to an honest signal, and fosters the evolution of perceiving those signals and responding to them.

Oviposition-Induced Synomones and/or Kairomones

What is the importance of host cues and oviposition-induced plant signals for host location? Fatouros et al. (2005a) did not detect an olfactory response of *T. brassicae* to volatiles from Brussels sprouts leaves with eggs. When studying the question of how this wasp locates host eggs from a distance, *T. brassicae* was found to locate mated host female butterflies by the anti-aphrodisiac benzyl cyanide, which the

butterfly male transfers to the female during mating (Andersson et al., 2003). After location of a mated female butterfly, the wasp mounts the female, hitchhikes with her to the oviposition site, and descends as soon as eggs are laid (Fatouros et al., 2005b).

If egg parasitoids do not hitchhike with a host female (Clausen, 1976), highly volatile host female sex pheromones may attract them from some distance (e.g., Zuk and Kolluru, 1998). However, these pheromones just “promise” the presence of laid eggs. Also, aggregation pheromones, chemical cues from scales and frass, which all are known to act as kairomones for egg parasitoids (Vet and Dicke, 1992), just indicate that a host is around, but do not indicate with high reliability the presence of eggs. In contrast, oviposition-induced volatiles released locally and systemically by a large plant biomass reliably indicate the presence of laid eggs. Oviposition-induced plant volatiles, however, are obviously not always induced as soon as eggs are laid (see Table 1). Furthermore, an additional capability of egg parasitoids to respond to kairomones from, e.g., host frass (*O. gallerucae*), host sex pheromones (*Chrysonotomyia ruforum*), or volatiles from host females in the preovipositional state (*T. basalis*, *T. brassicae*) can act as a backup system for the egg parasitoids in cases when plants do not respond or do not respond strongly enough to egg depositions (Meiners and Hilker, 1997; Colazza et al., 1999; Hilker et al., 2000, Fatouros et al., 2005b).

Conclusions

A plant responding to oviposition of herbivorous arthropods is possibly acting early enough to prevent feeding damage by the larvae hatching from these eggs. Such an “early alert” may be especially advantageous for annual plants, which have a much smaller time and biomass budget with which to face feeding damage than perennials (Hilker et al., 2002a). However, trees also respond to insect oviposition by induction of volatiles (Table 1). Recently, oviposition-induced *Pinus sylvestris* was shown to lower photosynthetic activity in needles adjacent to the oviposition site (Schroeder et al., 2005). Thus, induced resistance (affecting the herbivore) might be paid here by reduced tolerance (affecting the plant) (Herms and Mattson, 1992). Further studies are needed that elucidate the costs or benefits of oviposition-induced plant responses with plant reproductive parameters as currency (e.g., fruit set, seed production).

The plant’s investment in “early alert” responses to egg deposition might be wasted if the eggs were to suffer mortality due to unsuitable abiotic conditions, such as, for example, high dryness. We need to study whether a plant is able to perceive egg mortality and stop the inductive process. Further, a plant might be able to minimize costs of induction by limiting the induced response only to a narrow time frame when eggs are most acceptable to egg parasitoids. The study by Fatouros et al. (2005a) on induction of Brussels sprouts by *Pieris* eggs indicates such a narrow time window during which induction by egg deposition occurs. However, other studies, such as the one described above on induction of elm by leaf beetle eggs, show a much broader time frame of induction (Meiners, unpublished data). We do not yet understand which parameters drive the selection of narrow and broad time frames for induction by egg deposition.

When induction of plant defense by insect egg deposition is considered an “early alert” strategy, it might be objected that plants induced by volatiles released from their

neighbor plants respond even earlier, i.e., prior to being encountered by a herbivore. Up to now, chemical information transfer has only been shown between feeding-damaged and undamaged plants (Dicke and Bruin, 2001). However, if a plant could also “listen” to oviposition-induced cues of its neighbor plants, this “information” might be the earliest possible information on the danger of attack by an herbivore. Such “listening” to oviposition-induced cues of the neighbor plant might enhance the inducibility of the nonattacked plant in such a way that it could respond sooner or stronger when egg deposition occurs. We do not know yet whether cues from oviposition-induced plants are able to “inform” non-attacked plants next to egg-laden ones about danger. Future studies also need to elucidate whether oviposition-induced cues are able to act as an early alert in interplant communication.

Finally, we need to examine whether there is a trade-off between induction of plant defenses by oviposition and feeding, or whether these defensive strategies addressing different stages of the herbivore act effectively *in concert*.

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Oviposition in *Delia platura* (Diptera, Anthomyiidae): The Role of Volatile and Contact Cues of Bean

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Abstract The choice of a suitable oviposition site by female insects is essential for survival of their progeny. Both olfactory and contact cues of the oviposition site may mediate this choice. The polyphagous *Delia platura* (Diptera: Anthomyiidae), a severe agricultural pest of numerous crops, lays eggs in the soil close to germinating seeds. Maggots feed upon the cotyledons. Only little is known about the cues guiding oviposition behavior. In this study, the effects of both olfactory and contact cues of beans (*Phaseolus vulgaris*) on oviposition of *D. platura* females were tested. Egg deposition on germinated beans was preferred to egg deposition on ungerminated beans or on beans in different postgerminating developmental stages. Olfactory cues of germinating beans alone stimulated female flies to lay eggs. Additional contact cues of germinating beans seemed to enhance the response, but the difference was not significant. Surface extracts of germinating beans sprayed on surrogate beans showed that both polar and nonpolar substances stimulated oviposition of *D. platura* flies. Gas chromatography-electroantennographic detection recordings of head space samples of germinating beans showed positive response of females to different compounds. We conclude that olfaction plays a major role when *D. platura* females are searching for oviposition sites. Volatile compounds released from germinating beans such as 4-hydroxy-4-methyl-2-pentanone, 1-hepten-3-one, 1-octen-3-ol, and 3-octanone should be considered as key compounds that mediate oviposition behavior. The use of different sensory modalities by closely related species of *Delia* is discussed.

Keywords *Delia platura* Meigen · Diptera · Anthomyiidae · *Phaseolus vulgaris* · Germinating bean surface extract · Contact chemoperception · Olfaction · GC-EAD · Oviposition

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Introduction

Plant chemicals play an essential role in host plant selection of herbivorous insects and, in particular, in the oviposition choice of females (Städler, 2002). Active compounds include volatiles and contact chemicals with varying polarity that are perceived by different chemosensory organs (Bernays and Chapman, 1994; Städler, 2002). Visual cues such as the shape and color of plants also influence, to a lesser extent, the acceptance or rejection of a plant by insects (Harris and Miller, 1983; Bernays and Chapman, 1994; Schoonhoven et al., 1998). The choice of a plant by females to lay eggs on is essential for the survival of offspring, since the mobility of larvae is often limited (Thompson, 1988; Iyengar and Eisner, 1999; Harris et al., 2001). This is particularly true in some Diptera such as *Delia* spp. (Anthomyiidae) feeding upon plant roots in the soil. *Delia* flies use various sensory modalities to locate a suitable host for their progeny.

D. radicum feeding upon Brassicaceae relies on visual cues. The shape and color of the plant play a role in attracting females. In this species, olfaction is also important to locate oviposition sites. Isothiocyanate released from the host plants is highly attractive and used in traps to catch this pest (Finch and Skinner, 1982). However, olfactory cues alone are not sufficient for females to find and recognize a suitable oviposition site. Also, contact with the leaf surface of cabbage is essential in stimulating the oviposition. Glucosinolates and a thia-triaza-fluorene compound (CIF) are present on the surface of cabbage leaves and stimulate the egg-laying behavior of *D. radicum* females (Roessingh et al., 1997; Hurter et al., 1999; De Jong et al., 2000).

D. antiqua, the onion fly, relies mainly on visual cues and olfaction to lay eggs. A specific compound, dipropyl disulfide, plays an essential role in attracting and stimulating the oviposition in this species (Romeis et al., 2003; Gouinguéné et al., 2005). The determination of the compounds attracting these flies has led to the development of traps to monitor the pest distribution.

The bean seed fly *D. platura* is an important pest in agricultural systems. The maggots mine the roots of various *Fabaceae*, cereals, tubers, and tobacco seedlings (Griffiths, 1991), which can then be attacked by stem rot organisms. Crop yield is usually reduced by 10–30% because of *D. platura* infestation (Finch, 1989; Fleischer and Gesell, 1999; Whalen, 2002), but may even reach 75% when infestation levels are high (Stivers, 1997). In India, where moisture conditions are ideal for the development of the pest, Chaudhary et al. (1987) reported that up to 88% of corn fields were infested. Several pesticides are used to control this fly (Vea et al., 1976; Montecinos et al., 1986; Valenciano et al., 2004). Usually, seeds are treated with synthetic chemical protectants before sowing, thus reducing the risk of *Delia* attack during the germination stage. However, *D. platura* populations often develop resistance to these insecticides (Finlayson and Campbell, 1971; Harris and Turnbull, 1980; Montecinos et al., 1986). Different methods and periods of sowing have been recommended to prevent high infestation (Hough-Goldstein, 1987; Adams et al., 1990; Brust et al., 1997; Valenciano et al., 2004). Finch (1989) noted the importance of developing integrated approaches to control *Delia* pests in vegetable crops as the number of available and efficient insecticides decreases. To attain this aim, a better understanding of the interactions of *D. platura* with its environment is necessary.

Few studies have examined the cues used by *D. platura* females to locate a suitable habitat and host for progeny (Yu et al., 1975; Dindonis and Miller, 1980;

Ishikawa et al., 1983). Germinating peas, moist sand, decomposing organic material, organic fertilizer mix, and fish meal have been shown in behavioral assays to stimulate oviposition of *D. platura* (Reid, 1940; Barlow, 1965). Microorganisms associated with seeds have been suggested to stimulate oviposition, since sterilization of germinating seeds reduced the number of eggs laid by the fly (Eckenrode et al., 1975). The attractiveness of different germinating seeds and their acceptance by *D. platura* as oviposition sites was studied by Yu et al. (1975). They showed that snap beans were most effective in trapping male and female *D. platura* in the field. Under laboratory conditions, squash seeds, snap bean seeds, and lima beans were attractive and stimulated egg laying of *D. platura* females (Yu et al., 1975; Weston and Miller, 1989). However, volatiles from the germinating seeds were not collected to determine the active substances. Aqueous extracts of germinating seeds were shown to stimulate oviposition, but the active compounds were not identified. The authors only indicated that the compounds stimulating the oviposition were nondialyzable, nonlipolyzable, and unextractable with diethyl ester (Yu et al., 1975).

The aim of our study was to further elucidate which sensory stimuli mediate oviposition in *D. platura*. We compared the importance of olfaction and contact in the process of egg laying and determined the compounds in the volatile blend of germinating beans that are perceived by the *D. platura* females. The polarity of oviposition-stimulating contact cues was assessed by extracting beans with different solvents and testing the activity of the extracts. The results obtained are discussed in comparison with other *Delia* species and with respect to further use for monitoring *D. platura*.

Methods and Materials

Insects

Experiments were performed with *D. platura* from our continuous laboratory rearing that originated from pupae supplied by J. Whittlecraft (Agriculture Canada, Southern Crop Protection and Food Centre, London, Ontario, Canada) in 2000, collected in fields around London region. Flies were reared on germinated bean (*Phaseolus vulgaris*, var. Daisy; Schweizer AG, Thun, Switzerland) and fish food (Reid, 1940) on which females deposit eggs; the larvae developed until pupation in the bean roots in a climate chamber ($19 \pm 0.5^\circ\text{C}$, $90 \pm 5\%$ RH, 16:8 L/D). For the experiment, pupae were isolated from the rearing cage, so that new emerging flies never had any contact with beans before the experiments. Flies were provided with 10% sugar solution and a mixture of raw cane sugar, yeast hydrolysate, and water (4:1:1) applied on absorbent tissue strips. For experiments, flies were between 5 and 9 d old.

Plants

Beans (*P. vulgaris*) of the variety Daisy (Schweizer) were placed on wet vermiculite for germination. They were used after 3–4 d, when just the very small root shoot had emerged. Small plants with two leaves were also tested. For the extraction, beans were germinated on moist vermiculite for 4 d in a greenhouse at 20°C and 70% RH.

Extraction of Bean Surface

The method for the surface extraction of beans was adapted from Roessingh and Städler (1990). Beans were soaked in 300 ml chloroform (CHCl_3) for 15 min, and afterwards they were dipped twice in 300 ml methanol (MeOH). The two MeOH extracts were pooled together. CHCl_3 and MeOH extracts were filtered separately and evaporated almost to dryness (Rotavapor, Büchi Labortechnik AG, Flawil, Switzerland). Each dry, extract was diluted in its respective solvent to a specified volume related to the weight of the germinated beans. The extract was expressed in gram bean equivalent (gbe) or gbe/ml, which corresponded to the weight of germinated beans extracted per volume extract.

Oviposition Choice Experiments

Oviposition choice experiments took place in a climate chamber set up under conditions similar to the ones for rearing ($19 \pm 0.5^\circ\text{C}$, $90 \pm 5\%$ RH, 16:8 L:D).

Substrate Effects on Oviposition Behavior

Four substrates were tested simultaneously in a choice test: (1) sand only as control, (2) nongerminated beans, (3) germinating beans, and (4) fish food as organic substrate. Fish food was chosen because it stimulates the development of *D. platura* (Reid, 1940), but it is not clear if it primarily affects the oviposition of the female bean seed flies. The stimuli were placed in a glass cup (5 cm \times 9.5 cm diam) filled with foam (9.5 cm diam \times 2 cm) covered with plastic and a layer of moistened sand (Fig. 1A). Females were able to smell and contact each of the four stimuli tested. The glass cups were placed in a cage (70 \times 70 \times 70 cm) with about 70 female flies and 15 males. Females were allowed to oviposit for 24 hr, and then the eggs laid into the sand of cups with the different treatments were counted. Six replicates were conducted, and the position of the treatments was changed after each egg count.

Effect of Plant Stage on Oviposition Behavior

We tested the importance of the plant development for egg-laying behavior. Flies were given the choice among young germinating beans seeds (3–4 d), advanced germinating beans (5–6 d), young plants (7–9 d, 2 emerging leaves), and sand alone as control. Germinating seeds and young plants were removed from the vermiculite and placed on wet sand and foam in a glass cup. The same conditions with six replicates as in the previous experiment were used.

In all the above-described experiments, female flies could use both olfactory and contact cues.

Comparison of Importance of Olfactory and Contact Cues

To test if olfaction or contact with the sprouting seeds plays a role for oviposition, we gave the flies the choice between three stimuli: (1) germinated beans covered with a screen, so that the flies could not contact plants, (2) germinated beans with complete access to the plant, and (3) sand only.

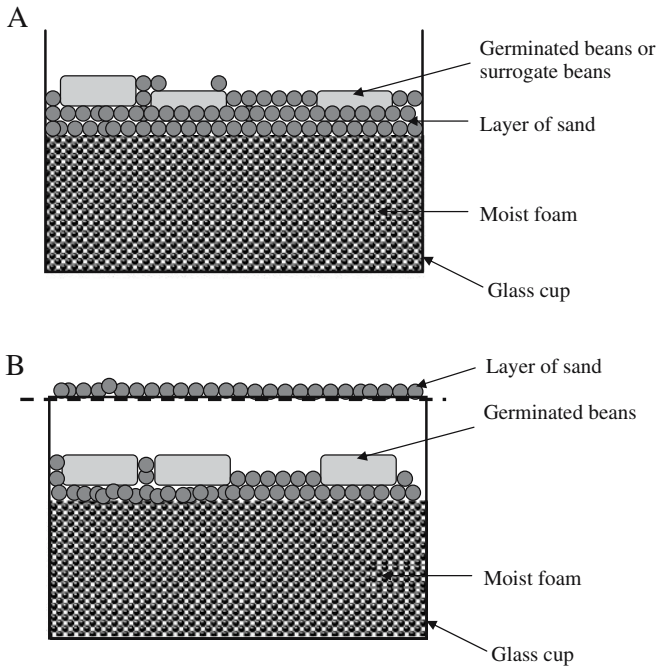


Fig. 1 Schematic diagram of the set-up used in behavioral experiments. (A) Set-up when *D. platyura* is allowed to contact the stimuli. (B) Set-up for testing the effect of olfaction on the oviposition of *D. platyura*

To provide oviposition sites (1), germinating beans were placed in a glass container with moistened foam to prevent desiccation and covered with a screen topped with a layer of moist sand (Fig. 1B). The screen prevented direct contact of flies with germinated beans. The same container with only wet foam and sand was used as a control [oviposition site (3)]. Oviposition site (2) allowing contact with the bean was provided by using the cups described in Fig. 1A. The eggs laid into the sand of these containers were counted after 24 hr. This experiment was replicated six times.

Effect of Bean Extract on Surrogate Beans on Oviposition Behavior

Extracts of plants were tested by using surrogate beans onto which the extracts were sprayed. The surrogate beans consisted of pieces of glass rods of 7 mm diam and 15 mm length. For experiments, they were placed on sand in glass cups. Before use, the surrogates were washed and rinsed with acetone and dried until all solvent trace evaporated.

The extract (see above) was sprayed on glass surrogate beans. Beans, either sprayed with the tested extract or control solvent, were placed on top of a 0.5-cm sand layer. Flies had the choice among a container with MeOH bean extract, CHCl₃ bean extract, MeOH only, CHCl₃ only, and sand only. The experiment ran for 24 hr, then the eggs laid on the differently treated surrogates were collected and counted.

Fractionation of the Preferred Bean Extract

The active MeOH extract was fractionated by using medium-pressure liquid chromatography, with an Rsil C-18 HL column (0.015–0.035 mm, pore size: 90 Å, length: 20 cm; RSL Chromatography Chemie, Vetikon, Switzerland). A volume of 25 ml of MeOH extract (1.73 gbe/ml) was evaporated to dryness, dissolved in 25 ml of water, and applied onto the top of the column. The solvent gradient was 100% water (H₂O), 50% water–50% methanol (MeOH/H₂O), and 100% methanol (MeOH). Eluates of 500 ml were collected with each solvent. The collected fractions were then evaporated under vacuum, and the residuals were dissolved in their initial solvent to 50-ml aliquots. The biological activity of the fractions obtained was tested in the previously described oviposition choice experiments with surrogate beans.

The most active fraction (fraction H₂O/MeOH) was further separated by HPLC equipped with a C-18 column (LiChrospher 100-5 RP-18, 230 mm, Machery-Nagel, Oensingen, Switzerland). Five fractions were collected by using a MeOH–water gradient (100% MeOH, 80:20, 50:50, 20:80, 100% H₂O). Each fraction was collected during 10 min of elution. The resulting five fractions were tested in the choice experiment.

Collection and Analysis of Volatiles From Germinating Beans

The method for collecting volatiles released by germinated beans was adapted from a push–pull odor collection system developed by Turlings et al. (1990). Eighty germinating beans were put in a glass container (described by Gouinguéné et al., 2003), with a filtered and humidified airflow passing over the beans. The air charged with the plant volatiles was pulled through a filter made of Super-Q® (Alltech, Switzerland) to trap the volatiles. Volatiles were desorbed by using 150 µl methylene l methylene chloride (HPLC grade; Biosolve BV, The Netherlands), and the extracts were analyzed with gas chromatography-electroantennographic detection (GC-EAD) as described below followed by gas chromatography-mass spectrometry to determine the identity of the compounds perceived by the flies.

Electrophysiology

GC-EAD recordings were made by using the female antennae. The insect was cooled (5°C) for 10 min. Then, its head was cut and mounted on the indifferent electrode, the tip of the recording electrode touching the tip of the antenna for contact. The GC was equipped with a 5% siloxane column (ZB-5, 30 m, 0.32 mm i.d., 0.25 µm film thickness, Brechbühler, Schlieren, Switzerland), with helium at 25 cm/sec as the carrier gas. The effluent was split with a deactivated three-way press fit splitter (0.32 × 0.32 × 0.32 mm i.d., BGB Analytik Ag, Anwil, Switzerland), followed by 60 cm of deactivated column (0.32 mm ID, BGB Analytik, Anwil, Switzerland). One arm was directed to the FID, and the other to the EAG preparation. The output of the column was heated at 300°C, to avoid condensation of the effluent when the column leaves the GC. The end of the column entered a glass tube blown over with humidified air at 3 cm before the antennal preparation. The airflow in the glass tube was set at 1 l/min. The EAG set up has been described in details by Marazzi and Städler (2004). Ten recordings were made, each with a different female. For compounds perceived by the flies, we calculated the percentage of occurrence of response.

Active compounds were identified by GC (Hewlett Packard HP 6890, Agilent 6890 Series GC system G1530A), coupled to a mass spectrometer (MS) operated in electron impact mode (Agilent 5973 Network Mass Selective Detector; transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33–280 amu). A 3- μ l aliquot of sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25 mm i.d., 0.25 μ m film thickness; Alltech Associates, Inc., USA). Helium at constant pressure (18.55 psi) was used as carrier gas flow. Following injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min, and subsequently to 200°C at 5°C/min, followed by a post-run of 5 min at 250°C. Compounds were identified by comparison of their mass spectra with those of the NIST 02 library and by comparison of their spectra and retention times with those of authentic standards.

Contact chemoreceptor recordings were made from sensilla present on the tarsi of the front legs of the flies. Stimuli were tested on the ventrolateral D-sensilla on the third (D_3), fourth (D_4), and fifth tarsomer (D_5) as well as in the ventromedial C-sensillum on the fifth tarsomer (C_5) (terminology according to Grabowski and Dethier, 1954). The technique was recently described by Gouinguené and Städler (2005). Briefly, the cooled female was placed without head in a small depression cut into a Plexiglas cube. The front legs were taped to the surface of the block to expose the ventral side of the tarsi. To avoid desiccation, a humidified air stream (0.5 m/sec, 19–25°C) was directed over the insect preparation. The indifferent glass electrode (tip diameter? 1 μ m) filled with Ringer solution was inserted into the tibia. The spike activity of the stimulated neurons was recorded and analyzed by using a computer and in-house software. All stimuli used in electrophysiological experiments were dissolved in a 1 mM solution of potassium chloride (KCl), which evoked only few spikes. The responses were compared by the total number of spikes, as only one type of spike could be determined in all the recordings. Eight replicates on each sensillum were realized.

Statistical Analyses

In behavioral experiments, the Friedman test was used to check for significant differences between treatments, as the assumptions of data independency may not be respected because of multiple choices offered to the flies (Iman and Davenport, 1980). We used *t*-test to determine if the number of spikes recorded from the tarsal sensilla stimulated with the MeOH extract was significantly different from the response recorded with the KCl control stimulus.

Results

Substrate Effects on Oviposition Behavior

Female flies laid more eggs on sand in which germinated beans were present, but this number was not significantly different from that on fish food (Fig. 2). Nongerminating seed did not stimulate oviposition, thus the visual stimuli (bean shape and color) were not used as oviposition stimuli (Fig. 2). The sand by itself did not stimulate oviposition.

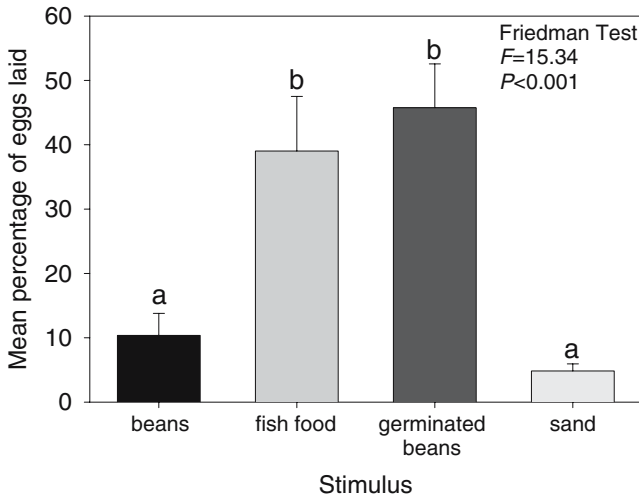


Fig. 2 Oviposition preference of *D. platyura* for different substrates. Friedman test was used to test for significant difference among treatments. Bars represent the mean proportion of eggs laid, and the error bar indicates the standard error. Pairwise comparisons were performed as described by Iman and Davenport (1980), and significant differences between treatments are indicated with letters above bars $N = 6$

Effect of Plant Stage on Oviposition Behavior

No significant difference in the number of eggs laid was found among the three stages of germination of the beans. Young germinating beans (3–4 d old) stimulated the oviposition of *D. platyura* females as much as advanced germinated seeds (5–6 d old) or young plants with already well-developed root systems (Fig. 3). In accordance with the previous experiment, sand alone was not stimulating.

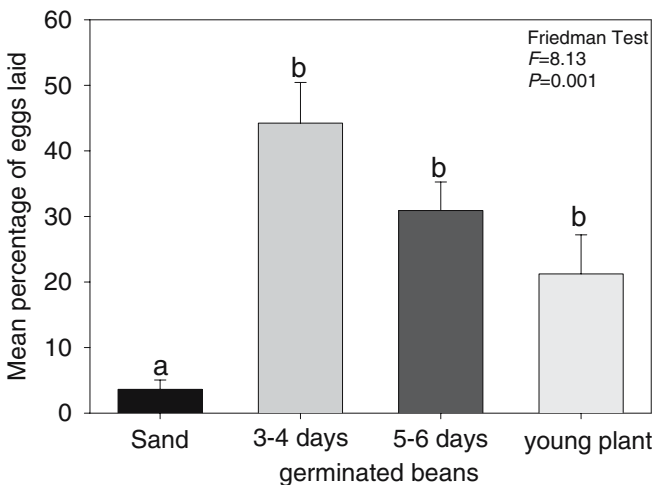


Fig. 3 Bean plant stage influencing oviposition preference of *D. platyura*. Bars and statistics as in Fig. 2. $N = 6$

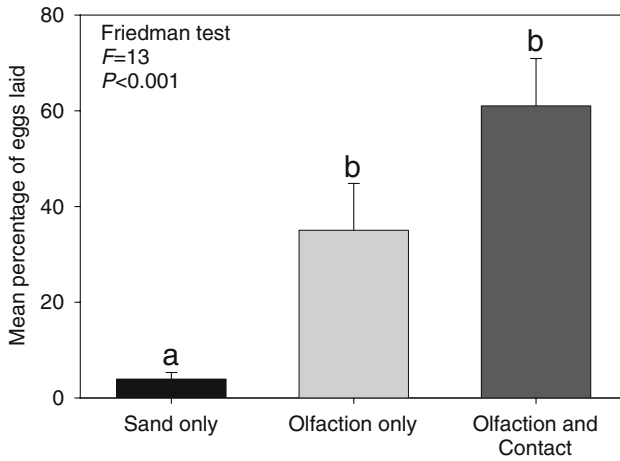


Fig. 4 Comparison of the effect of contact vs. olfactory perception of volatiles of germinating beans on the oviposition of *D. platura*. Bars and statistics as in Fig. 2. *N* = 6

Olfaction

Females were able to detect the germinating beans since the odor of the host alone stimulated the oviposition (Fig. 4). Contact with the germinated seed did not significantly increase the proportion of eggs laid.

Effect of Bean Extract on Surrogate Beans on Oviposition

The MeOH extract of germinated beans received the most eggs (Fig. 5), and few were deposited on surrogate beans treated with the CHCl₃ extract alone. Interestingly, more eggs were laid on untreated glass beans than on solvent treated controls, which may indicate that the solvents had a deterrent effect (Fig. 5).

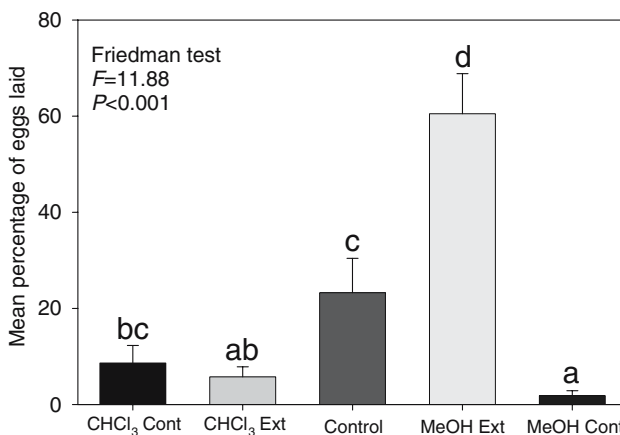


Fig. 5 Oviposition preference of *D. platura* for different extracts. Bars and statistics as in Fig. 2. *N* = 6

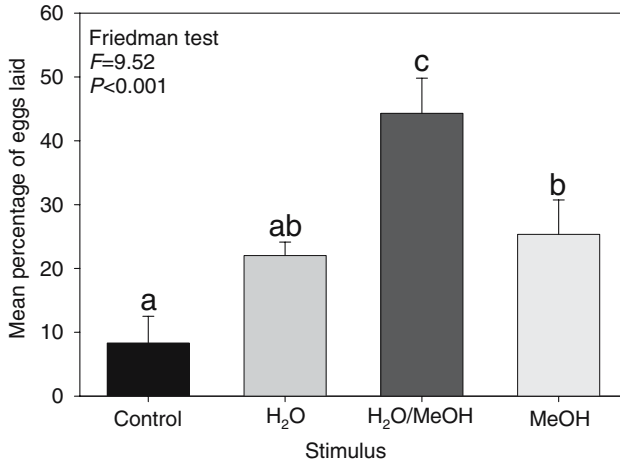


Fig. 6 Oviposition preference of *D. platura* for the different fractions of the MeOH extract from germinating beans. Bars and statistics as in Fig. 2. $N = 6$

Fractionation of the Preferred Bean Extract

All the three fractions were stimulatory, but significantly more eggs were laid on the surrogate beans treated with the H₂O/MeOH and MeOH fractions than on the control (Fig. 6). The fraction H₂O was not significantly more stimulatory than the control (Fig. 6).

Electrophysiology GC-EAD

Only a few compounds clearly stimulated the receptors (Fig. 7; Table 1). In 78% of the recordings, the response to 4-hydroxy-4-methyl-2-pentanone (d) was present and

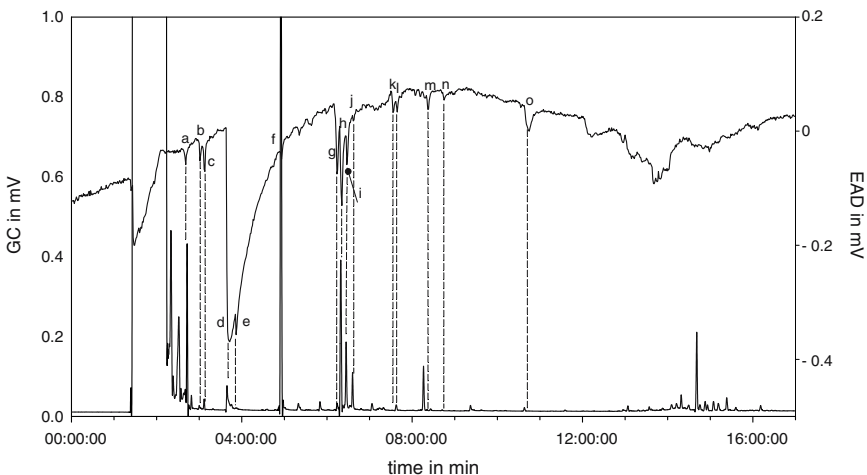


Fig. 7 GC-EAD recording from a *D. platura* female stimulated by the odor from germinating beans. This recording is representative of 10 recordings. Identities of active compounds are given in Table 1

Table 1 Identity of the compounds from the odor of germinating beans that were perceived by the antennae of *D. platura*

Peak	Identity	Occurrence of responses (%)	Retention index (ZB-5)
a	Dimethyl disulfide	56	746
b	Hexanol	44	773.2
c	2-Hexanol	33	783.4
d	4-Hydroxy-4-methyl-2-pentanone	78	822.7
e	Unknown	67	836.7
f	Styrene	56	895
g	1-Hepten-3-one	89	985.9
h	1-Octen-3-ol	100	991.2
i	3-Octanone	44	999
j	3-Octanol	22	1007.7
k	Unknown	22	1059.5
l	2-Methoxy-2,5-dimethylpyrazine	33	1064.8
m	Unknown	44	1105.4
n	Unknown	22	1121.2
o	Unknown	44	1236.5

Letters in the first column correspond to those in Fig. 7.

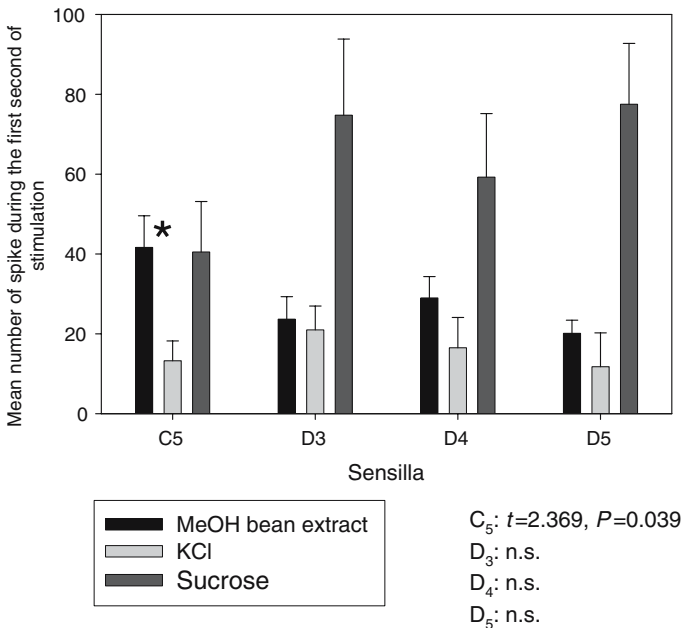


Fig. 8 Tip recordings from *D. platura* prothoracic tarsal sensilla. Bars represent the mean of the number of spikes recorded in the first second of stimulation. Star above bars indicates a significant difference (*t*-test) in the number of spikes recorded due a specific stimulus and the number of spikes from the KCl control. $N = 8$

gave large EAG amplitudes. Most consistent responses were obtained with 1-hepten-3-one (g) and 1-octen-3-ol (h). Responses to 3-octanone (j) were less consistent, but such results should be taken with care, as in many of the recordings the response to this compound may be covered by the large response to 1-octen-3-ol.

Contact Chemoreception

Only the neurons of the C₅ sensillum responded significantly to the MeOH extract. The neurons of the other tarsal sensilla were not stimulated by this extract, as the number of spikes measured was never different from that of the control KCl solution (Fig. 8). Analysis of spike classes revealed that only one class of spikes was recorded in the different recordings.

Discussion

Delia platura females can locate an oviposition site by relying on olfaction alone (Barlow, 1965; Weston and Miller, 1989). The response of the antennae of *D. platura* showed that the female flies responded to different volatiles released from germinating beans, and in particular to octenol and octanone. These two compounds are known to be characteristic for the odor of fungi (Pfeil and Mumma, 1993; Omura et al., 2002). Thus they may be produced and released by the activity of microorganisms associated with seeds or fungi present in the surroundings of germinating seeds. The presence of these organisms were demonstrated to stimulate oviposition in *D. platura* (Eckenrode et al., 1975) and also in *Megaselia halterata*, a phorid fly developing on compost (Pfeil and Mumma, 1993). Further experiments should test different species of plants in order to compare the profile of volatiles emitted by the seeds.

In this study, the same bean variety Daisy was used in the experiments and for rearing the fly colony. Using the same variety for experiments and rearing may have affected the preference of the insects. Collection of volatiles from another variety of beans (var. Cupidon; Schweizer) was also conducted (data not shown), but volatile profiles of var. Daisy and Cupidon revealed only minor differences. The oviposition behavior of flies on bean var. Cupidon did not differ from that on var. Daisy. The olfactory response of *D. platura* to 1-octen-3-ol is interesting. This may be a possible ancestral ability, since this compound has been found to be an attractant for other dipterans that are hematophagous such as mosquitoes or biting flies (Ndegwa and Mihok, 1999; Voskamp et al., 1999; Birkett et al., 2004).

Contact with the plant plays a moderate role in the egg-laying behavior of the females. Interestingly, the neurons sensitive to contact with the host plant seem to be at least partly located on the C₅ sensilla. The recordings show that only one neuron was stimulated by the MeOH extract of germinating beans. The neurons of the other sensilla did not respond to the extract. This is remarkable because in a closely related species, *D. radicum*, the C₅ sensilla also contains a specific neuron responding to a particular compound from cabbage (a thia-triaza-fluorene named CIF), which is also an effective oviposition stimulant of *D. radicum* females (Roessingh et al., 1997; Hurter et al., 1999; De Jong et al., 2000).

D. radicum, a closely related species to *D. platura*, relies on different sensory modalities to locate a suitable host, primarily vision, since the shape and color of

leaves play a role in the attraction of females (Roessingh and Städler, 1990). Olfaction is also important, as specific compounds emitted by Brassicacean plants (isothiocyanates) were shown to attract females (Finch and Skinner, 1982). Contact with the cabbage leaf surface, however, plays a major role in inducing oviposition in this species. The perception of glucosinolates and of the thia-triaza-fluorene compound (CIF) by sensilla located on the tarsae is important for the choice of oviposition sites in *D. radicum* females (Hurter et al., 1999; De Jong et al., 2000). In contrast, *D. antiqua*, which is phylogenetically more closely related to *D. platura*, relies mainly on olfaction (Harris and Miller, 1988; Romeis et al., 2003; Gouinguéné et al., 2005). Females of *D. antiqua* are stimulated to lay eggs only when smelling a volatile compound typical for onion, i.e., dipropyl disulfide. When considering the sensory modalities used to detect a suitable oviposition site, *D. platura* takes an intermediate position between *D. radicum* and *D. antiqua*, as it relies on olfaction to locate germinating beans, but it also perceives nonvolatile compounds from the bean surface to lay eggs, whereas visual cues seem to play no or a minor role. Weston and Miller (1989) mentioned that *D. platura* is under a selective pressure to oviposit on unapparent germinating seeds. Thus, olfaction may be favored over contact. Comparison of the sensory modalities used by closely related insect species could be interesting to determine if the number of modalities used corresponds with a certain degree of specialization.

Future experiments should aim to identify the compounds involved in the contact perception of germinating seeds. Furthermore, 1-octen-3-ol and octanone need to be tested for their effects on attraction and stimulation of the bean seed fly. If these compounds can be proven to be active, they may be used in traps to monitor this pest as is done for other *Delia* species (*D. antiqua*, *D. floralis*, *D. radicum*) that attack vegetable crops (Finch, 1991, 1995; Romeis et al., 2003). In addition to the application perspectives of 1-octen-3-ol to control *D. platura*, the evolutionary aspects of the perception of this compound by both phytophagous and hematophagous insects should require attention in further studies.

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Age-Related Shifts in Leaf Chemistry of Clonal Aspen (*Populus tremuloides*)

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Abstract Developmental changes in plant structure and function can influence both mammalian and arthropod feeding preferences for many woody plant species. This study documents age-related changes that occur in the leaf chemistry of trembling aspen (*Populus tremuloides* Michx., Salicaceae) and discusses implications for the herbivore community and ecosystem processes. We collected leaves from replicate ramets from six age classes (1–25+ yr) in each of seven aspen clones growing in south central Wisconsin, USA. Chemical analyses were conducted to determine concentrations of condensed tannins, phenolic glycosides (salicortin and tremulacin), nitrogen, starch, and soluble sugars. Each variable differed significantly among clones and among age classes. On average, condensed tannin concentrations doubled in the first five years and then remained fairly constant among older age classes. Combined phenolic glycoside (salicortin + tremulacin) concentrations were high in the youngest ramets (ca. 19%) and decreased sharply with age. Developmental changes in tannin, salicortin, and tremulacin concentrations exceeded those of nitrogen and carbohydrates. Developmental shifts of this magnitude, and the age-related tradeoff that occurs between condensed tannins and phenolic glycosides, are likely to have significant influence on the herbivore community of aspen and may influence leaf litter decomposition and nutrient cycling.

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Introduction

Plant characteristics, ranging from metabolic processes to morphology, often change through the process of maturation (Jones, 1999). Plant developmental changes can occur rapidly, for example, as leaves mature through a growing season, or slowly, as long-lived perennial plants reach reproductive maturity. These changes can have effects on community interactions. For example, many physical traits contributing to herbivore performance and preference (e.g., thorniness, leaf toughness, resin flow, leaf abscission) change in a predictable manner as plants mature (Brink, 1962; Kozłowski, 1971). Plant chemistry, which can be more important than physical characteristics in determining herbivore host ranges (Schultz, 1988), may also change as plants mature. Comparatively little is known, however, about developmental changes that occur in phytochemical composition as long-lived perennial plants mature.

Plant chemistry is influenced by both genotype and environment and can vary considerably both within species (Lindroth and Hwang, 1996a; Agrawal et al., 1999; Laitinen et al., 2000) and within individual plants (Gill et al., 1995; Orrians and Jones, 2001; Pavia et al., 2002). Within-plant differences in chemical defense can occur as a result of induction (Karban and Baldwin, 1997), leaf maturation (Lindroth et al., 1987; Riipi et al., 2002), somatic mutations in meristematic tissues (Suomela and Ayres, 1994; Gill et al., 1995; Tuskan et al., 1996), and through the process of ontogenetic maturation from juvenile to adult phase (Bryant and Julkunen-Tiitto, 1995; Karban and Thaler, 1999; Erwin et al., 2001; Boege and Marquis, 2005; Laitinen et al., 2005).

Within-species variation in plant chemistry can affect ecological interactions and ecosystem processes. For example, primary plant compounds such as protein and water are critical for herbivore growth and development (Awmack and Leather, 2002), and qualitative or quantitative variation in phytochemical defenses within a species also strongly influences herbivore behavior and performance (Crawley, 1983; Schultz, 1988; Shelton, 2000). At the ecosystem level, phytochemical variation can influence litter decomposition and nutrient cycling rates (Schimel et al., 1996; Hättenschwiler and Vitousek, 2000; Kraus et al., 2003; Madritch et al., 2006).

Chemical variation within a plant may have ecological consequences similar to those of within-species variation (Suomela and Ayres, 1994). Whereas relatively little is known about the effects of development on the chemical content of plants, many studies have found that developmentally based variation can significantly influence herbivore behavior and performance (Kearsley and Whitham, 1989; Swihart and Bryant, 2001; Lawrence et al., 2003). Studies that have measured within-year and between-year (Bryant and Julkunen-Tiitto, 1995; Erwin et al., 2001; Boege and Marquis, 2005; Laitinen et al., 2005) developmentally mediated changes in plant chemistry indicate that such changes can be significant and suggest that these chemical shifts are likely to be ecologically important.

Previous work has demonstrated comparatively high levels of allelochemicals in juvenile compared with mature ramets in some species of Salicaceae (e.g., Bryant, 1981; Reichardt et al., 1990; Martinsen et al., 1998; Brian Rehill,

unpublished data), and two studies have indicated that between-year developmental shifts in phytochemistry may occur in aspen (Basey et al., 1988; Erwin et al., 2001). Evidence also suggests that mammals show preferences for tissues from mature vs. juvenile aspen trees (Basey et al., 1988; Swihart and Bryant, 2001) and establishes reasonable expectations for arthropods and pathogens to be affected by developmental shifts in phytochemistry (Lindroth and Hwang, 1996a; Kearsley and Whitham, 1989, 1998).

This research assessed among- and within-clone variation in trembling aspen (*Populus tremuloides* Michx.) leaf chemistry, and identified developmental patterns of allocation to leaf chemical constituents important for growth and defense. By “developmental,” we refer to long-term or between-year changes in plant chemistry through the process of maturation. We also assessed how such developmental patterns vary among clones because the rate and magnitude of chemical shifts may significantly affect their potential to influence associated herbivore communities and ecological processes.

Trembling aspen is one of the most widely distributed and genetically variable plant species in North America (Mitton and Grant, 1996). Aspen reproduction is often vegetative, via suckering from previously established root systems. Individual ramets (stems) originating from a common root system often vary widely in age and maturity as the clone expands from the central genet (original seedling). After disturbances, such as windfall, fire, or clear-cutting, aspen produces new ramets from surviving root systems. Depending on disturbance types and frequencies, aspen stand demographics can vary greatly at the local and landscape scales. Thus, developmental-based changes in the chemical attributes of this important and widespread tree species may significantly influence its associated communities.

Methods and Materials

Clone Selection and Sampling Protocols

To assess background levels of among-clone variation in aspen leaf chemistry, we haphazardly selected 20 aspen clones from locations throughout Dane Co., Wisconsin, USA. In early June 2002, four reproductively mature trees (ramets >10 yr of age) from each of the 20 clones were sampled for chemical analyses. Using a pole pruner, we haphazardly collected 5–10 leaves from each of at least three locations in the canopy. These 20 clones were subsequently identified as genetically unique on the basis of microsatellite markers (Cole, 2005).

Seven of the 20 clones sampled were suitable (age-distribution) for extensive sampling from ramets of different age classes. For these seven clones, we haphazardly selected and sampled from three individual ramets within each of the following age classes: 0–1, 2–5, 6–10, 11–15, 16–20, and >20 yr. For one of the clones (clone 11), no trees were available over 20 yr old. Leaf samples and diameter and height measurements were taken for each ramet in June 2002. Samples were collected as described above, i.e., 5–10 leaves from at least three locations within the canopy. To avoid the potentially confounding effect of within year variation in chemistry (Lindroth et al., 1987; Riipi et al., 2002), only fully expanded source leaves were used in chemical analyses. Ramet age was determined by counting annual

growth rings on cut ends or, for larger ramets, by averaging ring counts from two tree cores made with an increment borer. Heights were measured with a measuring tape for felled ramets and a clinometer for large standing ramets. Stem diameters were measured with a hand caliper 2 cm above ground level for small ramets (<3 cm diam) and a diameter measuring tape 10 cm above ground was used for large ramets (>3 cm diam).

Phytochemical Analyses

Leaf samples were kept on ice while in the field and returned to the laboratory daily. Samples were flash-frozen in liquid nitrogen, vacuum dried, ground with a Wiley mill through a number 40 mesh, and stored at -20°C . We analyzed leaf tissues for chemical constituents likely to be important in influencing general host or litter quality (condensed tannins, phenolic glycosides, total nitrogen, starch, and sugars). Condensed tannins were assayed using a modified butanol–HCL method described by Porter et al. (1986). Purified aspen condensed tannins served as standards. The phenolic glycosides salicortin and tremulacin were quantified by HPLC as reported by Lindroth et al. (1993), using purified phenolic glycosides as standards. Samples were initially screened for salicin and tremuloidin, but contained little or none of those two compounds. Thus, the value for total phenolic glycosides is the sum of salicortin and tremulacin concentrations. Samples were analyzed for total N (an index of protein) with an elemental analyzer (LECO, St. Joseph, MI, USA). Glycine-*p*-toluenesulfonate was used as a standard in N analyses. Starch and sugar (sucrose + hexose) concentrations were assayed by using enzymatic hydrolysis and the dinitrosalicylic acid method as in Lindroth et al. (2002). All phytochemical variables are reported in the text and figures as percent dry weight.

Statistical Analyses

Statistical analyses were performed by using JMP IN version 4.0.4 (SAS, 2001) and SAS version 8.2. Because this study was conducted on naturally occurring aspen clones, it was not possible to replicate clones. Estimates of within-clone error were achieved by measuring chemistry of multiple ramets (“trees”) from within a clone, either from 1) four mature ramets within a clone (Fig. 1), or 2) multiple trees from within an age class (Figs. 2 and 3).

Phytochemical differences among the 20 mature clones were evaluated by an analysis of variance (ANOVA). The effects of plant age on phytochemical attributes were assessed in a mixed model (Proc Mixed; SAS, 1999). Ageclass was fitted as a fixed effect, and clone was fitted as a random effect. *Post hoc* means comparisons were made for phytochemical concentrations among age classes by using *t* tests and Bonferroni-adjusted probabilities (SAS, 1999). Two different analyses were used to determine whether developmental patterns varied among clones. First, we compared -2 log-likelihoods for models run with and without the random variable “clone.” Chi square tests were used to assess the significance of the clone effect (Zar, 1999). Second, an analysis of covariance (ANCOVA) was used to compare the trajectory (slopes) with which concentrations of phytochemicals differed as plants aged. Tree age (years) was used as the covariate. A significant clone by age interaction indicated that clones showed different developmental trajectories in allocation to phytochemical variables. In our assessments of age-related variation, a

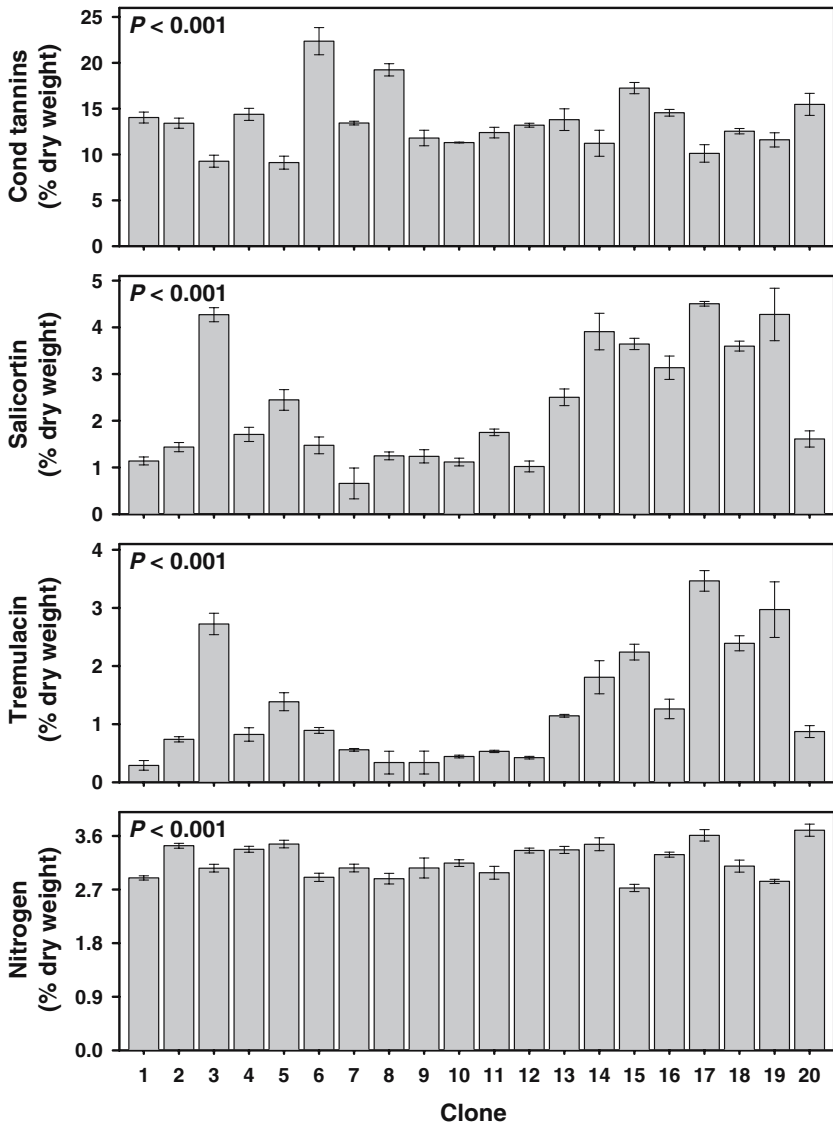
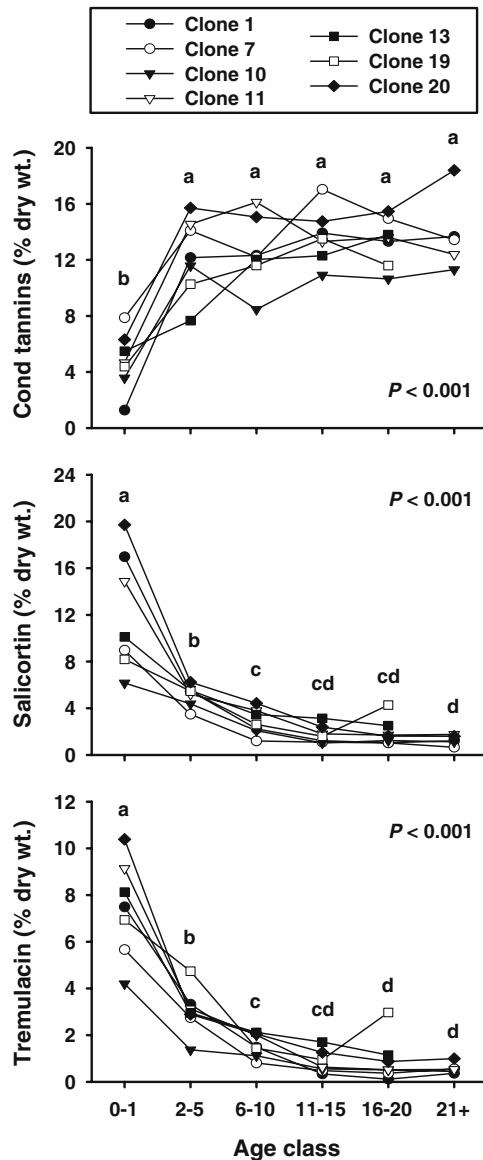


Fig. 1 Leaf concentrations of condensed tannins (cond tannins), salicortin, tremulacin, and nitrogen from mature trees of 20 aspen clones haphazardly selected throughout Dane Co., Wisconsin. Bar heights represent averages of four mature ramets per clone. Error bars indicate ± 1 SE

Box-Cox transformation was applied to salicortin and tremulacin values prior to statistical tests to improve normality and homogeneity of variance (Zar, 1999).

Pairwise correlations were examined to identify relationships among phytochemical constituents, size, and age variables (SAS, 2001). Correlations were assessed by using mean values for age classes within clones. The relationship between condensed tannin and phenolic glycoside concentrations was described using quadratic regression.

Fig. 2 Developmental shifts in aspen leaf concentrations of condensed tannins (cond tannins) and phenolic glycosides (salicortin and tremulacin). Symbols represent the average concentration for replicate ramets ($n = 3$) within each of six age classes for the seven clones, respectively. P values refer to the main effect of age class (Proc Mixed). Age classes with different letter are significantly different (Bonferonni-adjusted $P < 0.05$)

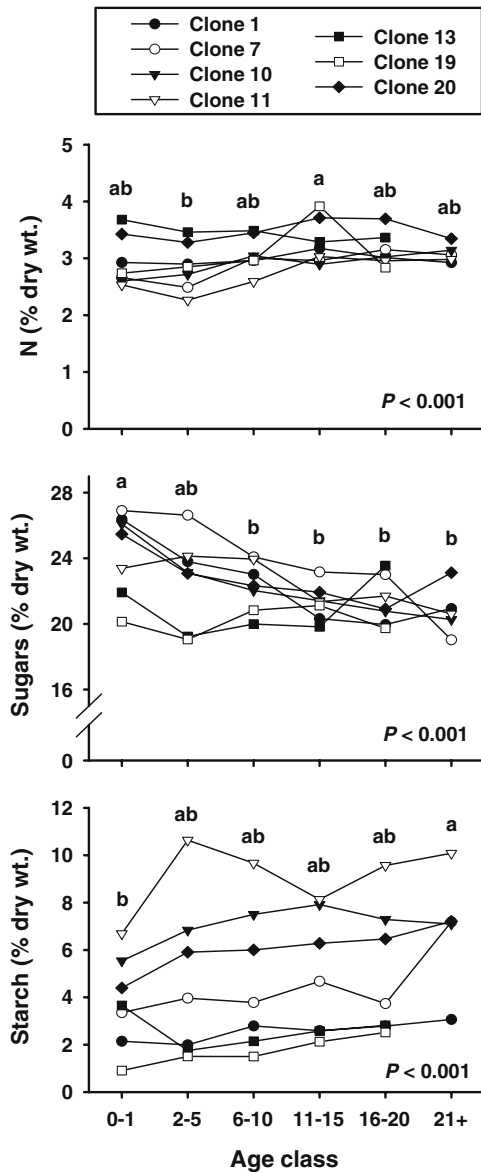


Results

Among-Clone Phytochemical Variation

Leaf chemical constituents of mature ramets varied among the 20 aspen clones (Fig. 1). Condensed tannin concentrations varied 2-fold among clones ($P < 0.001$). Concentrations of salicortin and tremulacin were highly correlated and more variable among clones than were tannins. Salicortin concentrations varied almost 7-fold and tremulacin varied 12-fold among clones ($P < 0.001$). Nitrogen

Fig. 3 Developmental shifts in aspen leaf concentrations of nitrogen (N), sugars, and starch. Symbols represent the average concentration for replicate ramets ($n = 3$) within each of six age classes for the seven clones, respectively. P values refer to the main effect of age class (Proc mixed). Age classes with different letters are significantly different (Bonferroni-adjusted $P < 0.05$)



(N) concentrations were less variable among clones, ranging from 2.8% to 3.6% ($P < 0.001$).

Developmental Patterns Within and Among Aspen Clones

Variation in Growth

Equal-aged ramets from the seven clones differed considerably with respect to height and diameter. An ANCOVA with tree age confirmed differences (height,

$P < 0.001$; diameter, $P < 0.001$). In general, tree size and growth trajectories varied among clones. Separate plots of height and diameter show similar patterns (data not shown).

Variation in Secondary Metabolites

Condensed tannin concentrations increased sharply with age (Fig. 1). Developmental changes occurred primarily between the “0–1” and “2–5” yr age classes, where condensed tannin concentrations increased from 4.5% to an average of almost 14% dry weight (Fig. 2). Phenolic glycoside concentrations also varied among age classes (Table 1), but in contrast to tannins, salicortin and tremulacin concentrations decreased with age, from an average of almost 13% to 1%, and 7% to less than 1%, respectively (Fig. 2). The developmental changes occurred in the first 10 yr, as evidenced by the lack of differences among age classes greater than 10 yr old. Among-clone variation for salicortin and tremulacin was much greater for juveniles than for mature ramets (Fig. 2).

Variation in Nitrogen, Sugars, and Starch

Aspen nitrogen and carbohydrate concentrations also differed among age classes, but the developmental patterns were weak relative to those of tannins and phenolic glycosides (Fig. 3). Nitrogen differed among clones and increased only slightly in more mature ramets (Fig. 3). Average sugar concentrations decreased from 24% in the youngest trees to 19.6% in mature trees. Average starch concentrations were highly variable among clones, ranging from 1.5% to over 8%, and tended to increase in mature trees (Fig. 3).

Variation in Developmental Trajectories

The effects of plant age on phytochemical concentrations varied among aspen clones. For each variable tested, removing the effect of “clone” from the mixed model significantly decreased the goodness of fit (χ^2 tests, $P < 0.001$ for each variable). Looking specifically at the slopes, or rates of concentration change in phytochemical variables, we found that the trajectories of developmental shifts in aspen secondary metabolites were consistent among clones for tannins, but not for

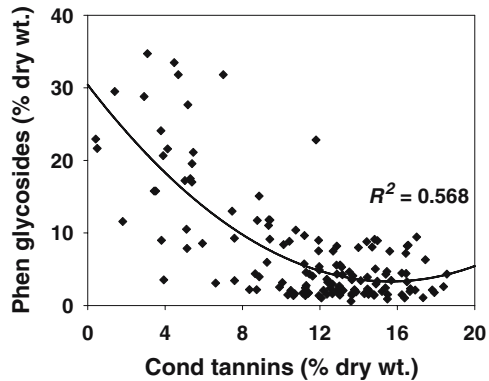
Table 1 Correlation matrix (Pearson) of mean age and aspen phytochemicals, including condensed tannins, total phenolic glycosides (box-cox transformed salicortin + tremulacin), nitrogen (N), sugars, and starch

	Ramet age	Condensed tannins	Phenolic glycosides	N	Sugars
Condensed tannins	0.60***				
Phenolic glycosides	-0.73***	-0.74***			
N	n.s.	n.s.	n.s.		
Sugars	-0.50***	n.s.	0.45**	-0.36*	
Starch	n.s.	n.s.	n.s.	n.s.	n.s.

n.s. = not significant.

n.s.: $P > 0.05$; * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$.

Fig. 4 The negative relationship between condensed tannin (cond tannins) and phenolic glycoside (salicortin + tremulacin; phen glycosides) concentrations in aspen leaves from ramets of different ages. Each point represents a single ramet. Quadratic regression provided the best fit ($P < 0.001$)



phenolic glycosides. Condensed tannin concentrations increased with age similarly for the seven clones (i.e., nonsignificant age \times clone interaction; ANCOVA). Clones that contained higher relative tannin concentrations in juvenile ramets also had higher concentrations in mature ramets. Again, the differences occurred in the first five years, after which tannin concentrations showed no consistent pattern of change. The developmental trajectories of salicortin and tremulacin, however, were markedly different among the clones (i.e., significant clone by age interactions; $P = 0.005$ and $P = 0.018$, respectively). The differences in trajectory were largely due to the variability in levels of phenolic glycosides in juvenile ramets. For example, juvenile ramets of clone 20 (0–1 age class) had almost 30% total phenolic glycosides (salicortin + tremulacin), compared with ca. 10% in clone 11. However, in both clones, ramets greater than 20 yr old had less than 2% total phenolic glycosides.

Correlations

Nitrogen was not correlated with tannins or total phenolic glycosides but was negatively correlated with sugar concentrations (Table 1). Sugar concentrations were positively correlated with total phenolic glycosides. A particularly strong negative correlation occurred between tannin and total phenolic glycoside concentrations when all age classes were considered together. The shape and strength of this relationship are described by a quadratic regression in Fig. 4 ($R^2 = 0.568$; $P < 0.001$).

Discussion

Our survey of phytochemical variation among aspen clones in south central Wisconsin indicates that clones occupying similar habitats and growing under similar conditions produce varying concentrations of condensed tannins, phenolic glycosides, and nitrogen. These patterns are consistent with those found in other natural aspen populations growing in northern lower Michigan (Lindroth and Hwang, 1996b) and in northern Wisconsin (Donaldson, 2005).

In common gardens, genetically based differences in condensed tannins and phenolic glycosides among clones are similar in magnitude to those observed here (Lindroth and Hwang, 1996a; Stevens and Lindroth, 2005; Donaldson et al., 2006). Nonetheless, in this study, we are unable to infer what proportion of the observed

variation may be due to genetics rather than to environment. However, phenolic glycosides (the most variable compounds) show relatively little phenotypic plasticity in common gardens (Hwang and Lindroth, 1997; Osier and Lindroth, 2001). Thus, most of the observed variation in concentration among clones may be due to genotype.

Our survey of developmentally based phytochemical variation indicates that stand demographics may add to the overall magnitude of spatial and temporal chemical variation occurring in aspen populations (Erwin et al., 2001). The strong inverse relationship between the developmental trajectories of tannins and phenolic glycosides in aspen creates strikingly different chemical phenotypes among mature and juvenile ramets within a clone that are likely to influence herbivory (Lindroth and Hwang, 1996a; Osier and Lindroth, 2001) and other chemically mediated processes (Lindroth et al., 2002; Madritch et al., 2006). The trend of increasing tannins with age is difficult to explain because we lack sufficient understanding of their functions in aspen (Lindroth and Hwang, 1996a). The biological activity of phenolic glycosides, however, is well established (Lindroth and Hwang, 1996a; Osier and Lindroth, 2001). The fact that leaves of young ramets contain much higher concentrations of these compounds compared with mature ramets may indicate that they have historically experienced more intense selective pressure than have mature ramets (discussed below).

The magnitude of developmentally based phytochemical shifts in condensed tannin and phenolic glycoside concentrations varied among the clones surveyed (i.e., clones showed different developmental trajectories). If this variation is genetically based and heritable, it would suggest that the same evolutionary factors involved in selecting for (or against) chemical traits in aspen may influence the degree to which developmental shifts occur. Spatial or temporal variation in costs and benefits of allocation to tannins and phenolic glycosides among clones and among age classes may explain the observed patterns. For example, temporally variable population densities of insect herbivores or browsing mammals, or spatially variable resource availability, all can potentially affect the relative costs and benefits of producing foliar allelochemicals (Donaldson et al., 2006; Osier and Lindroth, 2006).

Developmental patterns are also visible but far less pronounced for nitrogen, starch, and sugars. This comparative lack of change in primary compounds may reflect that their role in plant physiology is less variable over developmental time than is that of “secondary” compounds.

We recognize that the developmental patterns observed in this study cannot with certainty be attributed solely to ontogeny (genetically programmed developmental changes). Some portion of the variation between juvenile and mature ramets may be due to differences in their respective environments. For example, resource (light, soil nutrients) availability may differ across space occupied by a clone. As discussed above, the short stature of younger ramets may predispose them to browsing (historically and recently). However, in our selection of 0- to 1-yr-old ramets, we specifically selected individuals that were new root sprouts and that had not been previously browsed. Therefore, the most extreme differences between juvenile and mature clones appear not to be a function of recent browsing. Previous defoliation studies also argue against induction as a proximate explanation. Contrary to the patterns we observed, studies have failed to find substantial induction of phenolic glycosides, but do find significant induction of tannins (Osier and Lindroth, 2004; Stevens and Lindroth, 2005).

Another consideration we must make is that our data derive from differently aged ramets of clonally integrated plants. The importance of clonal integration for the chemical composition of ramets may be significant, as ramets may exchange photosynthate, nutrients, and perhaps even compounds signaling herbivore damage (Magyar et al., 2004). Clonal integration, therefore, may influence the costs and benefits of allocation to defense and contribute to both spatial and temporal variation in the chemical profiles of aspen populations. However, considering the number of studies that report strong herbivore preferences for (Basey et al., 1988; Swihart and Bryant, 2001) and phytochemical differences among (Erwin et al., 2001) juvenile and mature trees in this and other systems, we believe that the developmental changes evident in aspen tannin and phenolic glycoside concentrations may be largely a result of ontogenetic shifts in allocation.

Our findings have several potentially important evolutionary and ecological implications that require further investigation. The fact that leaf tissues of young aspen trees are better defended (i.e., higher levels of phenolic glycosides) suggests a greater selective pressure for defense in young trees compared with mature trees. Browsing pressure from mammals is a likely explanation for such patterns. Mammals exert tremendous selective pressure on aspen, particularly on resprouts and seedlings that are accessible to browsers (Swihart et al., 1994; Kay and Bartos, 2000). Furthermore, because the impacts of herbivory on plant survival, growth, and reproduction may be particularly strong during the juvenile phase (Watkinson, 1986), optimal defense theory (Rhoades, 1979) predicts that this selective pressure leads to relatively higher levels of defense in juvenile compared with mature tissues. Indeed, a meta-analysis conducted by Swihart and Bryant (2001) indicates that mammals almost always prefer mature tissues to juvenile tissues of forest trees, including aspen. Our data suggest that mammals may be a primary source of selection explaining the quantitative distribution of phenolic glycosides in aspen.

The most significant arthropod herbivores of aspen, eruptive outbreak species such as forest tent caterpillars, large aspen tortrix, and gypsy moths, generally cause the greatest damage in advanced even-aged stands. During outbreaks, trees are defoliated regardless of their level of commitment to chemical defenses (Mattson et al., 1991; Donaldson, 2005). As a result, defoliating arthropods may present less selective pressure for chemical defenses than previously thought. In fact, this study and other recent work suggest that advanced stands of aspen in the Great Lakes region generally have lower concentrations of phenolic glycosides than previously thought (Fig. 1; Donaldson, 2005), although levels in advanced stands of western aspen are typically high (Donaldson, Lindroth, and Wooley, unpublished data).

Regardless of the evolutionary outcomes of aspen–arthropod interactions, phenolic glycoside concentration is the best predictor of insect performance both among aspen clones (Lindroth and Hwang, 1996a) and among species of Salicaceae (Edwards, 1978; Tahvanainen et al., 1985). Given that trees undergo developmental shifts in phenolic glycoside concentrations that are greater in magnitude than the genotypic variation observed among adult clones, it is likely that these age-related changes have significant impacts on insect behavior, distributions, and populations (Kearsley and Whitham, 1989, 1998; Waltz and Whitham, 1997).

Explaining the marked increase in condensed tannins as aspen trees mature in terms of selective pressure exerted by herbivory is complicated. In fact, the role of condensed tannins in aspen (and many other plant species), although long assumed

to be defensive, is still not well understood (Ayres et al., 1997). Most studies have failed to find a relationship between aspen tannin concentrations and mammalian or arthropod preferences or performance (Lindroth and Hwang, 1996a). Tannins may play diverse “alternative” roles in plants, from defenses against photooxidative stress (Close and McArthur, 2002) and microbial pathogens (Kellam et al., 1992), to modifying local soil processes and nutrient cycling rates (Hättenschwiler and Vitousek, 2000). For example, variation in tannin concentrations can alter leaf litter quality (Lindroth et al., 2002; Madritch et al., 2006). Concentrations of both nitrogen and phenolics (especially tannins) play important roles in litter decomposition and soil nutrient dynamics in forest soils (Schimel et al., 1996; Northup et al., 1998; Hättenschwiler and Vitousek, 2000; Kraus et al., 2003; Schweitzer et al. 2004). In fact, an alternative hypothesis to the role of tannins as antiherbivore defenses posits that they may be adaptive in their capacity to regulate proximal nutrient cycling (Northup et al., 1998).

In summary, developmental shifts in chemical defenses within aspen clones appear to be greater in magnitude than the variation observed among mature clones. We believe that these developmental changes have likely been shaped by evolutionary factors related to herbivory and ecosystem function and hold importance for diverse ecological interactions today. Developmental shifts in chemical composition in this dominant clonal species are likely to contribute to temporal and spatial mosaics of host quality and to influence patterns of community structure and ecosystem function.

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Resistance and Tolerance of *Terminalia sericea* Trees to Simulated Herbivore Damage Under Different Soil Nutrient and Moisture Conditions

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Abstract Resource availability, degree of herbivore damage, genetic variability, and their interactions influence the allocation of investment by plants to resistance and tolerance traits. We evaluated the independent and interactive effects of soil nutrients and moisture, and simulated the effects of herbivore damage on condensed tannins (resistance) and growth/regrowth (tolerance) traits of *Terminalia sericea*, a deciduous tree in the Kalahari desert that constitutes a major component of livestock diet. We used a completely crossed randomized-block design experiment to examine the effects of nutrients, water availability, and herbivore damage on regrowth and resistance traits of *T. sericea* seedlings. Plant height, number of branches, internode length, leaf area, leaf mass for each seedling, combined weight of stems and twigs, and root mass were recorded. Condensed tannin concentrations were 22.5 and 21.5% higher under low nutrients and low soil moisture than under high nutrient and high water treatment levels. Tannin concentrations did not differ significantly between control and experimental seedlings 2 mo after simulated herbivore damage. Tannin concentrations correlated more strongly with growth traits under low- than under high-nutrient conditions. No trade-offs were detected among individual growth traits, nor between growth traits and condensed tannins. *T. sericea* appeared to invest more in both resistance and regrowth traits when

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grown under low-nutrient conditions. Investment in the resistance trait (condensed tannin) under high-nutrient conditions was minimal and, to a lesser degree, correlated with plant growth. These results suggest that *T. sericea* displays both resistance and tolerance strategies, and that the degree to which each is expressed is resource-dependent.

Keywords Fertilization · Defoliation · Herbivory · Condensed tannins · Compensation · Trade-offs · Plant defenses · Resistance

Introduction

Plants are subjected to a variety of stress factors that affect both their development and survival (Larcher, 1995). On the African savanna, where low nutrient availability and water scarcity are commonplace, herbivory by mammals is also a key factor that may cause significant deviations from conditions of optimal plant life (Du Toit, 1995). In response to these limitations, plants have evolved tolerance and resistance strategies to deal with stress (Mauricio, 2000). Tolerance is the ability of plants to maintain fitness after herbivory in the presence of potentially fitness-reducing stress (Pilson, 2000; Simms, 2000). Resistance traits such as thorns and chemical substances presumably reduce plant acceptability to herbivores (Cooper and Owen-Smith, 1985), thereby reducing the amount of damage that a plant suffers.

Plant defensive strategies are influenced by the availability of resources to the plant (Coley et al., 1985). Any effect on the carbon/nutrient ratio, be it herbivory, changes in soil nutrient condition, water availability, or exposure to light, can cause a change in the total defense level (see review by Stamp, 2003). When trees experience herbivory, a temporary nutrient deficiency is experienced (Luxmore, 1991), which may limit growth more than photosynthesis. Therefore, carbon in excess of growth demand may be shunted into storage and production of resistance traits (see review by Koricheva et al., 1998), while storage may aid tolerance (regrowth) ability (Stowe et al., 2000). Furthermore, excess carbon may increase the production of carbon-based defenses under conditions of low resources, but at little cost to plant fitness (Tuomi, 1992; Ruiz et al., 2002).

Mammalian Herbivory on *Terminalia sericea* Trees in the Kalahari Desert

Terminalia sericea (Combretaceae) is a major contributor of browse for both domestic cattle and goats during the hot-dry season under heavy grazing systems (Katjiua and Ward, 2006). We presumed that browsing on *T. sericea* would elicit carbon-based chemical defenses, given the high light conditions, and poor soil nitrogen and phosphorus concentrations of the Kalahari desert (Mendelsohn et al., 2002). Thus, we reasoned that *T. sericea* would have poor regrowth capacity upon damage because allocations to carbon-based chemical defenses may present an opportunity cost to regrowth capacity (Van der Meijden et al., 1988; Pilson, 2000).

The potential interactions of foliar damage, nutrient availability, and soil moisture content provide an opportunity for us to examine the effects of herbivore damage on the recovery and leaf quality of *T. sericea*. We investigated the effects of

simulated herbivory on the resistance and tolerance traits of *T. sericea* in a fully crossed randomized-block design experiment with 12 factor-level combinations (three levels of herbivory, two levels of fertilizer addition, and two levels of water treatment) across plants derived from five mother plants.

We made the following predictions regarding the levels of condensed tannin (resistance trait): (1) Under conditions of high resource availability, plants will reduce the synthesis of condensed tannin but increase growth rate. (2) Foliar damage to seedlings growing on poor soils will induce greater levels of condensed tannin than on rich soils. (3) Low water and nutrient availability will result in increased condensed tannin at no cost to plant growth. We also predicted the following regarding tolerance traits: (1) Compensation for herbivory is resource-dependent, i.e., compensatory ability will be enhanced under low-resource environments and reduced under high soil nutrient conditions. (2) Tolerance of herbivory is complex and involves trade-offs between tolerance traits that are likely to be expressed under low-resource conditions. (3) The seedling siblings (i.e., from the same mother plants) with high levels of resistance traits will have low levels of tolerance traits and *vice versa*.

Methods and Materials

We investigated condensed tannin levels (resistance trait) of *T. sericea* and its tolerance for partial simulated herbivory among seedlings taken from five mother plants under varying levels of nutrient and water availability in a nursery experiment at the Forestry Research Station in Okahandja, Namibia. Seedlings were raised from seeds collected from 13 individual trees (mother plants) in their natural environment and sown at the end of winter (August 2001) in sterilized river sand. Germination rates and establishment varied considerably among mother plants. Seedlings from five plants with the highest establishment were transplanted and grown in 10-l polythene bags with a surface area of about 0.047 m². Each bag was inoculated with 100 cm³ soil collected under *T. sericea* trees in the field to allow for growth of soil mycorrhizae. Seedlings were grown under nursery conditions for 12 mos, and hardened for 1 mo under reduced solar radiation, and then for 2 wks under full radiation. At this stage, all seedlings were watered to field capacity three times per week. The 2 wk of hardening was necessary because simultaneous exposure to direct radiation and supplementation of fertilizer resulted in the death of seedlings in the pilot phase. Thus, experimental treatments commenced after 2 wks of hardening the plants to direct solar radiation (third week of January 2003) on 15.5 mo-old seedlings and were terminated 3.5 mos later.

We used a completely crossed randomized-block design experiment with two levels of water treatment (low and high), two levels of fertilizer treatment (control and fertilizer addition), and three levels of herbivory (control, low and high). For details of treatment levels, see below. This constituted 12 treatment-level combinations across the five mother plants, with four replicates in each treatment-level combination. Thus, a total of 240 seedlings were used.

For the water treatment, plants were watered once a week (Monday) for the low water level, and three times per week for the high water level. A commercially available fertilizer (N/P/K, 2:3:2) was used for the fertilizer treatment. We applied approximately 4 g of the fertilizer fortnightly over a period of 3 mos (February–

April), which is considered an intermediate level of fertilization. This frequency of application did not only allow for the initial hardening, but also prevented excessive leaching of the nutrients from the sandy soil. The intermediate amount that was applied (30 g/m²) corresponded with those applied by Tilman (1988) and on field plantations in South Africa (J.M.T. Theron, personal communication). Simulated herbivory was administered 1 mo after the application of water and fertilizer treatments. For the low herbivory level, one out of three (33%) leaves was damaged and later removed by hand within an hour of damage, while two out of three (66%) leaves were damaged and also removed in the case of high simulated herbivory. The apical shoot was left intact, as some plants had poor growth and the removal of the apical shoot could have had detrimental effects. Thus, manual defoliation started just below the apical shoot.

The experiment was terminated at the end of April 2003, 1 mo before winter and senescence. Several plant growth parameters, viz. height, leaf area (length and width), number of branches, and internode length, were sampled. Twigs and stems (aboveground biomass), roots (belowground biomass), and leaves were oven-dried at 65°C for 48 hr to obtain dry mass. Whole leaves were counted before drying. Plant height was measured twice, before the experiment started and at the end of the experiment.

Condensed tannin in leaf samples was measured by using a standard acid–butanol protocol (Waterman and Mole, 1994). This method with modification by Hagerman (unpublished data) was applied to extract condensed tannin with 50% methanol. The extract was added to the acid–butanol reagent and heated at 95°C in a boiling bath for 1 hr, then cooled, and absorbance read at 550 nm. Two replicates were used. Condensed tannin concentrations were expressed in quebracho equivalents (% QE) (quebracho is used as a standard and was obtained from A. Hagerman; Hagerman and Butler, 1989). Quebracho tannin is the only readily available condensed tannin. Purified quebracho standard prepared from a single lot of the crude commercial mixture is recommended for use in tannin analysis to minimize differences that may exist among materials provided by even a single supplier (Hagerman and Butler, 1989).

To control the comparison-wise error (see, e.g., Zar, 1984), we performed an overall comparison of the seven dependent variables by using multivariate analysis of variance (MANOVA), with water, herbivory, fertilizer, and their interactions as fixed factors, and mother plants as random factors. The dependent variables used were tannin concentration, leaf area per unit final height, leaf mass per unit final height, change in height during the experiment (per unit initial height), above-/belowground mass, internode length per unit final height, and number of branches per unit final height. Leaf area, leaf mass, internode length, and number of branches were standardized by dividing by final plant height because of strong positive correlations between these variables and final height. Subsequent to the detection of significant effects, *post hoc* univariate analysis (ANOVA) tests were performed. These effects were analyzed in a mixed-model randomized block-design ANOVA (error terms as for model III ANOVA), using Statistica v. 6, GLM procedure. Where appropriate, analyses were followed by a multiple comparisons of means using a Scheffé *post hoc* test. Means are represented \pm 1 standard error, unless otherwise stated. Pearson product–moment correlations were used to determine trade-offs between resistance and tolerance traits and between the various tolerance traits investigated.

Table 1 Multivariate analysis of variance (Manova) of nursery experiment

Factor	Wilk's λ	<i>F</i>	Effect <i>df</i>	Error <i>df</i>	<i>P</i>
Water	0.747	10.55	7	218	<0.001
Herbivory	0.885	1.95	14	436	0.020
Fertilizer	0.531	27.50	7	218	<0.001
Water \times Herbivory	0.875	2.14	14	436	0.009
Water \times Fertilizer	0.978	0.69	7	218	0.678
Herbivory \times Fertilizer	0.885	1.96	14	436	0.019
Water \times Herbivory \times Fertilizer	0.924	1.25	14	436	0.233
Mother Plant	0.240	13.63	28	787.4	<0.001

Dependent variables used in this analysis were as follows: tannin concentration, leaf area per unit final height, leaf mass per unit final height, change in height during experiment (per unit initial height), above/below mass, internode length per unit final height, and no. branches per unit final height. Leaf area, leaf mass, internode length, and no. of branches were standardized by dividing by final plant height because of strong positive correlations between these variables and final height.

Results

MANOVA results revealed a significant overall multivariate effect for water ($df = 7, 218, F = 10.55, P < 0.001$), herbivory ($df = 14, 436, F = 1.95, P = 0.020$), and fertilizer ($df = 7, 218, F = 27.50, P < 0.001$) (Table 1). There were interaction effects for water \times herbivory ($df = 14, 436, F = 2.14, P = 0.009$) and herbivory \times fertilizer ($df = 14, 436, F = 1.96, P = 0.019$). The interaction effects of water \times fertilizer and water \times herbivory \times fertilizer were nonsignificant. The effect of mother plant was significant ($df = 28, 787.4, F = 13.63, P < 0.001$).

Resistance Trait and Growth

Fertilizer addition reduced the production of condensed tannin ($df = 1, 224, F = 26.91, P < 0.001$). Mean \pm SE condensed tannin in the low-resource environment was 22.86 ± 1.229 (% QE) compared to 17.73 ± 0.741 (% QE) on fertilized soils. Thus, tannin levels declined by 22.5% when fertilizer was added. In contrast, fertilizer positively affected all growth traits ($P < 0.005$), with the exception of internode length.

The mean growth of fertilized seedlings was higher than those grown on the control-fertilizer soils (Table 2). The biggest difference in parameters was in

Table 2 Significant ($P < 0.05$) effects of fertilizer treatment on seedling growth (Mean \pm SE)

Growth parameter	Fertilizer treatment	
	Control	Fertilizer addition
Plant height (cm)	17.5 \pm 6.49	46.8 \pm 7.70
Leaf area (mm ²)	913.1 \pm 31.38	1053.6 \pm 27.09
Leaf mass (g)	0.05 \pm 0.000	0.06 \pm 0.002
Branches/height ratio	0.01 \pm 0.000	0.02 \pm 0.001
Number of branches	5.1 \pm 0.23	7.5 \pm 0.26
Above-/belowground biomass ratio	0.2 \pm 0.01	0.3 \pm 0.01

seedling height. Seedlings treated with fertilizer grew 165% taller. Similarly, fertilized seedlings yielded a greater number of branches (48.2%), above-/belowground biomass ratio (31.3%), number of branches/height ratio (29%), leaf area (15%), and leaf mass (14%) than control seedlings.

We predicted that leaf damage on seedlings growing in low-resource environments would induce greater levels of condensed tannin to avoid future damage [resistance prediction (2)]. This was not the case. Although condensed tannin concentration was higher in the low-resource environment, simulated herbivory did not induce higher tannin concentration in the low-resource environment ($df = 2, 224, F = 2.77, P = 0.065$). The interaction of herbivory and fertilizer was also not significant ($df = 2, 224, F = 0.05, P = 0.953$). That is, resource availability did not influence the response of condensed tannin concentration to simulated herbivory.

Water level affected tannin concentration ($df = 1, 224, F = 24.84, P < 0.001$). Tannin concentration was 21.7% higher in the low water treatment ($22.76 \pm 1.092\%$ QE) than in the high water treatment ($17.82 \pm 0.935\%$ QE).

Trade-Offs between Resistance and Growth Traits

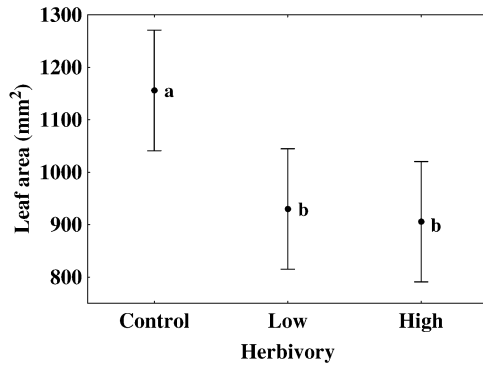
We considered trade-offs in seedlings grown in low-resource conditions (low water and control fertilizer, i.e., LW–CF treatment combination) and in high-resource conditions (high water and fertilizer addition treatment, i.e., HW–IF combination). Condensed tannins in the low-resource environments (LW–CF) correlated positively with the majority of growth traits, but negatively with the number of branches/height ratio ($r = -0.376, P = 0.003$) (Table 3). The correlations of condensed tannin with leaf area, leaf mass, seedling height, number of branches, and internode length were significant ($P < 0.05$) and positive, while above-/belowground biomass ratio did not correlate ($P > 0.05$) with tannin concentration. The positive correlation between condensed tannin synthesis and the majority of growth traits is consistent with resistance prediction (3), which predicted that tannin would accumulate at no cost to plant growth in the low-resource environment.

A similar trend was found in the high-resource environment (HW–IF treatment combination) (Table 3). Most correlations were significant ($P < 0.05$) and positive, except for a negative correlation involving the number of branches/height ratio.

Table 3 Correlations of condensed tannin concentrations (% QE) with seedling growth traits, grown under low and high resource environments

Tannin		Growth traits						Above-/below ground biomass
		Leaf area (mm ²)	Leaf dry mass (g)	Height (cm)	No. of branches	Internode length (cm)	No. of branches/height	
Low-resource environment (LW–CF)	<i>r</i>	0.70	0.80	0.44	0.43	0.73	-0.38	-0.10
	<i>P</i>	0.001	0.001	0.001	0.001	0.001	0.003	0.450
High-resource environment (HW–IF)	<i>r</i>	0.66	0.51	0.37	0.39	0.56	-0.35	0.37
	<i>P</i>	0.001	0.001	0.004	0.002	0.001	0.006	0.004

Fig. 1 Effect of simulated herbivory on leaf area. Error bars denote 95% confidence limits, and the different letters indicate significant mean differences at $P < 0.05$



Correlations were generally weaker in the high-resource environments than in the low-resource environment.

Tolerance Traits

We predicted that compensatory ability of previously herbivore-damaged seedlings is resource-dependent [tolerance prediction (1)]. However, there was no significant interaction effect of herbivory \times water \times fertilizer treatments for seedling height ($df = 2, 224, F = 0.61, P = 0.545$), number of branches/height ratio ($df = 2, 224, F = 0.77, P = 0.465$), above-/belowground biomass ratio ($df = 2, 224, F = 2.23, P = 0.110$), internode length ($df = 2, 224, F = 1.36, P = 0.259$), and the number of branches ($df = 2, 224, F = 1.92, P = 0.149$). Thus, no differences existed in the (re)growth rate of these traits among seedlings exposed to control, low, and high herbivory.

Leaf regrowth capacity was resource-dependent. Herbivory reduced leaf area ($df = 2, 57, F = 5.79, P = 0.005$) and leaf mass ($df = 2, 57, F = 8.89, P < 0.001$) when grown under conditions of low water and fertilizer addition levels. Both leaf area (Fig. 1) and leaf mass (Fig. 2) undercompensated (i.e., size upon regrowth was less than the original) equally under the low and high levels of herbivory treatment.

The interaction effect of herbivory \times water on the number of branches was significant ($df = 2, 224, F = 4.35, P = 0.014$). Both low and high simulated herbivory reduced the number of branches produced when seedlings were exposed to the high water treatment level (Fig. 3). The results also indicate that the reduced branches

Fig. 2 Effect of simulated herbivory on leaf dry mass. Error bars denote 95% confidence limits, and the different letters indicate significant mean differences at $P < 0.05$

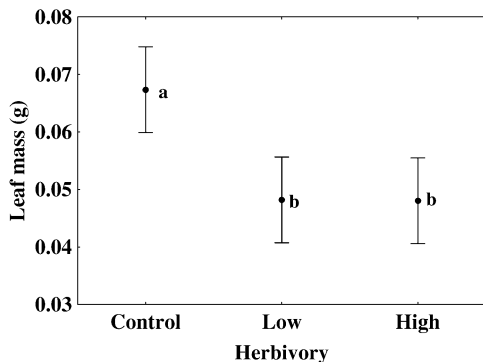
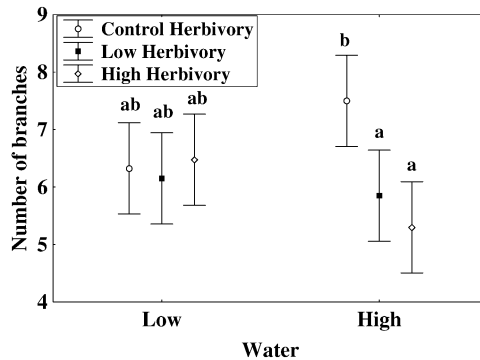


Fig. 3 Effect of simulated herbivory on the number of branches produced when seedlings were treated with high water levels. Error bars denote 95% confidence limits, and the different letters indicate significant mean differences at $P < 0.05$



produced under herbivory in the high water treatment level did not differ from those produced in the low water treatment level, irrespective of the degree of herbivory.

Trade-Offs among Growth Traits

Resource conditions affected trade-offs among tolerance traits (Tables 4 and 5). Under high stress conditions (LW–HH–CF), leaf mass correlated negatively with above-/belowground biomass ratio ($r = -0.471$, $P = 0.036$) and the number of branches/height ratio ($r = -0.491$, $P = 0.028$) (Table 4). No trade-offs were detected under the least stressful conditions (HW–CH–IF) (Table 5). There were stronger correlations under the high stress conditions (Table 4) than in the least stressful conditions (Table 5).

Negative correlations among growth traits were detected in the intermediate range of stress conditions. For example, leaf area correlated negatively with

Table 4 Correlations between growth traits of seedlings exposed to high stress conditions (LW–HH–CF), using bonferroni-adjusted values of α to control the comparison-wise error (Type I Error) for a large number of correlations

		Leaf area	Leaf mass	Height	Branches	Internode length	Branches/Height
Leaf mass	r	0.81					
	P	<0.001*					
Height	r	0.53	0.49				
	P	<0.001*	0.001*				
Number of branches	r	0.58	0.36	0.46			
	P	<0.001*	0.006	0.002*			
Internode length	r	0.85	0.76	0.51	0.37		
	P	<0.001*	<0.001*	0.001*	0.005		
Branches/height	r	-0.29	-0.49	–	–	-0.51	
	P	0.011	0.001*	–	–	0.001*	
Above/below	r	-0.28	-0.47	-0.10	-0.06	-0.28	0.33
	P	0.011	0.002*	0.032	0.038	0.011	0.007

* α was only considered significant if $P < 0.0024$.

Correlations involving height and the number of branches vs. the number of branches/height were not reported because they were autocorrelated.

Table 5 Correlations between growth traits in seedlings exposed to low stress conditions (HW-CH-IF), using Bonferroni-adjusted values of α to control the comparison-wise error (Type I Error) for a large number of correlations

		Leaf area	Leaf mass	Height	Branches	Internode length	Branches/Height
Leaf mass	<i>r</i>	0.60					
	<i>P</i>	<0.0021*					
Height	<i>r</i>	0.17	0.26				
	<i>P</i>	0.023	0.013				
Number of branches	<i>r</i>	0.48	0.19	0.30			
	<i>P</i>	0.002*	0.020	0.009			
Internode length	<i>r</i>	0.58	0.68	0.26	0.45		
	<i>P</i>	<0.001*	<0.001*	0.013	<0.001*		
Branches/height	<i>r</i>	0.11	-0.32	-	-	-0.42	
	<i>P</i>	0.031	0.008	-	-	0.003	
Above/below	<i>r</i>	0.21	-0.03	0.43	0.70	0.43	-0.10
	<i>P</i>	0.018	0.044	0.003	<0.001*	0.003	0.032

* α was considered significant if $p < 0.0024$.

Correlations involving the number of branches and height vs. the number of branches/height were not reported because they were autocorrelated.

branches/height ratio under HW-HH-IF ($r = -0.59$, $P = 0.006$), LW-LH-IF ($r = -0.51$, $P = 0.021$), and even under LW-CH-CF ($r = -0.51$, $p = 0.023$). Leaf mass correlated negatively with branches/height ratio under LW-CH-CF ($r = -0.56$, $P = 0.010$) and LW-LH-IF ($r = -0.46$, $P = 0.040$).

Mother Plant Resistance and Tolerance Traits

Tannin concentrations differed among mother plants ($df = 4, 180$, $F = 89.28$, $P < 0.001$). Differences in allocation to condensed tannins (resistance trait) among mother plants corresponded linearly with investments into growth traits such as leaf area ($r = 0.91$, $P = 0.03$), leaf mass ($r = 0.94$, $P = 0.02$), height ($r = 0.93$, $P = 0.02$), and internode length ($r = 0.97$, $P = 0.006$). These results contradicted tolerance prediction (3). Tannin synthesis was not correlated with above-/belowground biomass production ($r = -0.27$, $P = 0.657$).

Discussion

Resource Gradients

Seedlings in the resource-poor environments had 22.5% higher condensed tannin concentrations than those grown in high-resource environments. Consistent with this, other studies have shown that plants growing in resource-poor environments maintain a high resistance level against herbivore damage (Gebauer et al., 1998; Osier and Lindroth, 2001). Nutrient-poor soils combined with drought conditions constrain growth more than photosynthesis, and, subsequently, assimilates in excess of growth requirements are shunted into defense (Herms and Mattson, 1992) and

storage (see review by Koricheva et al., 1998). In this study, seedlings under limited soil moisture synthesized higher (21.7%) concentrations of condensed tannins than those in the high soil moisture environment. Such effects of drought conditions have been reported elsewhere (Kouki and Manetas, 2002). The current study showed that drought and poor soil nutrients affect condensed tannin synthesis in a similar manner.

Fertilizer addition to poor soils seems to reduce the ratio of carbon/nutrients available to defense, thereby reducing potential resistance (Kouki and Manetas, 2002), but promoting greater plant growth (Mutikainen et al., 2000). Along an environmental resource gradient, tolerant plants seem to be faced with the dilemma whether to grow faster or to compensate for damage (Hochwender et al., 2000). In this study, fertilizer addition increased seedling leaf area, leaf dry mass, height, number of branches, number of branches/height ratio, and above-/belowground biomass ratio, rather than enhancing compensatory responses to damage.

Inducible Resistance

The effects of herbivory or its interactions with either fertilizer or water treatments did not influence the synthesis of condensed tannins in seedlings. Expression of resistance traits in previously browsed species may take time to reach levels above controls (Larcher, 1995). Initially, browsed plants may show a decline in allocation to resistance traits (stress reaction), followed by mobilization of resources for resistance traits that, depending on the severity and persistence of stress, may recover and exceed the levels of the controls (= overcompensation of resistance), or may revert back to levels similar to that of controls (= full compensation) if stress was light and of short duration (Underwood, 1999), or if sufficient time was allowed without further herbivore damage over the growing season (Katjua and Ward, unpublished data). Severe and persistent herbivore damage may, however, reduce tannin concentrations to levels lower than that of control seedlings (= undercompensation) due to reduced resources (see review by Bergelson and Purrington, 1996). However, our results were not consistent with these studies. This may be because the high simulated herbivory was not frequent enough to deplete plant reserves needed for condensed tannin production.

Resistance vs. Growth Trade-Offs

This study showed no indication of trade-offs between investments into tannin synthesis and the majority of growth traits (leaf area, leaf dry mass, height, and internode length) in the low-resource environments. These results contradicted resistance prediction (3). The negative correlation between condensed tannin content and the number of branches/height ratio in the low-resource environment was also detected in the high-resource environment. Thus, resource availability alone could not explain this negative correlation.

Growth traits such as leaf area, internode length, and above-/belowground biomass ratio correlated positively with condensed tannin concentration. A comparison of these results (Table 3) suggests that investments into resistance traits may be more closely coupled with growth traits in low-resource environments than in high-resource environments. The cost of allocation to resistance traits is expected to

be more evident when plants are grown under stressful conditions (Bazzaz et al., 1987; Herms and Mattson, 1992). It appears that our plants under low-resource conditions had limited investment budgets available for growth and resistance traits, and that investment partitioning between growth and resistance traits could approximate unity with greater resource limitation. Thus, our results yielded no evidence of cost implications for allocations to resistance and tolerance traits. This is consistent with results reported by Ward and Young (2002) on *Acacia drepanolobium* in Kenya.

Tolerance Compensatory Responses

There was no significant interaction effect of water \times herbivory \times fertilizer treatment on seedling height, branches/height ratio, above-/belowground biomass ratio, internode length, and number of branches. The lack of a difference in the regrowth rates of the above growth traits between the control, low, and high simulated herbivory implies that either herbivory had little or no effect on these traits, or that growth capacity was fully compensated. In contrast, leaf compensatory ability was resource-dependent. Leaf area and leaf dry mass undercompensated for growth when grown on the low water and intermediate fertilizer treatments. However, both leaf area and leaf dry mass fully compensated for growth in low-resource environments (= control fertilizer treatment). Hochwender et al. (2000) argued that, because compensatory growth and reproduction are more prevalent in low-resource environments, it contributes greatly to the survival and reproduction of plants in these environments.

In this study, a 31.3% lower above-/belowground biomass ratio was found in seedlings grown on the low-resource environment than in the high-resource environment. Prior investments into storage structures in the low-resource environments are reallocated to sites where new growth is required, thereby enhancing compensatory regrowth (Hochwender et al., 2000). In high-resource environments, compensatory ability is limited or may even be absent, because investments into storage structures are presumably limited by high growth rates. Indeed, plants invest more in aboveground growth than in belowground structures when growing in high-resource environments (Kouki and Manetas, 2002).

Herbivory did not affect the number of branches, possibly because the apical shoots were not damaged during experimentation. There was a significant water \times herbivory interaction effect on the number of branches. Seedlings exposed to low and high herbivory treatments produced fewer branches when given the high water treatment. This is consistent with tolerance prediction (1) that compensatory ability is resource-dependent. Among all growth traits investigated, leaf area and leaf dry mass were most sensitive to herbivore damage.

Trade-Offs among Tolerance Traits

Allocations among growth traits in high-resource environments seem to be flexible and less coupled to the competing demands of the different growth traits (Table 5), than in the low-resource environments (Table 4). In general, our results indicate that: (1) resource conditions, internal or external to the plant, resulting from variability in soil moisture, herbivory, and soil nutrients determine the proportional

allocation of investments among competing growth traits; (2) partitioning of investment appears to be more strongly correlated among competing growth traits in the low-resource environments than in the high-resource environments; and (3) leaf area and leaf mass are the most sensitive traits to allocation shifts in *T. sericea*.

Mother Plant Resistance and Tolerance

The concentrations of condensed tannin (= resistance trait) differed in the same way as the regrowth capacity (= tolerance traits) among the five mother plants. That is, plants with high vigor invested heavily in both resistance and tolerance traits, whereas weak plants invested equally poorly in resistance and tolerance traits. Thus, there was a positive genetic correlation between the levels of resistance and tolerance traits among *T. sericea* mother plants.

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Effects of Elevated Carbon Dioxide and Ozone on Foliar Proanthocyanidins in *Betula platyphylla*, *Betula ermanii*, and *Fagus crenata* Seedlings

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Abstract Proanthocyanidins (PAs) or condensed tannins are a major group of phenolic compounds in the leaves of birch trees and many other woody and herbaceous plants. These compounds constitute a significant allocation of carbon in leaves and are involved in plant responses to environmental stress factors, such as pathogens or herbivores. In some plants, PA concentrations are affected by atmospheric carbon dioxide (CO₂) and ozone (O₃) levels that may influence, for example, species fitness, community structure, or ecosystem nutrient cycling. Therefore, a study on the quantitative response of PAs to elevated concentrations of carbon dioxide (CO₂) and ozone (O₃) was undertaken in seedlings of *Betula platyphylla*, *Betula ermanii*, and *Fagus crenata*. Seedlings were exposed to ambient or elevated O₃ and CO₂ levels during two growing seasons in the Kanto district in

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Japan. Ten open-top chambers were used for five different treatments with two replicates: filtered air (FA), ambient air (ambient O₃, 43 ppb; ambient CO₂, 377 ppm), elevated O₃ (1.5 × ambient O₃, 66 ppb), elevated CO₂ (1.5 × ambient CO₂, 544 ppm), and elevated O₃ and CO₂ combined. In addition, seedlings growing in natural conditions outside of chambers were studied. Leaf samples were analyzed for total PA concentrations by butanol–HCl assay and for polymeric PA concentrations by normal-phase high-performance liquid chromatography. Total PA concentrations in leaves of all species were similarly affected by different treatments. They were significantly higher in seedlings treated with elevated CO₂ and O₃ combined, and in seedlings growing outside chambers compared with the FA controls. *F. crenata* contained only traces of polymeric PAs, but significant species × treatment interaction was observed in the polymeric PA concentrations in *B. ermanii* and *B. platyphylla*. In *B. platyphylla*, leaves treated with elevated CO₂ + O₃ differed significantly from all other treatments. It was suggested that the strongest effect of elevated CO₂ and O₃ combined on leaf PA contents resulted from the additive effect of these environmental factors on phenolic biosynthesis.

Keywords Beech · Birch · Butanol–HCl assay · Carbon dioxide · HPLC · Ozone · Proanthocyanidins

Introduction

Background levels of greenhouse gases, ozone (O₃) and carbon dioxide (CO₂), have continued to increase because of human activities. The total amount of tropospheric O₃ has been estimated to have increased by 36% since 1750. Atmospheric CO₂ has increased more than 30% over the same time (IPCC, 2001). O₃ inhibits plant growth by reduction in net photosynthesis (inhibition of carbon assimilation), causes an imbalance of carbon allocation to plant organs (decreasing yield and nutritive quality of biomass), and increases sensitivity to other environmental stresses such as plant diseases (Reich, 1983; Darral, 1989; Manning and Tiedemann, 1995; Fuhrer and Booker, 2003). CO₂ has direct effects on plant photosynthesis and growth, and enriched CO₂ can alter the concentrations of primary and secondary metabolites in leaves (Lindroth et al., 1993). Furthermore, O₃ stimulates phenylpropanoid metabolism and thus induces accumulation of phenolic compounds, such as flavonoids and tannins in leaf tissues (Jordan et al., 1991; Kangasjärvi et al., 1994; Eckey-Kaltenbach et al., 1994; Booker and Miller, 1998).

Proanthocyanidins (PAs) (also known as condensed tannins) form a class of natural polyphenolic compounds. They are oligomeric and polymeric end products of the biosynthetic flavonoid pathway consisting of chains of flavan-3-ol units linked together mainly by carbon–carbon bonds at C4→C8 or C4→C6. PAs can be classified to six subclasses (procyanidins, prodelphinidins, proflisetinidins, propelargonidins, prorobinetinidins, and proguibourtinidins) based on the substitution pattern of the single flavan-3-ol unit. Birch leaf PAs are procyanidins and prodelphinidins (Ossipova et al., 2001). PAs are the main phenolic compounds in the leaves of certain woody plants and in herbaceous plants. For example, in the mature leaves of mountain birch, PA concentration can be as high as 120 mg g⁻¹ of dry mass that corresponds to approximately 80% of their total phenolic concentration (Ossipova et al., 2001).

PA concentrations typically increase in response to enriched CO₂. For example, enriched CO₂ increased total PA concentrations in *Populus tremuloides*, *Quercus rubra*, and *Acer saccharum* leaves (Lindroth et al., 1993; Kinney et al., 1997). PA concentrations were also increased by elevated CO₂ in *Salix myrsinolia* (Julkunen-Tiitto et al., 1993), *Eucalyptus tereticornis* (Lawler et al., 1997), *Pinus palustris* (Entry et al., 1998), *Pinus taeda* (Gebauer et al., 1998; Booker and Maier, 2001), *Lotus cornutus* (Carter et al., 1999), and *Gossypium hirsutum* (Booker, 2000; Booker et al., 2000). The effect of elevated CO₂ or O₃ levels on total PA concentration in *Betula* sp. leaves has also been investigated (Lavola et al., 1994, 2000; Traw et al., 1996; Lindroth et al., 2001). Experiments with *B. allegheniensis*, *B. populifolia*, *B. pendula*, and *B. papyrifera* seedlings showed that total PA concentrations in leaves of seedlings growing under elevated concentration of CO₂ were significantly higher than those in control leaves (Traw et al., 1996; Lavola et al., 2000; Lindroth et al., 2001; Kuokkanen et al., 2003). In contrast, enriched O₃ did not affect total PA concentrations in birch leaves (Lavola et al., 1994; Lindroth et al., 2001). The strongest effect was observed with combined CO₂ and O₃ treatments. However, in *Gossypium hirsutum*, O₃ treatment did not have a significant effect on leaf PA concentration, but a CO₂–O₃ interaction was observed (Booker, 2000). Jordan et al. (1991) and Booker et al. (1996) have reported that treatment with high O₃ concentrations increases PA concentration in *Pinus taeda* needles.

PAs influence plant–herbivore interactions. They bind and precipitate proteins and polysaccharides and may reduce the suitability of foliage of woody plants for herbivores (Haslam, 1988; Herms and Mattson, 1992). Concentrations of PAs are generally used to quantify resistance levels of woody plants to insects and other herbivores. The total PA concentrations in birch leaves correlate negatively with growth rates in later larval stages of *Epirrita autumnata* (Kause et al., 1999; Ossipov et al., 2001). Therefore, induction of phenolic biosynthesis by elevated levels of CO₂ and O₃, single or combined, may also regulate plant–herbivore relationships.

PAs are commonly quantified as total PA concentrations by using colorimetric methods. These methods are inexpensive and fast, but lack specificity (Rohr, 2002). The butanol–HCl (BH) and vanillin assays are the most commonly used methods. The BH assay uses hydrochloric acid catalyzed depolymerization of PAs in butanol to yield colored anthocyanidins that can be detected spectrophotometrically (Waterman and Mole, 1994). Various limitations for this method have been reported (see, for example, Porter et al., 1986; Scalbert, 1992; Waterman and Mole, 1994; Schofield et al., 2001). The most important are the proportion of water in the reaction mixture and the choice of PA standard. The BH assay should be used with caution for quantification (Schofield et al., 2001). The vanillin method uses the condensation reaction of PAs with vanillin (Rohr, 2002). The reliability of this method can be affected by many factors as listed in Schofield et al. (2001): the type of solvent used, the nature and concentration of acid, the vanillin concentration, the reaction time and temperature, and the PA standard used. Chromatographic methods enable more specific determination and quantification of PAs. We have used reversed-phase high-performance liquid chromatography (RP-HPLC) for analysis of individual shorter-chain PAs (less than tetramers) and normal-phase HPLC (NP-HPLC) for analysis of higher PAs separated by their degree of polymerization (DP; Karonen et al., 2004). PAs in birch leaves are mainly in polymeric form (DP >10), and their concentrations can be easily determined with NP-HPLC (Karonen et al., 2006).

The principal aim of this research was to test some biochemical mechanisms involved in the adaptations of woody plants to an atmospheric pollutant (such as tropospheric O₃) possibly involved in forest decline and to increasing concentrations of atmospheric CO₂. The separate and combined effect of CO₂ and O₃ on the concentrations of total and polymeric PAs in leaves of three woody plant species, *Betula platyphylla*, *Betula ermanii*, and *Fagus crenata*, were evaluated and compared.

Methods and Materials

Study Site

Experiments were carried out at the Akagi Testing Center of the Central Research Institute of Electric Power Industry (100 km north–northwest of Tokyo, Seta county, Gunma prefecture, Japan), 540 m above sea level on the south slope of Mt. Akagi.

Plant Material and Experimental Design

In May 2002, 2-yr-old *B. platyphylla* Sukatchev var. *japonica* seedlings, 3-yr-old *B. ermanii* Cham. seedlings, and 4-yr-old *F. crenata* Blume seedlings were planted individually in pots filled with 12 l of a volcanic ash soil. Trees were grown outdoors at the experimental site until transfer into the chambers. Twenty-four seedlings per species were put into each open-top chamber or ambient plot (AP; outdoor) at the beginning of July 2002. To reduce positional effects, each chamber was divided into four square sections, and seedlings were placed in a fully randomized design within each of these sections. Each spring and summer, all pots were fertilized at a rate of 80–80–80 kg/ha of N–P–K. Seedlings in chambers received subterranean water (pH 7.7) by drip irrigation to the soil, as well as deionized water to the top of plants via mist generating systems.

Exposure to O₃ and CO₂ was conducted by using modified square-greenhouse-type open-top chambers (3.6 × 3.6 m). Advantages of the open-top chamber systems are the ability to control exposure dynamics, an easy definition of the experimental unit, and the possibility to exclude a proportion of ambient O₃ by charcoal filtering of ambient air (Reich, 1987). Chamber walls were transparent glass. Each open-top chamber was equipped with a fan box. Fan boxes were fitted with two fans and two filters containing activated charcoal. Filtered air and treatment gases were fed into a 7.8-m³ space beneath the chamber floor where a thorough mixing of gases occurred before injection into the chamber. Treatment air was introduced into the chamber through holes in the interior. Air supply was sufficient to achieve four changes per min. Injected air was passed through activated charcoal filters. Chamber fans operated continuously (24 hr per day) over the experimental period. Wind velocity at a height of 1.0 m above the floor of chamber ranged from 0.06 to 0.21 m/sec and averaged 0.15 m/sec. Air temperature and gas concentrations were not uniformly distributed within chambers, but deviations were small (about 5%). During the experiment, increases in annual average of mean air temperature inside the chambers as compared with outside were <1°C. Photosynthetic photon flux density inside the chambers was 75% of full sunlight.

Ten open-top chambers were used for five different treatments (two replicates of each): filtered air was passed through activated charcoal (FA), ambient air ($1.0 \times$ ambient O_3), elevated O_3 ($1.5 \times$ ambient O_3), elevated CO_2 ($1.5 \times$ ambient $CO_2 + 1.0 \times$ ambient O_3), and elevated O_3 and CO_2 combined. In addition, two non-chambered APs were established outdoors to test the effect of the chamber. Treatments were conducted from 12 July to 31 October in 2002 and from 26 March to 31 October in 2003. Mean O_3 concentration was 43 ppb in ambient air and 66 ppb in the elevated O_3 treatment during the entire exposure time. Mean CO_2 concentration was 377 ppm in ambient air and 544 ppm in the elevated CO_2 treatment. Additional experimental parameters are shown in Table 1. Ozone was added to the chambers in the $1.0 \times O_3$ and $1.5 \times O_3$ treatments 24 hr per day. Ozone was generated by O_3 generator (model OZ-24-UA; Ebara Corp., Kanagawa, Japan) that used oxygen-enriched dry air as the source gas. Ozonated air was passed through a water scrubber to remove highly reactive by-products such as N_2O_5 before introduction into the air-mixing space. Gas cylinders containing pure liquid CO_2 were used as a source of CO_2 gas. The supply of CO_2 to the chambers in the $1.5 \times CO_2$ treatment was interrupted during the night. Ozone and CO_2 concentrations inside the chambers and in the ambient air were monitored at 3-min intervals by O_3 analyzers (Model 9810; Monitor Labs, Inc., USA) and infrared gas analyzer (Model ZRH; Fuji Electric Corp., Tokyo, Japan), respectively. The air sampling tube was 0.8 m above the floor in the center of the chamber.

Table 1 Average daily CO_2 and O_3 concentrations, mean daily and maximum air temperatures, and mean daily photosynthetic photon flux densities (PPFD) from 21 July to 31 October 2002 and from 26 March to 31 October 2003

	2002	2003
CO_2 concentration (ppm) ^a		
Amb	362	384
$1.5 \times$ Amb	534	549
AP	375	383
O_3 concentration (ppb) ^b		
FA	16	16
Amb	41	44
$1.5 \times$ Amb	61	68
AP	46	46
Mean daily temperature ($^{\circ}C$)		
In the open-top chambers	21	18
Outside the chambers	20	17
Mean maximum temperature ($^{\circ}C$)		
In the open-top chambers	26	23
Outside the chambers	24	21
Mean PPFD ($mol\ m^{-2}\ day^{-2}$)		
In the open-top chambers	17	15
Outside the chambers	22	20

^a Daily 12 hr (0600–1800) average CO_2 concentration in open-top chambers: in ambient CO_2 concentration (Amb) and in the elevated $1.5 \times CO_2$ concentration ($1.5 \times$ Amb); and in an ambient plot outside of the chambers (AP).

^b Daily 12 hr (0600–1800) average O_3 concentration in open-top chambers: in charcoal-filtered air (FA), in ambient O_3 concentration (Amb), and in the elevated $1.5 \times O_3$ concentration ($1.5 \times$ Amb); and in the ambient plot outside of the chambers (AP). O_3 was added to nonfiltered air for 24 hr daily.

Leaf Sampling

Mature leaves were sampled 70 d after the end of bud break in all individual trees in summer 2003: short shoot leaves of *B. ermanii* on 13 June and of *B. platyphylla* var. *japonica* on 20 June, and first-flushed leaves of *F. crenata* seedlings on 8 August. Leaves were collected from two or three lateral branches at the upper part of the seedling from six seedlings per species chamber for a total of 12 samples (seedlings) per treatment. Leaves were frozen in liquid nitrogen and lyophilized. Dry leaf material was ground to powder with a Retsch MM200 Mixer mill and stored at -20°C .

Extraction

Leaf material (30 mg) was extracted in Eppendorf tubes for 30 min with 0.65 ml of 80% aqueous acetone in a planar shaker. The homogenate was centrifuged for 10 min at $8800 \times g$, the clear supernatant was collected, and the insoluble residue was re-extracted once more with 0.65 ml of 80% aqueous acetone and twice with 100% acetone. Extracts were combined, and acetone was removed in a vacuum concentrator (Concentrator 5301, Eppendorf AG, Germany) prior to lyophilization. Lyophilized extracts were dissolved in 1.0 ml of chloroform/80% aqueous methanol mixture (1:1, v/v) and shaken for 25 min in a planar shaker. Following this treatment, 0.5 ml of 20% aqueous methanol was added, and the sample was mixed again for 20 min and centrifuged for 10 min at $8800 \times g$. The aqueous methanol fraction containing polar phenolic compounds was collected, and the methanol was removed by vacuum concentrator prior to lyophilization. The lyophilized fraction was dissolved in 0.7 ml of water, centrifuged for 10 min at $8800 \times g$, filtered through a 0.45- μm PTFE filter, and kept frozen until PA analyses.

Analysis of Proanthocyanidins

Total PA concentrations were analyzed as previously described (Ossipova et al., 2001). A 0.1-ml sample of birch leaf extract and 0.6 ml of water were added to 6 ml of 1-butanol/hydrochloric acid (95:5, v/v) solution. The reaction mixture was heated at 95°C for 2 hr and then cooled to room temperature. Absorbance was measured at 555 nm on Perkin-Elmer Lambda 12 UV/VIS spectrophotometer (Norwalk, CT, USA).

Polymeric PA concentrations were analyzed by using an HP 1090 Liquid Chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with an autosampler, binary pump, column oven, diode array detector, and HP ChemStation data system. The column and chromatographic conditions were previously described (Karonen et al., 2004, 2006). A Merck LiChrospher Si 60 column (250 \times 4 mm i.d., 5 μm) equipped with a precolumn at 37°C was used. Two solvents, dichloromethane–methanol–water–acetic acid (82:14:2:2, v/v) (A) and methanol–water–acetic acid (96:2:2, v/v) (B), were used. An injection volume of 5 μl and a flow rate of 1 ml/min were used. The elution profile was as follows: 0–30 min, 0–18% B in A (linear gradient); 30–45 min, 18–31% B in A; 45–50 min, 31–88% B in A; 50–60 min, 88% B (isocratic). The UV trace was recorded at 280 nm.

Total and polymeric PA concentrations were quantified against purified PA standard, which was isolated and purified from mountain birch leaf extract by column chromatography on Sephadex LH-20, as previously described (Ossipova et al., 2001).

Statistical Methods

The effects of treatments on total and polymeric PA concentrations were tested with mixed analyses of variance where treatments and tree species were included as fixed effects and growth chamber as a random effect. *F. crenata* contained only traces of polymeric PAs and was omitted from the analysis. Mixed models were analyzed with PROC MIXED in SAS 8.2 (SAS Inst. Inc. 1999–2001). The Kenward-Roger Method was used for degrees of freedom and variance components for the type of variance-covariance matrix. The desired pairwise comparisons were made with contrast statements according to the significance of the effects in the model. With significant species \times treatment interaction, the differences between treatments within species and the differences between species within treatments were tested. The normal distribution of model residuals was checked visually and with *Kolmogorov-Smirnov* test. Both TPA and PPA concentrations were log-transformed to meet the assumption of normal distribution of model residuals. Back-transformed least-squares means and their 95% confidence limits were used in the figures.

Results and Discussion

Betula platyphylla, *B. ermanii*, and *F. crenata* seedlings were exposed to ambient or elevated O₃ or/and CO₂ during two growing seasons using the open-top chambers.

Table 2 Effect of tree species and treatment on total proanthocyanidin concentration using mixed model analysis of variance and the required pairwise comparisons

	<i>df</i>	<i>F</i>	<i>P > F</i>
Effect			
Spec	2	0.43	0.654
Treat	5	5.56	<0.001
Spec \times Treat	10	1.33	0.216
Pairwise comparisons			
AP vs. Amb	1	9.71	0.002
AP vs. CO ₂	1	3.61	0.059
AP vs. FA	1	8.44	0.004
AP vs. O ₃	1	5.61	0.019
AP vs. CO ₂ + O ₃	1	0.96	0.328
Amb vs. CO ₂	1	1.48	0.225
Amb vs. FA	1	0.04	0.833
Amb vs. O ₃	1	0.56	0.455
Amb vs. CO ₂ + O ₃	1	16.79	<0.001
CO ₂ vs. FA	1	1.01	0.313
CO ₂ vs. O ₃	1	0.22	0.640
CO ₂ vs. CO ₂ + O ₃	1	8.30	0.004
FA vs. O ₃	1	0.29	0.592
FA vs. CO ₂ + O ₃	1	15.11	<0.001
O ₃ vs. CO ₂ + O ₃	1	11.22	0.001

Amb, ambient air; AP, outside of the chamber; CO₂, elevated CO₂; CO₂ + O₃, elevated levels of combined CO₂ and O₃; FA, air filtered through the activated charcoals; O₃, elevated O₃; Spec, species; Treat, treatment.

Leaves of seedlings were collected and extracted, and the soluble PAs were analyzed with two different methods, i.e., colorimetric and chromatographic. Total PA concentrations were analyzed by the colorimetric BH assay. This assay reveals the presence of PAs reliably, but should be used with caution for quantification (see above). There was a significant difference in total PA concentrations between treatments, but no difference between species or significant species \times treatment interaction (Table 2). Total PA concentration was highest in CO₂ + O₃ treatment that differed significantly from all other treatments except AP. Total PA concentration was 29% higher in CO₂ + O₃ treatment compared with the FA control. The AP, in turn, had significantly higher total PA concentrations compared to Amb (24% higher), FA (23% higher), and elevated O₃ (19% higher; Table 2, Fig. 1). Earlier, it was noticed that shading of mountain birch trees reduced the concentrations of soluble PAs in leaves (Henriksson et al., 2003). In this study, light intensity inside the chambers was only 75% of the full sunlight, and the reduced levels of PAs in control seedling leaves inside the chambers (Amb and FA) compared to controls outside the chambers (AP) could be a result of the shading effect. Thus, AP seedlings cannot be used as a control for determining effects of elevated CO₂ or O₃ levels on PA concentrations in the leaves.

The chromatographic methods enable more specific determination and quantification of PAs. In birch species, which have grown in Finland, PAs are mainly in the polymeric form (DP > 10), and only traces of oligomeric PAs can be detected in mature leaves (Karonen et al., 2006). Polymeric PA concentrations can be easily determined with NP-HPLC. The clear peak of polymeric PAs was detected in NP-HPLC traces of *B. ermanii* and *B. platyphylla* extracts (Fig. 2) corresponding to our previous studies (Karonen et al., 2006). Beech leaves contained high amounts of oligomeric PAs, but only traces of polymeric PAs (Fig. 2). The amount of polymeric PAs in beech leaves was below the quantification limit of the NP-HPLC method, and, thus, quantification could not be conducted. The effects of tree species and treatment on polymeric PA concentrations are presented in Table 3 and Fig. 1. Significant species \times treatment interactions were observed. In *B. ermanii*, the polymeric PA concentration was highest in the AP seedlings growing outside of chambers, presumably because of the shading effect of the chamber (see above). The polymeric PA concentration was 13% higher in AP seedlings compared with the FA control. In *B. platyphylla*, the highest polymeric PA concentration was found in the leaves of seedlings exposed to elevated CO₂ + O₃. These leaves differed significantly from all other treatments including AP seedlings. Polymeric PA concentration was 27% higher in CO₂ + O₃ treatment compared with the FA control. In comparison of species within treatments, *B. platyphylla* had significantly higher polymeric PA concentration in seedling leaves exposed to elevated CO₂ + O₃ (27% higher) than *B. ermanii*, whereas in the leaves of AP seedlings, the opposite was true (40% lower; Table 3 and Fig. 1). TPA concentrations were always higher compared to PPA concentrations (Fig. 1). This can be a result of different factors. Various limitations have been reported for the BH assay (see above). PA polymers theoretically produce more anthocyanidins than PA oligomers, but the yield does not stay constant when the chain lengthens. It is likely that even if the birch leaf PAs were mainly PPAs, the leaves also contained shorter-chain PAs. These oligomeric PAs were included in TPA concentrations quantified with the BH assay, but not in PPA concentrations quantified with the normal-phase HPLC method.

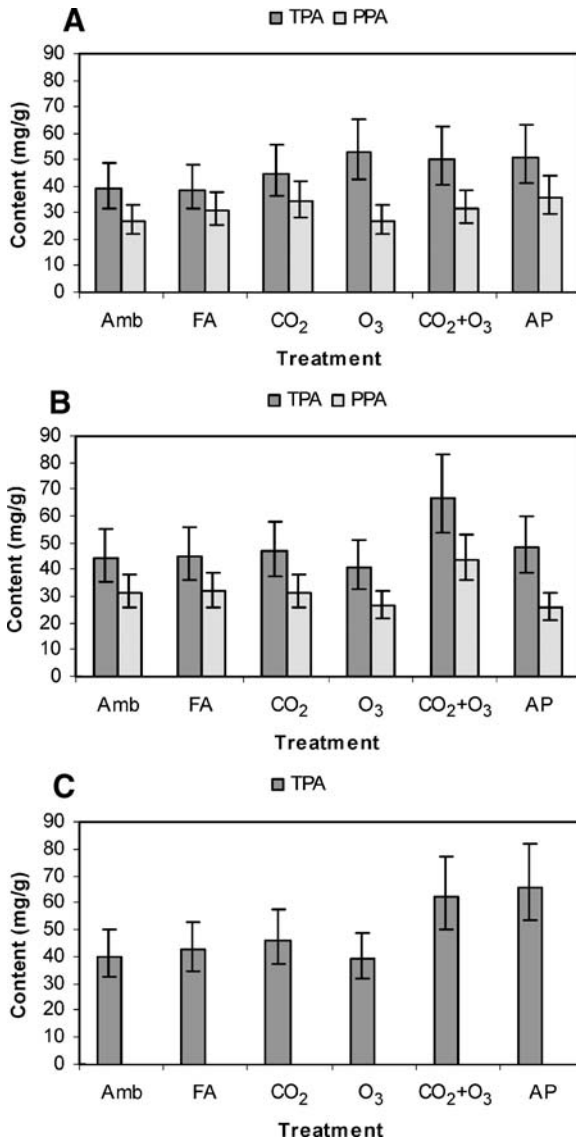


Fig. 1 Total (TPA) and polymeric (PPA) proanthocyanidin concentrations (mg g⁻¹ of dry weight) in the *Betula ermanii* (A), *B. platyphylla* (B), and *Fagus crenata* (C) leaves under atmospheres of ambient air (Amb), air filtered by activated charcoal (FA), elevated CO₂ (CO₂), elevated O₃ (O₃), and elevated levels of combined CO₂ and O₃ (CO₂ + O₃) in open-top chambers, as well as in the seedlings growing outside of the chamber (AP). Data are presented as means with 95% confidence interval

Previously, Lavola et al. (1994, 2000) and Lindroth et al. (2001) reported that total PA concentrations were higher in CO₂-exposed leaves than in control leaves in *B. pendula* and *B. papyrifera*, but fumigation with O₃ did not affect total PA concentrations. However, in our study, only combined CO₂ + O₃ treatment increased PA levels significantly. In the leaves of *B. ermanii*, the positive effect of CO₂

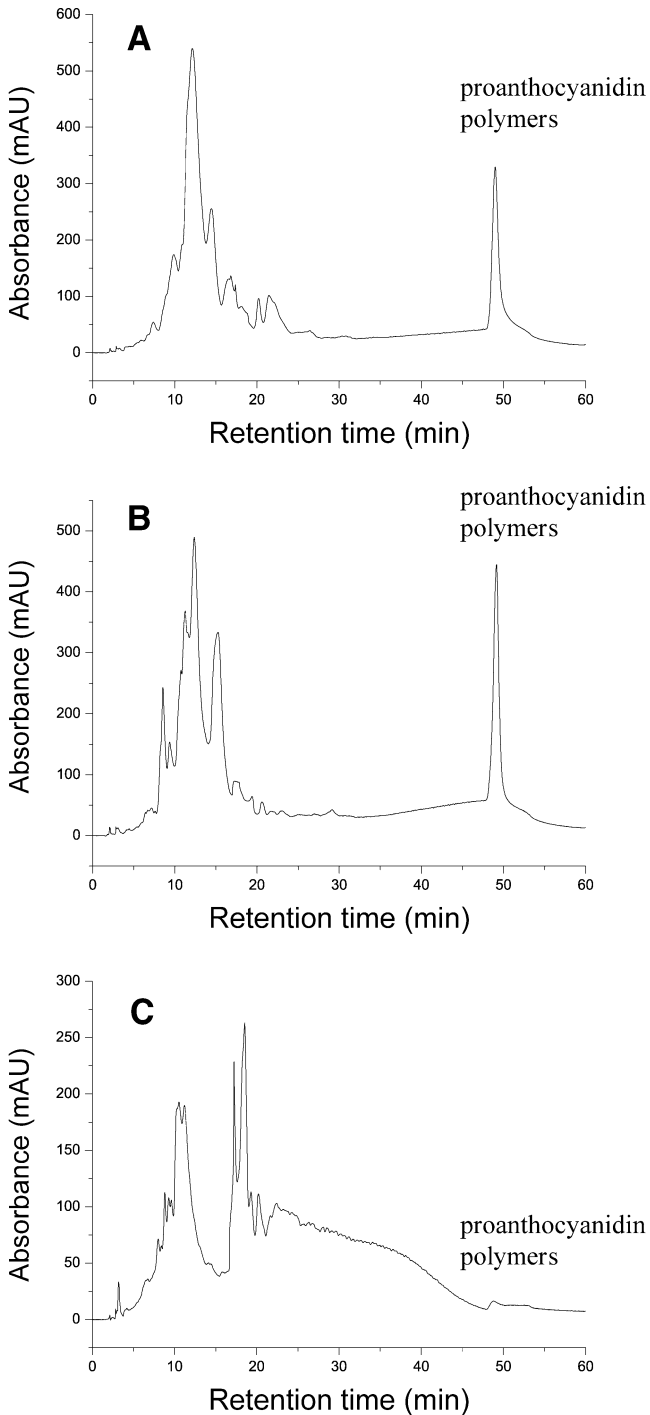


Fig. 2 Normal-phase HPLC traces of soluble phenolics for leaf extracts of *B. ermanii* (A), *B. platyphylla* (B), and *F. crenata* (C)

Table 3 Effect of tree species and treatment on polymeric proanthocyanidin concentration using the mixed model analysis of variance and the required pairwise comparisons

	<i>df</i>	<i>F</i>	<i>P > F</i>
Effect			
Spec	1	0.02	0.876
Treat	5	2.55	0.031
Spec × Treat	5	2.48	0.035
Pairwise comparisons			
<i>Betula ermanii</i>			
AP vs. Amb	1	4.17	0.043
AP vs. CO ₂	1	0.11	0.744
AP vs. FA	1	0.97	0.326
AP vs. O ₃	1	4.18	0.043
AP vs. CO ₂ + O ₃	1	0.78	0.379
Amb vs. CO ₂	1	2.94	0.089
Amb vs. FA	1	1.11	0.293
Amb vs. O ₃	1	0.00	0.997
Amb vs. CO ₂ + O ₃	1	1.34	0.249
CO ₂ vs. FA	1	0.43	0.511
CO ₂ vs. O ₃	1	2.95	0.088
CO ₂ vs. CO ₂ + O ₃	1	0.31	0.579
FA vs. O ₃	1	1.12	0.292
FA vs. CO ₂ + O ₃	1	0.01	0.918
O ₃ vs. CO ₂ + O ₃	1	1.35	0.248
<i>Betula platyphylla</i>			
AP vs. Amb	1	2.06	0.154
AP vs. CO ₂	1	2.20	0.140
AP vs. FA	1	2.33	0.129
AP vs. O ₃	1	0.07	0.792
AP vs. CO ₂ + O ₃	1	14.29	<0.001
Amb vs. CO ₂	1	0.00	0.961
Amb vs. FA	1	0.01	0.927
Amb vs. O ₃	1	1.37	0.244
Amb vs. CO ₂ + O ₃	1	5.50	0.021
CO ₂ vs. FA	1	0.00	0.967
CO ₂ vs. O ₃	1	1.49	0.225
CO ₂ vs. CO ₂ + O ₃	1	5.27	0.023
FA vs. O ₃	1	1.59	0.209
FA vs. CO ₂ + O ₃	1	5.08	0.026
O ₃ vs. CO ₂ + O ₃	1	12.36	<0.001
Difference between species within treatments			
Be vs. Bp (AP)	1	5.75	0.018
Be vs. Bp (Amb)	1	1.16	0.283
Be vs. Bp (CO ₂)	1	0.34	0.558
Be vs. Bp (FA)	1	0.01	0.909
Be vs. Bp (O ₃)	1	0.01	0.929
Be vs. Bp (CO ₂ + O ₃)	1	5.13	0.025

Amb, ambient air; AP, outside of the chamber; Be, *B. ermanii*; Bp, *B. platyphylla*; CO₂, elevated CO₂; CO₂ + O₃, elevated levels of combined CO₂ and O₃; FA, air filtered through the activated charcoals; O₃, elevated O₃; Spec, species; Treat, treatment.

treatment on polymeric PA concentration was close to significant. These differences could be a result of experimental conditions (open-top chambers instead of field and greenhouse experiments), different sensitivity of birch species to elevated concentrations of CO₂ and O₃, or different methods used for PA quantification. Furthermore, leaf dry mass can be influenced by starch levels in tissues, especially when plants are treated with elevated CO₂, and PA concentrations are calculated on dry mass basis. This so-called dilution effect might partly explain why PA concentrations were not increased at elevated CO₂ levels. Moreover, these data were obtained with small individuals grown under almost optimal light and moisture conditions, and plant stresses other than elevated O₃ or CO₂ were minimized. Therefore, the experimental seedlings were not exposed to a variety of interacting stresses, as is the case in nature. As Reich (1987) stated, we do not know if, or how, water stress, herbivores, pathogens, plant–plant competition, nutrition, shading, temperature fluctuations, or other factors influence the O₃-dose or O₃-uptake relations.

Elevated atmospheric CO₂ levels stimulate photosynthesis and increase carbohydrate availability for synthesis of carbon-based secondary metabolites such as phenolics (Booker, 2000). In contrast to CO₂, O₃ affects primary metabolism by inhibition of photosynthesis, which decreases carbohydrate availability for phenolic biosynthesis (Booker, 2000). However, O₃ may exhibit more specific and positive effects on phenolic metabolism by activating the signaling pathways of defensive mechanisms (Rao et al., 2000). Treatment of plants with elevated O₃ increases the biosynthetic activity of the phenylpropanoid pathway, which plays a significant role in plant defense responses by synthesizing compounds potentially protective against phytopathogens and herbivores (Kangasjärvi et al., 1994). Therefore, both elevated CO₂ and O₃ can activate or affect phenolic biosynthesis in different branches of a plant's metabolic network (both primary and secondary metabolism). Thus, the combined influence of elevated CO₂ and O₃ on the PA level in woody plants was the strongest.

Even if the enriched CO₂ or O₃ level alone did not affect PA concentrations, they still affected the growth of *B. platyphylla*, *B. ermanii*, and *F. crenata* seedlings. It was noticed that at ambient CO₂ concentration with increasing level of O₃, the shoot, root, and total biomass of *F. crenata* were decreased. Decreases in shoot or total biomass were also observed in *B. platyphylla* and *B. ermanii*. Predictably, at ambient O₃ concentration, the increased level of CO₂ caused positive effects on the growth of all three species. The total biomass of *B. platyphylla* and *F. crenata* increased significantly and likewise for the shoot biomass of *B. ermanii*. The interactive effects of enriched O₃ and CO₂ on growth of these tree species were species-specific. Significant interaction was found on the biomass of *B. platyphylla*, but not on the biomass in *B. ermanii* or *F. crenata* (Matsumura et al., 2005). Elevated CO₂ is known to increase biomass production, whereas elevated O₃ suppresses it. Increase or decrease of biomass because of the various gas treatments will affect PA inputs to the soil and thus decomposition and nutrient-cycling processes (Booker et al., 2005).

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Toxicity of Aflatoxin B1 to *Helicoverpa zea* and Bioactivation by Cytochrome P450 Monooxygenases

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Abstract Infestation of corn (*Zea mays*) by corn earworm (*Helicoverpa zea*) predisposes the plant to infection by *Aspergillus* fungi and concomitant contamination with the carcinogenic mycotoxin aflatoxin B1 (AFB1). Although effects of ingesting AFB1 are well documented in livestock and humans, the effects on insects that naturally encounter this mycotoxin are not as well defined. Toxicity of AFB1 to different stages of *H. zea* (first, third, and fifth instars) was evaluated with artificial diets containing varying concentrations. Although not acutely toxic at low concentrations (1–20 ng/g), AFB1 had significant chronic effects, including protracted development, increased mortality, decreased pupation rate, and reduced pupal weight. Sensitivity varied with developmental stage; whereas intermediate concentrations (200 ng/g) caused complete mortality in first instars, this same concentration had no detectable adverse effects on larvae encountering AFB1 in fifth instar. Fifth instars consuming AFB1 at higher concentrations (1 µg/g), however, displayed morphological deformities at pupation. That cytochrome P450 monooxygenases (P450s) are involved in the bioactivation of aflatoxin in this species is evidenced by the effects of piperonyl butoxide (PBO), a known P450 inhibitor, on toxicity; whereas no fourth instars pupated in the presence of 1 µg/g AFB1 in the diet, the presence of 0.1% PBO increased the pupation rate to 71.7%. Pupation rates of both fourth and fifth instars on diets containing 1 µg/g AFB1 also increased significantly in the presence of PBO. Effects of phenobarbital, a P450 inducer, on

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AFB1 toxicity were less dramatic than those of PBO. Collectively, these findings indicate that, as in many other vertebrates and invertebrates, toxicity of AFB1 to *H. zea* results from P450-mediated metabolic bioactivation.

Keywords Aflatoxin B1 · Bioactivation · Cytochrome P450 monooxygenase · *Helicoverpa zea* · Mycotoxin · Piperonyl butoxide

Abbreviations

AFB1	aflatoxin B1
P450	cytochrome P450 monooxygenase
AFBO	AFB1-8,9-epoxide
AFM1	aflatoxin M1
AFL	aflatoxicol
AFQ1	aflatoxin Q1
AFB2a	aflatoxin B2a
AFG1	aflatoxin G1
PBO	piperonyl butoxide
PB	phenobarbital

Introduction

Helicoverpa zea, the corn earworm, is a major pest of corn and a number of other crops throughout the USA; current estimates indicate that this species alone is responsible for losses exceeding 10% in field corn and 50% in sweet corn (Wiseman, 1999). Losses inflicted by *H. zea* infestation can be exacerbated by fungal infections that may accompany *H. zea* damage (Dowd and White, 2002). Among the most devastating are the ear molds that produce aflatoxins, including some of the most active natural carcinogens known (Lillehoj, 1992; Dowd, 1998). Because the maximum tolerance levels for aflatoxins are in the 0- to 20-ppb range for corn grown for human consumption, aflatoxin contamination in the USA is responsible for millions of dollars of crop losses every year [U.S. Department of Agriculture (USDA), 1999].

Aflatoxins are coumarin derivatives that can be classified as either dihydrofurocyclopentenones or dihydrofurolactones. These compounds owe their toxicity to their ability to form irreversible adducts to nucleic acids with the concomitant inhibition of DNA replication and DNA-dependent transcription (Murray et al., 1982). Chronic effects of exposure include decreases in food consumption in pigs, poultry, cattle, and a variety of livestock as well as decreases in milk yields in cattle. Acute and chronic exposures result in a variety of clinical symptoms in humans, including pulmonary edema, abdominal pain, vomiting, convulsions, and, in some cases, coma and death (Newberne and Butler, 1969).

One means of reducing the risk of aflatoxin contamination of corn is to reduce the likelihood of insect damage, which predisposes the plant to fungal infection. Reduction of insect damage through insecticide application and the use of insect-resistant germ plasm (e.g., tight-husked varieties) has had limited success owing to the evolution of resistance to both forms of control (Dowd et al., 1997). Transgenic Bt corn has long been touted as another means of reducing crop damage, but it is unclear how effective it will be (Windham et al., 1999), given that *H. zea* displays a wide range of sensitivity to Bt (Stone and Sims, 1993), and that any level of insect

infestation can facilitate mold establishment leading to mycotoxin contamination of corn (Dowd, 2001).

Although aflatoxins are toxic to a broad range of arthropods (Al-Adil et al., 1972; Wright et al., 1982; Chinnici and Bettinger, 1984; Lillehoj, 1992; Sadek et al., 1996), comparatively little is known about metabolic transformations of aflatoxin in insects that regularly encounter them. Among the enzyme systems responsible for metabolizing these compounds in many vertebrate and invertebrate taxa are the cytochrome P450 monooxygenases (P450s; <http://drnelson.utm.edu>; <http://www.biobase.dk/P450/>). Whereas in some species P450s metabolize aflatoxin B1 (AFB1) to hydroxylated metabolites, including aflatoxin M1 (AFM1), aflatoxin G1 (AFG1), and aflatoxin Q1 (AFQ1), in other species, P450s are responsible for bioactivation of these compounds, catalyzing the epoxidation of the terminal furan ring of AFB1 to yield AFB1-8,9-epoxide (AFBO), a highly genotoxic metabolite (Murray et al., 1982). AFBO owes its toxicity to its ability to bind to DNA to form the predominant 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB1 adduct (Iyer et al., 1994). Hydroxylated metabolites have lower genotoxic or toxic activities than AFB1 in most organisms (Eaton et al., 1988; Ramsdell and Eaton, 1990). The Hikone-R strain of *Drosophila melanogaster*, which is resistant to DDT and other insecticides, produces aflatoxicol (AFL) as a principal metabolite, along with AFM1 and aflatoxin B2a (AFB2a) (Foerster and Wurgler, 1984). Saner et al. (1996) demonstrated that bioactivation of AFB1 was catalyzed by CYP6A2. Lee and Campbell (2000) compared metabolism of AFB1 in larvae of *Amyelois transitella* (navel orangeworm), which are frequently exposed to aflatoxin-contaminated tissue, and *Cydia pomonella* (codling moth), which are rarely exposed to aflatoxins. These authors found that AFL is the major metabolite produced *in vitro* by navel orangeworms; in contrast, codling moths collected in the field produced only trace amounts of AFL, and a laboratory strain produced no detectable metabolites. Neither species produced AFBO as a metabolite, in contrast to vertebrates, which produce this highly mutagenic biotransformation product quite readily. Lee and Campbell (2000) have suggested that the absence of this particular bioactivation is indicative of coevolution between kernel-feeding insects and these toxin-producing fungi.

Although *H. zea* is likely to encounter aflatoxins in corn, as well as in several other host plants (including soybeans *Glycine max*), the degree to which this species has coevolved with mycotoxin-producing *Aspergillus* species is unknown. In this study, we examined the toxicological impact of AFB1 ingestion throughout the larval developmental period and tested the effects of a known inhibitor of cytochrome P450 metabolism, piperonyl butoxide (PBO), as well as a known P450 inducer, phenobarbital (PB; Brun et al., 1996; Maitra et al., 1996; Dunkov et al., 1997; Waxman 1999; Stevens et al., 2000; Li et al., 2002a; Brown et al., 2003), to determine the contributions of these enzymes to aflatoxin toxicity in this species.

Methods and Materials

Chemicals

Aflatoxin B1 and PB were obtained from Sigma (St. Louis, MO, USA). Analytical grade dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific, and PBO was from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Insects

Helicoverpa zea were individually reared in 30-ml creamer containers on a semisynthetic diet containing wheat germ (Waldbauer et al., 1984) and maintained in an insectary kept at 23–26°C with a photoperiod of 16-hr light/8-hr dark. Bioassays were conducted under the same environmental conditions. At each developmental stage tested, caterpillars were collected within 12 hr of molting to insure age comparability.

Bioassays

Stock solutions of AFB1 were prepared in DMSO at concentrations of 1 ng/μl, 20 ng/μl, 200 ng/μl, 1 μg/μl, and 20 μg/μl; 100 μl of each stock solution were added to 100 g of the semisynthetic diet to obtain final concentrations of 1 ng/g, 20 ng/g, 200 ng/g, 1 μg/g, and 20 μg/g AFB1. Control larvae were fed with the semisynthetic diet containing 100 μl DMSO per 100 g; pilot experiments determined that concentrations of DMSO at levels as high as 0.5% of the diet do not affect development or survivorship (data not shown).

To determine the toxicity of aflatoxin to *H. zea*, artificial diets containing AFB1 at four concentrations were administered in separate bioassays to larvae at three developmental stages—first, third, and fifth instars; 20 larvae were tested in each bioassay. Because pilot studies established that early instars display higher sensitivity to AFB1 (data not shown), first instars were exposed to diets containing AFB1 at concentrations of 1 ng/g, 20 ng/g, 200 ng/g, and 1 μg/g, and third and fifth instars were exposed to diets containing AFB1 at concentrations of 20 ng/g, 200 ng/g, 1 μg/g, and 20 μg/g. The proportions of larvae at each stage of development and the number of dead larvae were recorded daily through pupation, and pupal weights were measured within 48 hr of pupation. Each set of bioassays was replicated three times.

To elucidate the effects of cytochrome P450-mediated metabolism on aflatoxin toxicity, a series of bioassays was conducted whereby AFB1 toxicity was assessed in the presence of an inducer (PB) or an inhibitor (PBO). Stock solutions of PB were prepared in DMSO at a concentration of 0.25 mg/μl PB, and 400 μl of this stock were added to 100 g of the semisynthetic diet to obtain a final concentration of 0.1% PB in the diet. An aliquot of 105 μl commercial PBO (90% purity, 1.06 density) was added directly to 100 g of the diet for a final concentration of 0.1% PBO. Newly molted fourth or fifth instars were assayed separately, with 20 larvae per treatment. Caterpillars were placed on diets containing either 0.1% DMSO (control), 0.1% PBO, 0.1% PB, 1 μg/g AFB1, 1 μg/g AFB1 + 0.1% PBO, or 1 μg/g AFB1 + 0.1% PB and were reared and evaluated as described.

Statistical Analyses

All data were evaluated by one-way analysis of variance with treatment differences among means tested at $P = 0.05$ with Duncan's multiple range test. All data for larval development, pupation rate, and mortality were means from three replicates, with 20 caterpillars/treatment. Because significant differences were found among replicates in pupal weights, this parameter was analyzed independently for each replicate, and representative values from one replicate are reported.

Safety

Aflatoxin B1 is a highly toxic mycotoxin that should be handled with care. Solid AFB1 for calibration should be handled in a biohood, using a nose and mouth mask. Any material and equipment with possible contamination should be handled carefully and with surgical gloves.

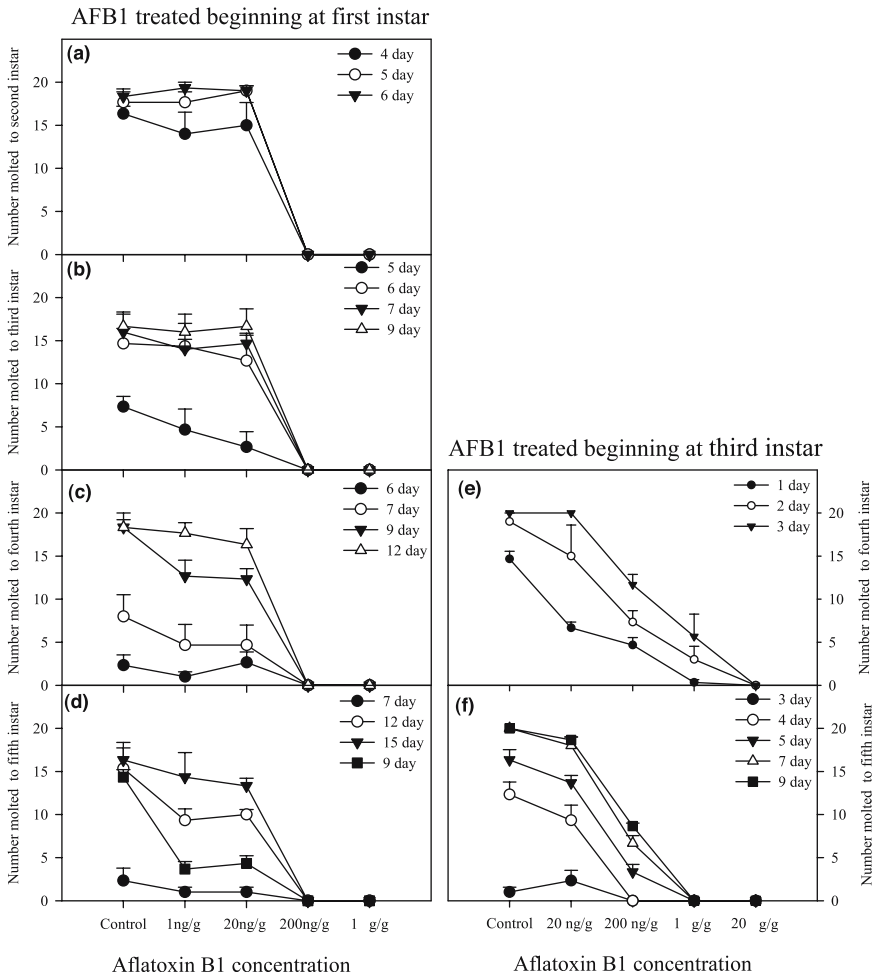


Fig. 1 Development of *H. zea* after exposure to different concentrations of aflatoxin B1 in diet beginning at first instar (a, b, c, d) and third instar (e, f). (a) Number of larvae that have molted to second instar after 4, 5 and 6 days of treatments, (b) number of larvae that have molted to third instar after 5, 6, 7 and 9 days of treatments, (c) number of larvae that have molted to fourth instar after 6, 7, 9 and 12 days of treatments, (d) number of larvae that have molted to fifth instar after 7, 9, 12 and 15 days of treatments, (e) number of larvae that have molted to fourth instar after 1, 2 and 3 days of treatments, (f) number of larvae that have molted to fifth instar after 3, 4, 5, 7 and 9 days of treatments. Each treatment contained 20 caterpillars and each experiment was repeated three times. Values are means + standard errors. Points on each line represent effects of different concentrations of AFB1 on a given day after AFB1 exposure

Results

Toxicity to First Instars

AFB1 at high concentrations strongly inhibited larval growth and development. At a concentration of 200 ng/g, larval mortality was 55% after 9 d ($P = 0.02$) and 100% after 15 d ($P < 0.001$; Figs. 1a–d and 2a). Most larvae died before molting to second instar, and only about 10% of larvae survived to third instar after 12 d. AFB1 at a concentration of 1 $\mu\text{g/g}$ completely inhibited larval development, and all larvae had died within 12 d without molting to second instar ($P < 0.001$; Fig. 2a).

AFB1 at the lower concentrations of 1 and 20 ng/g did not detectably delay the timing of the molt to second instar (Fig. 1a) but did delay developmental progress at all subsequent stages (Fig. 1b–d). Low concentrations of AFB1 also significantly reduced pupation rate ($P = 0.004$ at 1 ng/g and $P < 0.001$ for 20 ng/g) and pupal weight ($P = 0.001$ for 1 ng/g and $P < 0.001$ for 20 ng/g; Fig. 3a and b); after 15 d, mortality was 46.6% on diets containing 20 ng/g AFB1 compared with 18.3% on control diet ($P = 0.029$; Fig. 2a).

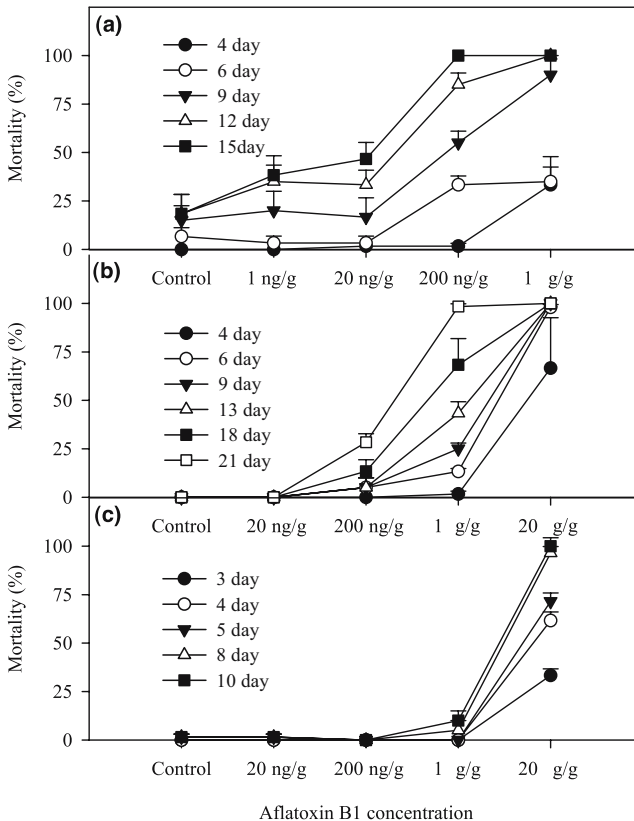


Fig. 2 Mortality of *H. zea* after exposure to different concentrations of aflatoxin B1 in diet beginning at first instar (a), third instar (b), and fifth instar (c). Values are means + standard errors from 20 caterpillars/treatment with three experimental replicates for each

Toxicity to Third Instars

Third instars display greater tolerance to AFB1 than do first instars. Administered to third instar larvae, the lowest concentration (20 ng/g) of AFB1 delayed development as well as pupation (Figs. 1e,f and 3c); pupal weight was also significantly reduced (Fig. 3d), although pupation rate and mortality were unaffected (Figs. 2b and 3c). The intermediate concentration of 200 ng/g AFB1 significantly delayed development (Figs. 1e,f and 3c) and reduced pupal weight to the same extent as did diets containing 20 ng/g AFB1 ($P = 0.411$; Fig. 3d). Mortality rates assessed after 21 d of treatment were 28.5% compared to no mortality for control larvae ($P < 0.001$; Fig. 2b) and were significantly lower than the mortality rate of 55% for first instars consuming AFB1 at this concentration ($P < 0.001$) for 9 d. Pupation rates assessed after 28 d of treatment were 66.7% compared to 100%

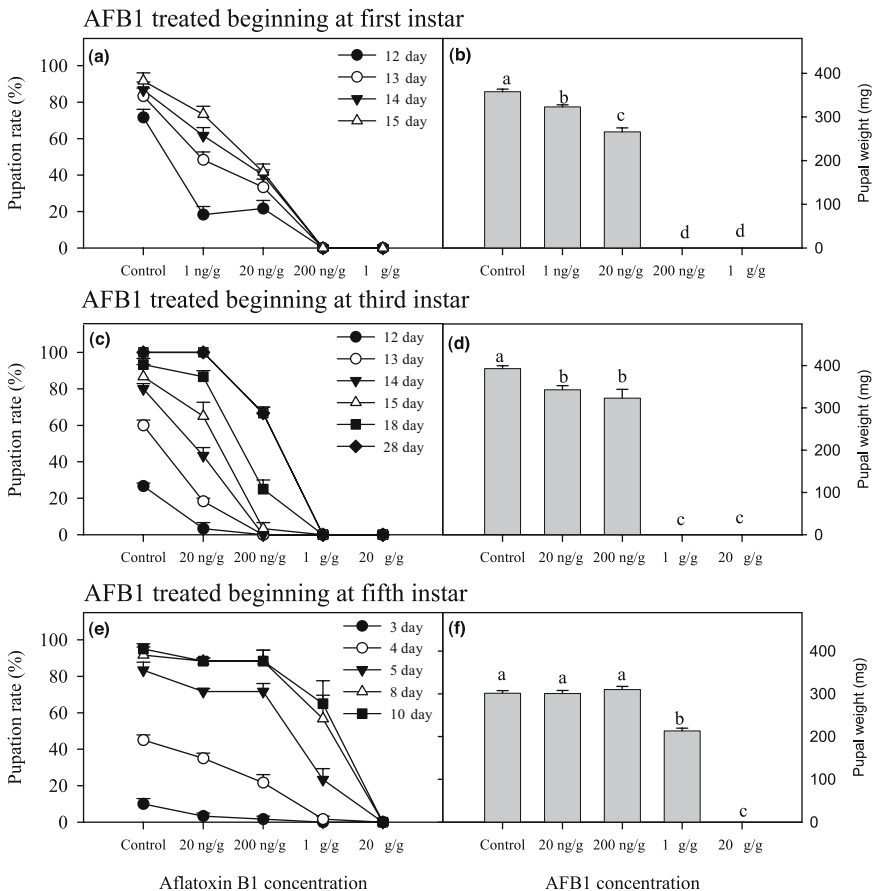


Fig. 3 Pupation of *H. zea* after exposure to different concentrations of aflatoxin B1 in diet beginning at first instar (a, b), third instar (c, d), and fifth instar (e, f). Values for pupation rate are means + standard errors from 20 caterpillars/treatment with three experimental replicates for each. Values for pupal weight are representative of one of the three series of replicates with the number of insects that have pupated for this replicate shown above each bar. Significant differences ($P < 0.05$) in pupal weights among treatments are indicated by different letters above the bars

for control larvae (Fig. 3c). Of the caterpillars that survived the growth delays associated with the treatment of third instars with 200 ng/g AFB₁, several reached pupal weights as high as those on control diets in all three replicate experiments.

Concentrations of 1 µg/g AFB₁ in the diet significantly interfered with development; only about 5% of larvae reached fourth instar, and of these, none developed into fifth instars (Figs. 1e, f and 3c). After 13 d, the larval mortality rate was 44.3%, and after 3 wk, no larvae remained alive (Fig. 2b). At the highest concentration (20 µg/g), no larval development occurred, and mortality was 100% after 6 d of treatment (Figs. 1e, f and 2b).

Toxicity to Fifth Instars

Fifth instar *H. zea* display substantially greater tolerance to AFB₁ than do earlier stages. Administered to fifth instars, the lowest concentrations (20 and 200 ng/g) of AFB₁ delayed pupation only after five or more days of treatment (Fig. 3e) without affecting pupation rate ($P = 0.479$ and 0.499), pupal weight ($P = 0.454$ and 0.245), or mortality ($P = 0.479$ and 0.499 ; Figs. 2c and 3e, f). At concentrations ≥ 1 µg/g AFB₁, however, fifth instar growth and development were affected. At a concentration of 20 µg/g, AFB₁ effected 100% mortality after 10 d (Fig. 2c). Whereas most larvae (83.3%) on control diets had pupated after 5 d, less than one third of larvae on diets containing 1 µg/g AFB₁ had pupated over that time period ($P = 0.011$). In addition, pupal weights on diets containing 1 µg/g AFB₁ were reduced by 29.3% relative to control diets ($P < 0.001$).

Morphological examinations indicated that malformations of pupae occurred when fifth instars consumed diets with 1 µg/g AFB₁ (Fig. 4); in these cases, the pupal molt is arrested so that only the anterior portion of the pupa, from the maxillary region to the anterior abdominal segments, protrudes dorsally from the thoracic and anterior abdominal areas of the persistent larval cadaver. These deformed pupae failed to produce adult moths.

PBO and PB Effects

To determine the role of P450s in the activation and/or detoxification of AFB₁ in *H. zea*, bioassays were conducted by administering AFB₁ alone, AFB₁ in combination with PBO, a P450 inhibitor, or AFB₁ in combination with PB, an inducer of some P450 monooxygenases. As shown in Fig. 5a and b, exposure of fourth instars to 0.1% PBO significantly improved the pupation rates ($P < 0.001$) and pupal weights



Fig. 4 Pupae of *H. zea* without (control) and with aflatoxin B₁ (1 µg/g) in diet beginning at fifth instar

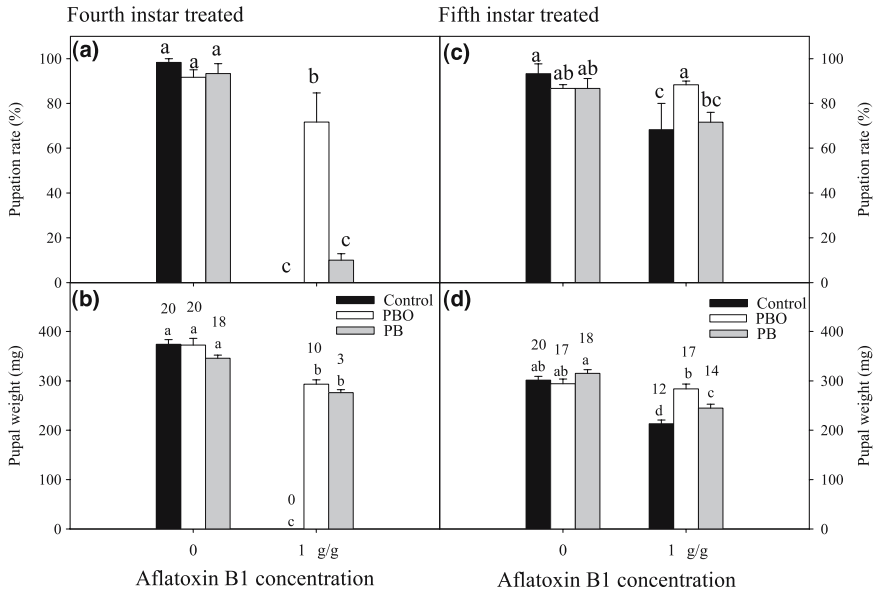


Fig. 5 Effects of piperonyl butoxide (PBO) and phenobarbital (PB) on pupation of *H. zea* exposed to aflatoxin B1 beginning at fourth instar (a, b) and at fifth instar (c, d). Values shown for pupation rate are means + standard error from three replicates with 20 caterpillars/treatment. Values for pupal weight are representative of one of the three series of replicates with the numbers of pupae weighed for this replicate shown above each bar. Significant differences ($P < 0.05$) among treatments are indicated by different letters above the bars

($P < 0.001$) of *H. zea* fed on diets containing 0.1% PBO and 1 $\mu\text{g/g}$ AFB1 compared to *H. zea* on diets containing AFB1 alone. Whereas no fourth instars on diets containing 1 $\mu\text{g/g}$ AFB1 succeeded in pupating, addition of PBO to the diet increased pupation rate of fourth instars to 71.7% ($P < 0.001$; Fig. 5a). In contrast, exposure of fourth instars to 0.1% PB resulted in a pupation rate of only 10% ($P = 0.256$). Pupal weights were not significantly changed by the addition of PB or PBO.

In contrast with fourth instars, which did not successfully pupate on diets containing 1 $\mu\text{g/g}$ AFB1, over 68% of fifth instars on this diet succeeded in pupating. Inclusion of PBO in the diet increased pupation success to 88.3% ($P = 0.044$); at the same time, PBO increased pupal weights to an average of 283.7 mg, compared with an average of 213.0 mg on control diets ($P < 0.001$). Pupation success and pupal weights in the presence of AFB1 and PBO were not significantly different from pupation success and pupal weight of larvae on control diets (Figs. 5c and d; $P = 0.55$ for pupation rate and $P = 0.14$ for pupal weight). In contrast with PBO, addition of PB did not affect pupation rate, although addition of PB to diets containing AFB1 increased pupal weight significantly.

Discussion

Just as insects may possess biochemical mechanisms that confer resistance to insecticides and plant allelochemicals, they may also possess mechanisms for circumventing the toxicity of mycotoxins such as aflatoxins. In one study, resistance

levels among species varied with the likelihood of ecological exposure to these compounds (Lee and Campbell, 2000). Resistance levels may even vary intraspecifically; differences in AFB1 sensitivity were found in wild-type strains of *D. melanogaster* (Llwellyn and Chinnici, 1978; Chinnici and Melone, 1985). The Crimea strain was extremely sensitive, with no adults produced at concentrations of 0.4 mg/l AFB1, whereas eclosion rates exceeded 50% in three other resistant strains in the presence of the same concentrations of AFB1 (Llwellyn and Chinnici, 1978).

Although *H. zea* has a remarkable capacity for metabolizing a broad diversity of plant allelochemicals, as befits its polyphagous diet (Li et al., 2004), its ability to tolerate the mycotoxin aflatoxin is limited, despite its decidedly frequent ecological exposure. Although AFB1 was not acutely toxic to *H. zea* at any stage of development, even at the highest concentration tested (20 µg/g), chronic exposure led to developmental delays, reductions in pupation rates and pupal weights, and higher mortality. The chronic effects of AFB1 ingestion varied depending on the stage of larval exposure, with first instars the most susceptible to AFB1 and fifth instars the most resistant. Exposure of first instars to low levels (1 ng/g) of AFB1 significantly affected development time and pupation rate, whereas pupation rates of third and fifth instars were affected only at concentrations of 200 ng/g and 1 µg/g of AFB1. Exposure of first instars to concentrations of 20 ng/g, representing the maximum levels permitted in grain (USDA, 1998), resulted in prolonged development and reduced pupation rates. Similar developmental effects have been documented in other insects; growth of *D. melanogaster* on AFB1-containing media caused significant protraction in egg-to-adult developmental time (Lalor et al., 1976). In contrast, exposure of fifth instars to substantially higher levels (200 ng/g) of AFB1 did not affect pupation rate, pupal weight, or mortality. Developmental abnormalities in the form of pupal deformities, however, were observed at these higher concentrations, preventing eclosion. AFB1 has previously been reported to cause developmental aberrations, including decreased wing length, in adult *D. melanogaster* (Lalor et al., 1976).

With respect to the mode of action of AFB1, PBO, an effective inhibitor of P450s in many different phyla, significantly decreases the toxicity of AFB1 to *H. zea*, a result consistent with bioactivation of aflatoxin to more toxic derivatives by one or more P450s expressed in fourth and fifth instars. Bioactivation of this sort has been documented for AFB1 in human P450s, with CYP3A4 the predominant protein bioactivating AFB1 to its highly toxic AFB1 *exo*-8,9-epoxide derivative (Ueng et al., 1995); CYP2A6 contributes to some extent in the bioactivation process (Crespi et al., 1990; Yun et al., 1991). In the mouse (*Mus musculus*), bioactivation of AFB1 is mediated by CYP2A5, which is closely related to human CYP2A6 (Pelkonen et al., 1994).

Our results also demonstrate that PB, which is capable of inducing some mammalian (Waxman, 1999), insect (Brun et al., 1996; Maitra et al., 1996; Dunkov et al., 1997; Stevens et al., 2000; Li et al., 2002a), and plant (Batard et al., 1998; Persans et al., 2001) P450s, has only a marginal ability to induce the P450(s) capable of bioactivating aflatoxin in fourth instars and some ability to induce P450s, possibly responsible for AFB1 detoxification, that allow larvae to survive and gain weight on diets containing aflatoxin. In humans, CYP3A4 and, to a lesser extent, CYP1A2 contribute to the transformation of AFB1 to less toxic hydroxylated AFM1 and AFQ1 derivatives (Ueng et al., 1995; Guengerich et al., 1998). Among candidate

P450s in *H. zea* that may be involved in these bioactivation processes are CYP6B8, which is induced by a range of plant allelochemicals and signaling compounds as well as PB (Li et al., 2000, 2002a,b), CYP6B9 and CYP6B27, which are induced by plant allelochemicals and signaling molecules (Li et al., 2002a,b), and CYP321A1, which at present is known to be induced only by xanthotoxin (Sasabe et al., 2004). Of these, CYP6B8 and CYP321A1 are both capable of metabolizing a wide range of toxic allelochemicals and insecticides (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). P450s in corn earworm appear to be involved in both bioactivation and detoxification of AFB1. In that these P450s are induced in response to exposure to plant allelochemicals, bioactivated mycotoxins such as aflatoxins, which co-occur with plant allelochemicals that are detoxified by P450s, present a distinct toxicological challenge to *H. zea*. In view of the fact that insect damage predisposes plants to fungal infection, and that mycotoxins other than aflatoxins depend on P450-mediated metabolism for their toxic effects (e.g., ochratoxin A, Obrecht-Pflumio et al., 1999), bioactivation may account at least in part for negative effects of phytopathogens on herbivorous insects documented in many plant–insect interactions (Hatcher, 1995).

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Selected Ectomycorrhizal Fungi of Black Spruce (*Picea mariana*) can Detoxify Phenolic Compounds of *Kalmia angustifolia*

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Abstract Allelopathy has been implicated as a factor contributing toward failure of black spruce (*Picea mariana*) regeneration in *Kalmia angustifolia*-dominated sites in eastern Canada. Several phenolic acids of *Kalmia* origin inhibit primary root growth of black spruce. We tested the hypothesis that some well-adapted conifer ectomycorrhizae can degrade and detoxify water-soluble phenolic compounds produced by *Kalmia* and use the degraded products as a carbon source to stimulate growth. We found that hyphal growth of *Paxillus involutus*, a common ectomycorrhizal fungus of black spruce, was stimulated by water leachates of *Kalmia* leaf and litter. An equimolar mixture of three phenolic acids (ferulic, *o*-coumaric, and *o*-hydroxyphenylacetic acid), commonly found in *Kalmia*, had no negative effects on fungal growth at 1 mM concentration. The *o*-hydroxyphenylacetic (*o*-HPA) acid, which is known to be toxic to black spruce, was found to stimulate the growth of *Laccaria laccata*, *L. bicolor*, and *P. involutus* (isolates 211804 and 196554) by 38.4, 29.3, 25.0, and 18.9%, respectively, at 1 mM. Pure ferulic, *o*-coumaric, and *o*-HPA acids were degraded by 100, 98, and 79.5%, respectively, within 10 d in the presence of *P. involutus* 211804. However, *L. laccata* could not tolerate high concentrations of the *Kalmia* leachates. *P. involutus* and *L. bicolor* used *o*-HPA acid as a carbon source when cultured in noncarbon nutrient medium. The 0.5 and 0.2 mM *o*-HPA acid inhibited the root growth of black spruce. However, after solutions had been exposed to a culture of *P. involutus*, they had no significant effect on seedling growth of black spruce. We concluded that some ectomycorrhizal fungi, such as *P. involutus* and *L. bicolor*, are able to degrade *Kalmia* phenolics. Our findings point to

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a mechanism by which ectomycorrhizal species can control species interactions in higher plants by changing the rhizosphere chemistry.

Keywords Ectomycorrhizal fungi · Phenolic compounds · Detoxification · *Picea mariana* · *Kalmia angustifolia*

Introduction

Naturally regenerating and planted conifers in nutrient-poor boreal forests of eastern Canada often suffer from growth inhibition due to ericaceous understory shrubs (Mallik, 1987, 2001; Mallik and Newton, 1988; Thiffault et al., 2004). Following clear cutting and fire, sheep laurel (*Kalmia angustifolia* L. var. *angustifolia*, hereafter referred to as *Kalmia*), a common ericaceous understory plant in eastern Canada, can seriously hinder conifer regeneration, especially black spruce [*Picea mariana* (Mill.)]. One cause has been attributed to allelopathic effects of *Kalmia* phenolics (Mallik, 1987; Thompson and Mallik, 1989; Zhu and Mallik, 1994). Phenolic acids are released into the soil as leaf and litter leachates, root exudates, or as decaying plant residues (Siqueira et al., 1991; Gallet and Pellissier, 1997). Several are reported to act as signal molecules, and some may influence signal transduction pathways in symbiotic systems (Lynn and Chang, 1990). Fries et al. (1997) reported that certain phenolic acids stimulate arbuscular mycorrhizal (AM) colonization, which may promote growth of host plants. The mechanism of conifer growth inhibition in the presence of ericaceous plants is complex and the role of *Kalmia* phenolics in black spruce growth inhibition is unclear (Wallstedt et al., 2002).

Reduced ectomycorrhizal (ECM) colonization of Norway spruce [*Picea abies* (L.) Karst] by *Cenococcum graniforme*, *L. laccata* (Pellissier 1993), and *Hebeloma crustuliniforme* (Souto et al., 2000) in the presence of *Vaccinium myrtillus* L. (hereafter referred to as *Vaccinium*) has been suggested as a cause of its regeneration failure in subalpine forests in Europe. Handley (1963) found few mycorrhizal fungi associated with Norway spruce root systems when grown in the presence of heather (*Calluna vulgaris* L.). Yamasaki et al. (1998) reported significantly lower stem height, root–shoot biomass, and reduced mycorrhization of black spruce grown near (<1 m) *Kalmia* than those grown away (>1 m) from *Kalmia* under field conditions. *Kalmia* leaf leachates have been found to be inhibitory to mycorrhizal formation and mycelial growth of some ectomycorrhizal fungi of black spruce (Mallik et al., 1998).

Ectomycorrhizal fungi often experience chemical stress from forest floor phenolics (Bending and Read, 1997). The role of phenolic acids in ectomycorrhizal associations is not well understood. Black spruce–*Kalmia* forests of eastern Canada and Norway spruce–*Vaccinium* forest of subalpine France and Fennoscandia produce large amounts of litter on the forest floor (Inderjit and Mallik, 1999; Berg and Dise, 2004). Ericaceous plants, such as *Kalmia* and *Vaccinium*, produce large amounts of phenolic acids, which may accumulate on the forest floor through leaching from leaves (Zhu and Mallik, 1994), litter, humus (Pellissier 1994), and possibly root exudates. Many of these phenolic acids inhibit primary root growth in black spruce (Zhu and Mallik, 1994), seed germination and seedling growth in

Norway spruce, and mycorrhizal fungal growth (Pellissier 1993). Several authors have reported inhibitory effects of ericaceous litter on growth and respiration of spruce mycorrhiza (Boufalis et al., 1994; Boufalis and Pellissier, 1994). Souto et al. (2000) showed that humus phenolics of *Vaccinium* were more toxic to the spruce mycorrhizal fungus, *H. crustuliniforme*, than to the ericoid (*Vaccinium*) mycorrhizal fungus, *Hymenoscyphus ericae*.

In ectomycorrhizal symbioses, plant roots and fungi function together as a unit. Formation of ECMs in plants often allows them to establish in habitats that neither symbiont may be able to occupy individually (Nehls et al., 2000). Ectomycorrhizae are able to alleviate toxic effects of heavy metal cations in host plants (Denny and Wilkin, 1987; Colpaert and Van Assche, 1993). They may have additional or related mechanisms to deal with phenolic allelochemicals on the forest floor. From a controlled experiment, Mallik et al. (1998) showed that the growth inhibitory effects of *Kalmia* leachate on black spruce could be overcome by inoculating black spruce with selected mycorrhizal fungi. Because mycorrhizal fungi are located at the interface between the soil-allelochemical reservoir and roots of the host plants, we hypothesized that if certain spruce ECM fungi can degrade *Kalmia* phenolics, and use the degraded product(s) as a carbon source, then these ectomycorrhizal fungi would be able to protect black spruce against *Kalmia* phenolics and enhance their growth.

The objectives of our study were (1) to identify certain ECM fungi that can withstand the toxicity of *Kalmia* leaf, litter, and humus leachates and their associated phenolic compounds, and (2) to test whether these ECM fungi can degrade and detoxify the phenolic acids of *Kalmia*.

Methods and Materials

Plant and Ectomycorrhizal Fungal Materials

Black spruce seeds were obtained from Hills Nursery, Thunder Bay, Canada. *P. involutus* (Batsch. Ex Fr.) Fr. 211804, 196554, and *L. laccata* (Scop.:Fr.) Cooke 211651 were obtained from the Canadian Collection of Fungal Cultures, Ottawa. *Pisolithus tinctorius* Coker and Couch 99132 was obtained from Prof. Mingqin Gong at the Research Institute of Tropical Forestry in Guangzhou, Forestry Academy of China. *Laccaria bicolor* (Maire) P.D. Orton 229559 was collected from Thunder Bay. *P. tinctorius* 99132 was used to compare responses of ECM fungi from ericaceous-dominated and nonericaceous-dominated areas.

Chemicals

Ferulic, *o*-coumaric, and *o*-hydroxyphenylacetic (*o*-HPA) acids were purchased from Sigma (St. Louis, MO, USA). Malt extract and granulated agar were bought from Becton Dickinson Co. All solvents used were analytical or HPLC grade.

Seed Surface Sterilization

Black spruce seeds were surface-sterilized with 1% NaClO₃ for 10 min and then rinsed with sterile water × 5 to remove surface borne pathogens. Sterilized seeds were dried with autoclaved filter paper and kept at 4°C for subsequent use.

ECM Fungal Cultures

Modified Melin–Norkrans' nutrient solution (MMN) was used for growing the ectomycorrhizal fungi (Marx, 1969). Petri dishes containing the MMN, *Kalmia* leachates, and phenolic acids were inoculated with 4-mm-diam agar plugs cut from the edge of 2-wk-old ectomycorrhizal fungal colonies. The inoculated plates were incubated at room temperature (20–25°C). Diameters of the fungal colonies were measured every 5 d. All experiments consisted of at least three replicates.

Kalmia Leachates

Kalmia fresh leaves (mature, 1-yr-old), litter (dry leaves on the ground), and humus (0–10 cm) were collected from 10 random locations in a *Kalmia*-dominated heath originating from a natural fire 25 yr ago near the village of Terra Nova just outside the boundary of Terra Nova National Park, Newfoundland. Composite samples of fresh leaves, litter, and humus were made by mixing the 10 samples. Coarse roots and branches were removed from the humus samples prior to mixing. Twenty g of fresh leaves and litter were soaked separately in 100-ml distilled water for 24 hr at room temperature (20–25°C). The concentrations obtained for leaf and litter leachates were 0.2 g FW equivalent of leaves or litter per ml of leachate. *Kalmia* humus leachate was obtained by draining 200 ml distilled water at a slow rate for 24 hr through 100 g fresh humus in a glass chromatography column (30 mm) at room temperature. The filtrate of humus leachate was adjusted to 100 ml, and its concentration was 1 g FW equivalent of humus per ml of leachate. The water leachates of leaves, litter, and humus were passed through Whatman No. 42 filter paper and then sterilized by passing through two layers of 0.45- μ m Millipore filters.

Experiment 1: Effects of *Kalmia* Water Leachates on ECM Fungi

We measured the growth responses of two fungal isolates, *P. involutus* 211804 and *L. laccata* 211651, to three concentrations of leaf and litter leachates and two concentrations of humus leachate. The 0.1 g FW/ml leachate was prepared by adding 50 ml original leachate (0.2 g FW/ml) into 50 ml autoclaved MMN medium containing 1.5 g agar at 50–60°C. The 0.05 g FW/ml leachate was prepared by adding 25 ml original leachate and 25 ml sterilized water into 50 ml autoclaved MMN agar medium. The 0.01 g FW/ml leachate was prepared by adding 5 ml original leachate and 45 ml sterilized water into 50 ml autoclaved MMN medium. Concentrations of 0.25 and 0.05 g FW/ml of humus leachate were used. The original leachates of humus (25 ml, 1 g FW/ml) and 25 ml sterilized water were mixed with 50 ml autoclaved MMN medium containing 1.5 g agar at 50–60°C to obtain 0.25 FW/ml of humus leachate. The 0.05 g FW/ml humus leachate was prepared by adding 5 ml original leachate and 45 ml sterilized water into 50 ml autoclaved MMN agar medium. The control was made from half-strength MMN medium. The medium was poured into 9-cm-diam Petri dishes (20 ml medium in each dish). A disk of inoculum (diam 4 mm) from each of the five ECM fungi was cut from the edge of a 2- to 3-wk-old colony and inoculated in a Petri dish. After they were cultured for 28 d at 20–25°C, diameters of the fungal colonies were measured.

Experiment 2: Effect of Pure Phenolics on ECM Fungi

o-HPA, ferulic, and *o*-coumaric acids were the major phenolic acids in *Kalmia* leaf leachates (Zhu and Mallik, 1994) and humus (Mallik, unpublished data). Therefore, we measured the responses of five ECM fungal isolates, *P. involutus* (211804, 196554), *L. laccata* 211651, *P. tinctorius* 99132, and *L. bicolor* 229559 to these compounds. *o*-HPA acid was directly dissolved in sterile water to obtain a 2-mM solution. Ferulic and *o*-coumaric acids were dissolved in sterile hot water (80°C). Phenolic solutions were sterilized by passing through two layers of 0.45- μ m Millipore filters. Equivalent volumes of a 2-mM phenolic solution and a double concentration MMN agar were mixed to obtain a medium with 1 mM phenolic acid and full-strength MMN. The equimolar mixture of the three phenolic acids was obtained by mixing equivalent volumes of their respective 1 mM media for a final concentration of 0.33 mM for each acid. The control consisted of only MMN medium. Petri dishes containing the above-mentioned media were inoculated with the ectomycorrhizal fungi employed in Experiment 1, with four replicates for each treatment. The inoculated plates were incubated at 20–25°C and the diameters of the fungal colonies measured after 20 d.

Experiment 3: Phenolic Acids as Carbon Source for ECM Fungi

In this experiment, we investigated whether *P. involutus* 211804 and *L. bicolor* 229559 could grow with *o*-HPA acid as the only source of carbon. To prepare fungal inocula, *P. involutus* 211804 and *L. bicolor* were cultured on MMN agar for 16–20 d. Mycelial disks (diam 10 mm) were cut from the edges of fungal colonies and transferred to dishes containing water agar. Large-diameter inocula (10 mm as opposed to standard 4 mm) were used to provide nutrition for fungal growth. Water agar was used to exclude the possible nutrient effect of inoculated plugs on noncarbon nutrient experiment. These fungi were cultured in water agar for 22 d. After 22 d, a plug of inoculum (diam 4 mm) cut from the colony edge on water agar was inoculated on the noncarbon MMN medium (MMN agar medium without sucrose and malt extracts) without *o*-HPA acid (control) and noncarbon MMN medium with 1 mM *o*-HPA acid. There were four dishes for each fungus and treatment combination. The experiment was conducted at room temperature (20–25°C). Diameters of the fungal colonies were measured after 25 d.

Biomass Assay in Liquid Media

Because mycelial biomass in the noncarbon medium was very small, half strength of MMN liquid medium was used to culture *P. involutus* 211804 and *L. bicolor*. Each Petri dish (9 cm) contained 12 ml liquid medium with or without 1 mM *o*-HPA acid, prepared as described above. There were 10 dishes for each treatment. After autoclaving, a U-shaped glass rod (diam 2 mm) wrapped with a piece of 4 \times 4 cm unbleached paper towel was added to each dish and was inoculated with two plugs (diam 4 mm) of *P. involutus* 211804 inocula on the surface of the unbleached paper. The glass rod and unbleached paper towel facilitated the culturing of the ECM fungi in liquid media and subsequent collection of mycelia. Five ml of sterile water were added after 15 d. After 30 d culture, the mycelia were harvested from the unbleached paper. Ten dishes were divided into five groups, and mycelia from two dishes were combined, dried at 60°C for 24 hr, and weighed to ± 0.1 mg.

Experiment 4: Phenolic Reduction by ECM Fungi in Pure Culture

We tested whether *P. involutus* 211804 could metabolize phenolics. *P. involutus* 211804 was cultured on MMN agar medium. The Petri dish was inoculated at the center with one piece of 4 mm mycelial plug. Cultures were maintained at 20–23°C for 20 d. Then two U-shaped glass rods (diam 2 mm) were placed on the surface of the medium. Acetone solutions of the phenolic compounds (10 mM, 200 µl) were spotted on a piece of Whatman No. 3 filter paper (diam 12 mm). When the acetone had completely evaporated, 200 µl sterile water were added to the filter paper. Filter papers were placed over each rod. Each treatment consisted of three replicates. The filter papers were taken out 10 d later and they were soaked in 5 ml methanol for 24 hr. The methanol extracts were concentrated to 2.5 ml at 40°C under reduced pressure and passed through Whatman No. 1 filter paper. Phenolic compounds in the filtrates were analyzed by using a Varian Prostar HPLC equipped with a Chrompak column (250 × 4.6 mm) and PDA detector (Model 330) monitoring the absorbance of the elution at 280 nm. The solvent system was as follows: 0–9 min, 10% methanol and 90% water containing 2.5% formic acid; 9–20 min, 40% methanol and 60% water containing 2.5% formic acid; 20–25 min, 60% methanol and 40% water containing 2.5% formic acid; 25 min, 100% methanol. Flow rate was 1.5 ml/min, and temperature was 32°C. Pure compounds were used as standards, and phenolic acids were identified by comparison of retention times and UV spectrum.

Experiment 5: Detoxification Bioassay

We investigated whether inoculation of spruce seedlings with ECM fungus reduced the phytotoxicity of *o*-HPA acid. Because *o*-HPA acid was the most toxic phenolic acid in *Kalmia* leaf leachates affecting black spruce seedlings (Zhu and Mallik, 1994), it was used to conduct further detoxification studies. Since full-strength MMN medium itself inhibits seedling growth of black spruce, all media used were half-strength MMN. *P. involutus* 211804 was cultured in MMN liquid medium (half strength) containing 0.2 and 0.5 mM *o*-HPA acid. Each Petri dish (10 cm) contained 15 ml liquid medium. *P. involutus* 211804 was inoculated and cultured as described in Experiment 4. Noninoculated culture was used as the control. Plates were incubated at room temperature (20–23°C). Five ml sterile water were added after 15 d. The culture solution was decanted from the dishes after 30 d and passed through Whatman No. 42 filter paper. Filtrates were adjusted with sterile water to initial volume and used for the phytotoxicity test with black spruce seedlings. Each dish contained 20 surface-sterilized black spruce seeds placed on a piece of Whatman 3 filter paper to which 5 ml culture solution were added. The half-strength MMN medium was used as negative control, and half-strength MMN medium with 0.2 and 0.5 mM *o*-HPA acid were used as positive controls. The bioassay was conducted at 20–25°C with a 16-hr photoperiod. Two ml sterile water were added to each Petri dish during incubation. The lengths of black spruce primary root and shoot were measured after 15 d.

Experiment 6: Phenolic Degradation by Inoculated Black Spruce

Black spruce seedlings were inoculated with *P. involutus* 211804 following the method described by Marx and Kenney (1982) with some modifications to

determine whether the ECM inoculated seedlings are able to degrade *o*-coumaric, ferulic, and *o*-HPA acids and enhance the root–shoot growth of black spruce.

ECM Inoculum Preparation

Vermiculite (approximately 600 ml) and MMN liquid medium (300 ml) were added to 1-l glass jars. The jar lids had holes tightly plugged with cotton. After autoclaving for 30 min, each jar was inoculated with 10 mycelial disks. After 45 d incubation, the inoculum was wrapped with four layers of cheesecloth and irrigated with tap water for 5 min to remove any remaining nutrient. This material was used for black spruce seedling inoculation for the following experiment.

Seedling Inoculation

Autoclaved (121°C for 30 min) vermiculite and peat/moss mixture (1:1) was added to multipots (120 ml/cell) for seedling culture. Pregerminated black spruce seedlings (5 d) developed from surface-sterilized seeds were sown in multipots and kept in a growth incubator maintained at 26°C with a 16-hr photoperiod, 150 Md/m²/s PAR and 60% relative humidity. Seedlings were watered daily and fertilized once a week with 20–20–20 NPK. After 60 d incubation, the seedlings were removed to new, larger multipots (250 ml/cell) that were filled with the mycorrhizal fungal inocula and vermiculite/peat moss mixture at the ratio 1:15. Each cell contained one seedling. Inoculated seedlings were grown in the same incubator and under the same environmental conditions as in the other experiments. The roots of inoculated seedlings were surrounded by dense mycelia after 135 d inoculation. Noninoculated (control) seedlings were prepared and grown in the same manner without inoculation. No mycorrhizal infection was found in control seedlings. At this point, 30 ml of 1 mM phenolic acid solution were added to each cell. There were six seedlings per treatment. Five d later, the growing media (peat/vermiculite mixture) of seedlings were extracted with 300 ml distilled water × 3. The water extracts were pooled and partitioned against hexane × 3 to remove lipids, then extracted with ethylacetate × 3. Ethylacetate extracts were combined and concentrated to dry form at 40°C under reduced pressure, and then dissolved with 5 ml methanol/ethyl acetate (4:1). The phenolic acids were analyzed with HPLC.

Statistical Analysis

All data were normally distributed. One-way analysis of variance (ANOVA) was used, and treatment differences among means were tested at $P = 0.01$ with Duncan's multiple range test.

Results

Experiment 1: ECM Fungal Response to *Kalmia* Leachates

Growth response of the ECM fungi to *Kalmia* leachates depended on the type of leachate (leaf, litter, or humus), its concentration, and the species of ECM fungus. Higher concentrations of leaf leachates and lower concentrations of litter leachates

had stimulatory effects on *P. involutus*. The 0.1 and 0.05 g FW/ml leaf leachates significantly stimulated the growth of *P. involutus* by increasing the colony diameter of the fungus by 101.0 and 60.1%, respectively (Table 1). However, these two concentrations inhibited *L. laccata*, whose colony diameters were 12.6 and 11.4% smaller with 0.1 and 0.05 g FW/ml leaf leachates, respectively, relative to controls. The 0.1 g FW/ml litter leachate had no effect on colony diameter of *P. involutus*. However, the 0.05 and 0.01 g FW/ml litter leachates stimulated colony diameter by 72.6 and 49.0%, respectively. The 0.1 and 0.05 g FW/ml litter leachate had little effect on colony diameter of *L. laccata*, but with 0.01 g FW/ml litter leachate, there was a increase of 8% compared to control. The 0.25 and 0.05 g FW/ml humus leachate had no effects on colony diameter of either *P. involutus* or *L. laccata* (Table 1).

Experiment 2: ECM Response to Pure Phenolic Acids

The response of fungal isolates to pure phenolic acids also differed depending on the type of phenolic acid and its concentration. *P. involutus* 211804 was the most tolerant fungal isolate to the three phenolic acids and their equimolar mixture (Fig. 1). Indeed, the colony diameter of this isolate was increased by 25% in the presence of 1.0 mM *o*-HPA acid, and the mixture of the three phenolic acids did not inhibit growth at either concentration. However, *P. involutus* 211804 was inhibited by ferulic acid at 1 mM. Both the 1.0 and 0.1 mM phenolic mixtures inhibited colony diameter of *P. involutus* 199554 and *L. laccata* 211651. Colony diameter of *L. bicolor* 229559 was reduced by 41.7% on a 1 mM phenolic mixture, but it was increased (by 17.8%) in the presence of the 0.1 mM mixture. Colony diameter of both *L. bicolor* and *L. laccata* was increased by 1.0 mM *o*-HPA acid. Colony diameter of *L. laccata* was also increased by 0.1 mM *o*-HPA acid. *P. tinctorius* was the most sensitive of the fungal isolates to the phenolic mixture at 1.0 mM, and its colony diameter was inhibited by 80.5% on the 1.0 mM mixture.

With respect to the effect of individual phenolic compounds on the mycorrhizal fungi, ferulic acid was the most toxic, strongly inhibiting growth of all tested ECM fungi at 1 mM. The decrease in colony diameter of *P. involutus* 211804, 199554, and

Table 1 Effect of *Kalmia* leaf, litter, and humus leachates on colony diameter of *P. involutus* 211804 and *L. laccata* 211651

Leachates	Concentrations (g FW/ml)	Colony diameters [mm (mean ± SE)]	
		<i>P. involutus</i> 211804	<i>L. laccata</i> 211651
Control	0	39.8 ± 2.8c	50.0 ± 1.0bc
Leaf	0.1	80.0 ± 0.0a	43.7 ± 1.2e
Leaf	0.05	63.7 ± 4.0b	44.3 ± 1.5de
Leaf	0.01	33.3 ± 3.2c	49.0 ± 1.7bc
Litter	0.1	36.7 ± 0.6c	48.3 ± 0.6bc
Litter	0.05	68.7 ± 6.7b	47.3 ± 1.5cd
Litter	0.01	59.3 ± 11.4b	54.0 ± 1.0a
Humus	0.25	33.3 ± 0.6c	48.0 ± 1.7c
Humus	0.05	36.0 ± 1.7c	51.3 ± 0.6ab

Values are mean ± standard error ($N = 4$), means followed by the same letter(s) in the same column are not significantly different ($P < 0.01$)

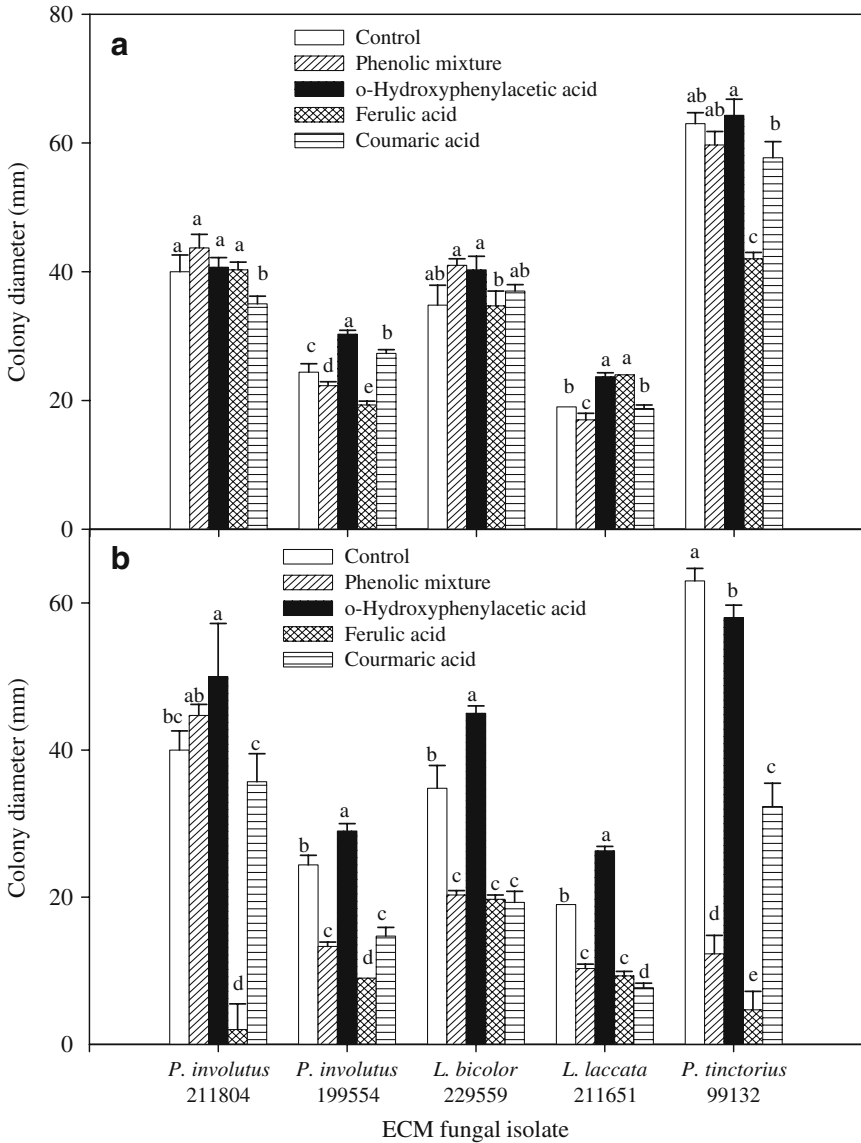


Fig. 1 Effect of three common *Kalmia* phenolic acids and their equimolar mixture on colony diameter of five ectomycorrhizal fungi at (a) 0.1 mM and (b) 1 mM. Values are mean \pm standard error ($N = 4$). Significant differences ($P < 0.01$) among treatments in each group are indicated by different letters

P. tinctorius was 95.0, 72.5, and 92.5%, respectively (Fig. 1). The compound also inhibited growth of *P. involutus* 199554 and *P. tinctorius* 99132 at 0.1 mM, but it stimulated the growth of *L. laccata* at this concentration. The *o*-HPA acid stimulated the colony diameter of *L. laccata*, *L. bicolor*, and the two isolates of *P. involutus* by 38.4, 29.3, 25.0, and 18.9%, respectively, at 1.0 mM; it also stimu-

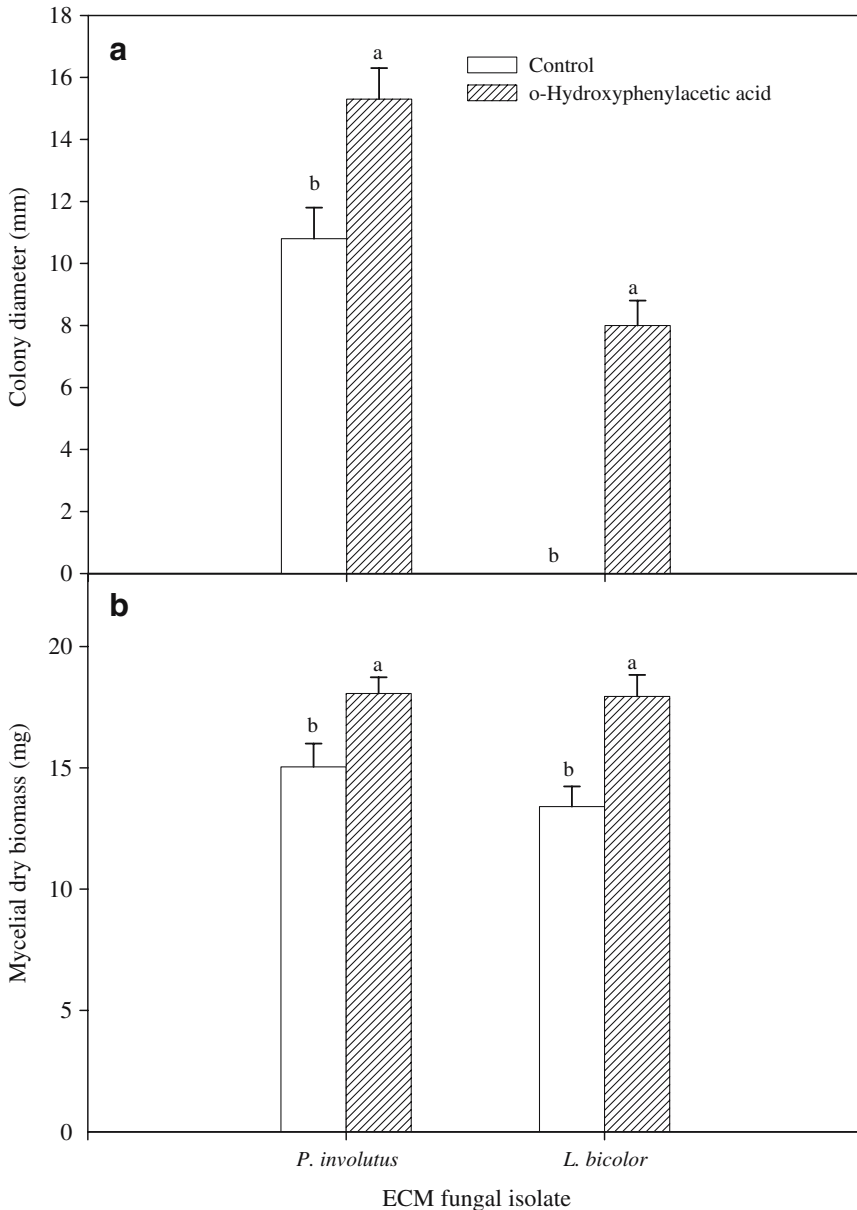


Fig. 2 Effects of 1 mM *o*-hydroxyphenylacetic acid on (a) colony diameter and (b) mycelial dry biomass of *P. involutus* 211804 and *L. bicolor* 229559 in carbon-free liquid MMN medium. Values are mean \pm standard error ($N = 5$). Significant difference ($P < 0.01$) among treatments in each group are indicated by different letters above bars

lated the colony growth of *P. involutus* 199554 and *L. laccata* at 0.1 mM. It did not stimulate colony growth of *P. tinctorius*. Coumaric acid inhibited the colony growth of ECM fungi except *P. involutus* 211804 at 1 mM. At 0.1 mM, coumaric acid did not inhibit any fungus, and stimulated the growth of *P. involutus* 199554 (Fig. 1).

Experiment 3: Phenolic Acids as Carbon Source for ECM Fungi

When *P. involutus* 211804 was cultured on noncarbon MMN agar medium, its colony diameter increased by 41.7% in the presence of 1 mM *o*-HPA acid (Fig. 2a). The hyphal density of the fungus in the medium with *o*-HPA acid was much higher than that of the control. Control plates had little mycelia. *L. bicolor* did not grow at all on the noncarbon MMN medium, but did in the presence of 1 mM *o*-HPA acid. *L. laccata* did not grow either on control or *o*-HPA acid medium.

The mycelial biomass of *P. involutus* 211804 and *L. bicolor* was increased by 20.1 and 33.9%, respectively when the two fungi were cultured separately in MMN liquid medium containing 1 mM *o*-HPA acid (Fig. 2b).

Experiment 4: Phenolic Degradation by ECM Fungus in Pure Culture

The concentrations of all three phenolic compounds, ferulic, coumaric, and *o*-HPA acids were reduced in the presence of *P. involutus* 211804 in pure culture. While the recovery of coumaric and *o*-HPA acids was reduced by 97.9 and 79.5%, respectively, we could not detect any ferulic acid following exposure to the fungal culture on the medium (Fig. 3).

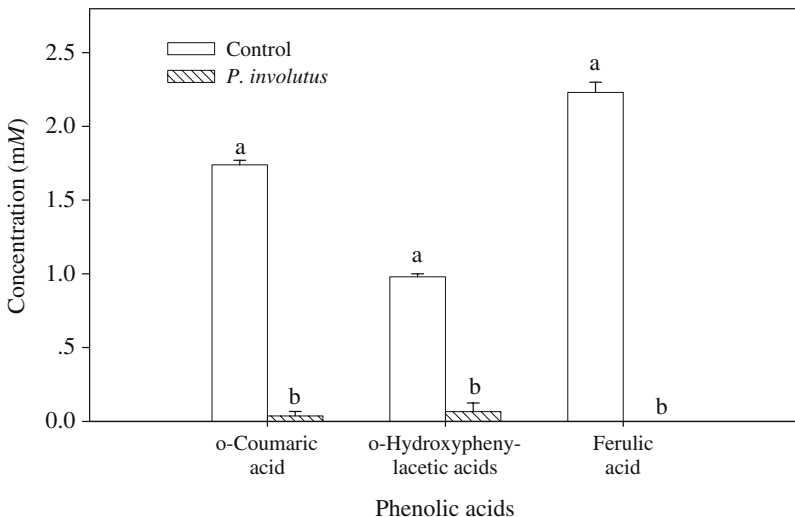


Fig. 3 Concentrations of *o*-coumaric, *o*-hydroxyphenylacetic, and ferulic acids in filter paper extract without (control) or with *P. involutus* 211804 culture. Values are mean \pm standard error ($N = 4$). Significant differences ($P < 0.01$) among treatments in each group are indicated by different letters above bars

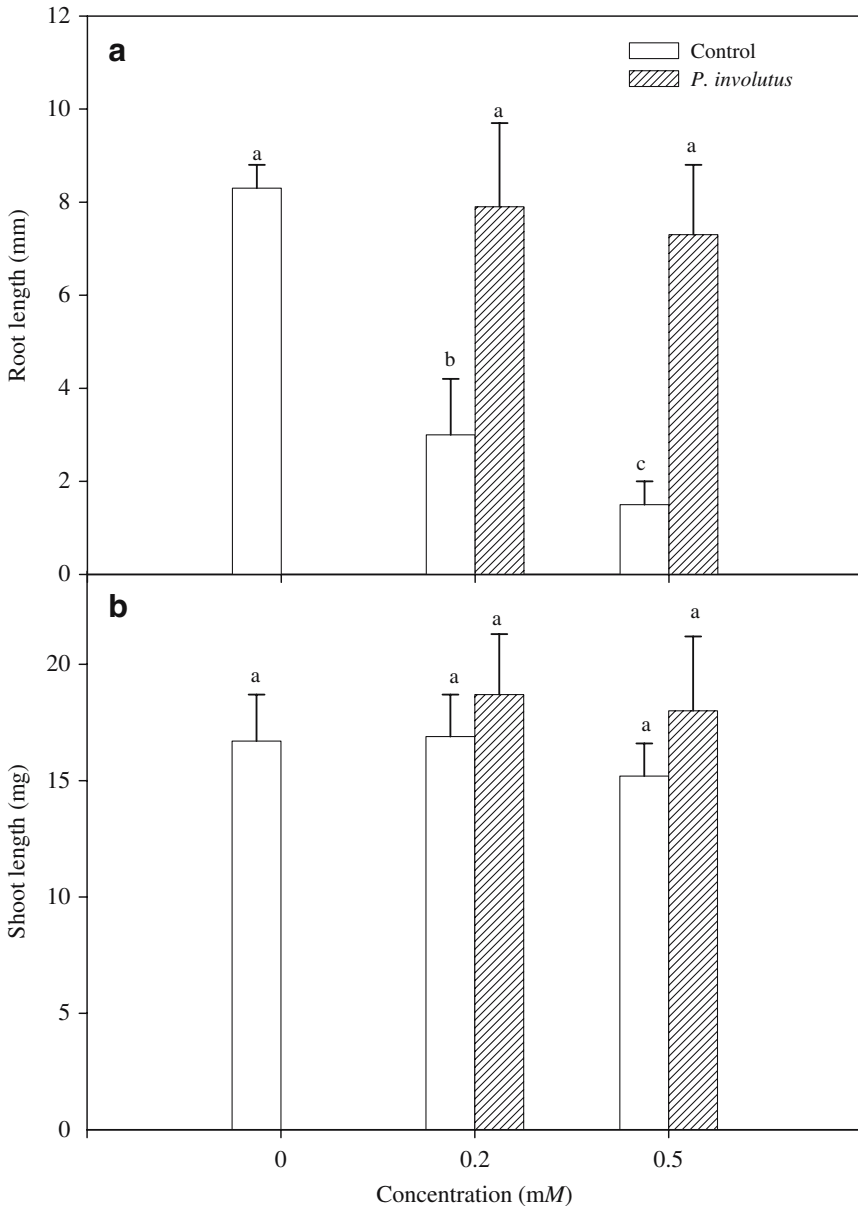


Fig. 4 Phytotoxic effects of *o*-hydroxyphenylacetic acid at concentrations of 0.2 and 0.5 mM with and without *P. involutus* culture on (a) root length and (b) shoot length of black spruce. Values are mean \pm standard error ($N = 15$). Significant differences ($P < 0.01$) among treatments are indicated by different letters above bars

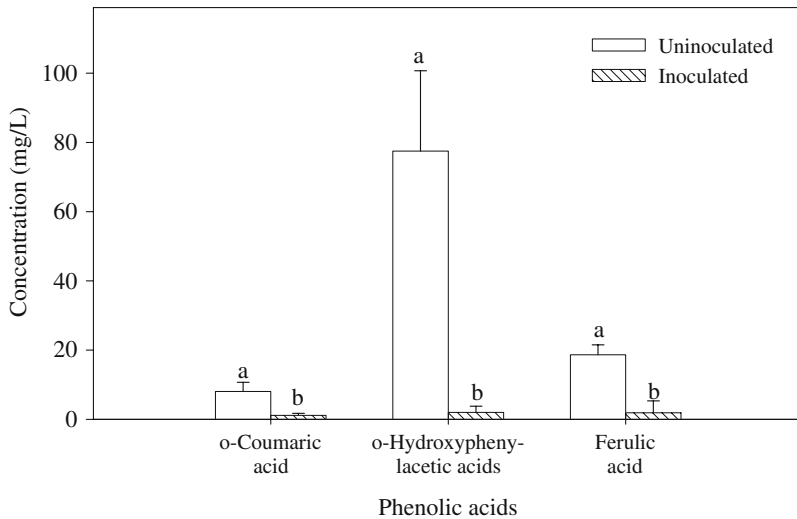


Fig. 5 Concentrations of *o*-coumaric, *o*-hydroxyphenylacetic, and ferulic acids in growing media containing uninoculated (control) and inoculated black spruce seedling with *P. involutus* 211804. Values are mean \pm standard error ($N = 3$). Significant differences ($P < 0.01$) among treatments in each group are indicated by different letters above bars

Experiment 5: Detoxification of *o*-HPA acid by ECM Fungi

Root growth of black spruce seedlings was inhibited by 82.0 and 63.9%, respectively, in the presence of *o*-HPA acid at 0.5 and 0.2 mM (Fig. 4a). However, no significant effect of *o*-HPA acid on root and shoot length was found when seedlings were grown with *P. involutus* 211804 culture broths (Fig. 4a,b).

Experiment 6: Phenolic Degradation by Inoculated Black Spruce

The concentrations of phenolic acids in peat/vermiculite mixture with mycorrhiza inoculated black spruce were much lower than those containing noninoculated black spruce (Fig. 5). Five d after adding *o*-HPA acid to the growing medium containing the ECM inoculated black spruce, less than 3% of the compound was recovered. Similarly, the recovery of *o*-coumaric acid and ferulic acid was only 13.3 and 10.4%, respectively, in the peat–vermiculite mixture that had *P. involutus* 211804-inoculated black spruce.

Discussion

Our results show that the different ECM fungal isolates had differential growth response to *Kalmia* leachates and phenolic compounds. *L. laccata* was less resistant than *P. involutus*. The growth of *P. involutus* 211804 was significantly stimulated by leaf and litter leachates even in the presence of full-strength leaf leachate (0.1 g FW/ml). This growth enhancement may be caused either by additional nutrients in the leaf and litter leachate and/or the degradation products of *Kalmia* phenolics in the

leachate that the ECM fungi can use as a carbon source. Earlier, Mallik and Zhu (1995) reported that growth of one isolate of *P. involutus* (NF4) was stimulated in the presence of *Kalmia* leaf leachate. Coté and Thibault (1988) found an isolate of *P. involutus* (0077) to be more sensitive to leaf leachate toxicity of raspberry (*Rubus idaeus*) compared to other ECM fungi. In this study, water leachate of *Kalmia* humus showed no significant effect on colony diameter of *P. involutus* and *L. laccata*. Phenolic profiles of leaf and litter leachates were different from that of humus. It is possible that by the time leaf and litter were decomposed into humus, many of the influential alleochemicals were degraded by microorganisms. It is also possible that degradation and use of humus phenolics is more difficult for ECM fungi than those of leaf and litter.

Because different isolates of the same species of ECM fungus responded differently to a particular phenolic acid, our results suggest that coadaptation may influence ECM fungal tolerances to phenolics. *P. tinctorius* was the most sensitive fungus to the phenolic mixture; its colony diameter was inhibited by 80.5% in the presence of 1.0 mM mixture. This isolate was collected from the subtropical region of Guangdong, South China, whereas the other four fungal isolates were collected from boreal forests of Canada. In South China, we know of no report of high concentrations of phenolic acids in soils, presumably because of high precipitation, high litter quality, rapid decomposition, and little organic matter accumulation. Therefore, this isolate may not have mechanisms to deal with high concentrations of phenolic compounds, whereas the fungi collected from Canada may possess this adaptation (Callaway and Ridenour, 2004; Vivanco et al., 2004).

In the noncarbon growing medium, both *P. involutus* and *L. bicolor* grew significantly better with respect to colony diameter and mycelial biomass in the presence of *o*-HPA acid than those without the phenolic acid (Fig. 2). Since *o*-HPA acid was the only carbon source in the medium, we conclude that the fungus must have used *o*-HPA acid as a carbon source to increase its biomass. Our phenol reduction experiment (Experiment 4) conducted with three pure phenolic acids confirmed the idea that *P. involutus* is able to reduce or oxidize phenolic acids quite easily within a short time (Fig. 3). However, high concentration (1 mM in the media) of ferulic acid inhibits the growth of *P. involutus* (Fig. 1b).

After *P. involutus* 211804 was cultured in 0.5 and 0.2 mM *o*-HPA, its phytotoxicity disappeared (Fig. 4). *o*-HPA acid, at 0.2 mM, was toxic to black spruce seedlings in the absence of *P. involutus*, but the 0.5 mM *o*-HPA acid solution became nontoxic in the presence of the fungal culture. From this result, we conclude that the concentration of 0.5 mM *o*-HPA acid was reduced to less than 0.2 mM, as a result of fungal culture. In other words, *P. involutus* detoxified the 0.5 mM *o*-HPA acid to a level lower than 0.2 mM, and allowed the seedlings to grow. Zhu and Mallik (1994) have shown that black spruce seedlings can grow in the presence of ferulic and coumaric acids but cannot tolerate high concentrations of *o*-HPA acid. From this study, we suggest that the symbiotic ECM fungi of black spruce can not only tolerate high concentrations of *o*-HPA, but can also degrade the compound and use it as a carbon source.

Most of the added phenolic acids in the peat/vermiculite mixture containing *P. involutus* inoculated black spruce disappeared within 5 d, whereas substantial amounts of the added acids were recovered from the growing medium of the noninoculated (control) seedlings. We suggest that the *P. involutus*-inoculated

seedlings are able to degrade *Kalmia* phenolics in the potting medium. However, the incomplete recovery of *o*-coumaric and ferulic acid in the uninoculated (control) medium suggests that preinoculation of black spruce seedlings with suitable ectomycorrhizal fungi may promote conifer regeneration in *Kalmia* dominated sites.

The problem of *Kalmia*-induced growth inhibition in black spruce is complex and the relationship between *Kalmia* phenolics and mycorrhizae is still unclear (Wallstedt et al., 2002). From a field study that showed poor growth, foliar N and P concentrations and mycorrhization of black spruce seedlings growing closer to *Kalmia* than those growing away, Yamasaki et al. (1998) concluded that *Kalmia* limits the growth of black spruce in ways other than direct nutrient competition. They suggested that since mycorrhizae and plant nutrition are closely related, fewer mycorrhizal short roots in black spruce growing near *Kalmia* means less nutrient acquisition due to poor mycorrhizal symbiosis, thus resulting into poorer growth in these seedlings. Another cause of poor growth was attributed to the susceptibility of black spruce near *Kalmia* to a potential pathogenic fungus, *Phialocephala dimorphospora*. Yamasaki et al. (1998) suspect that higher amount of mycorrhizal short roots in seedlings growing away from *Kalmia* may have protected them from the pathogenic fungus and consequently allowed better growth. One could also suspect long-term dominance of *Kalmia* in the absence of an appropriate host (in this case, black spruce) that the beneficial ECM would be reduced in soil inoculum. Our previous laboratory studies have shown that out of 51 fungal isolates tested, the growth of 41 isolates was reduced in the presence of *Kalmia* leaf leachate in liquid MMN agar. Yamasaki et al. (1998) suspect that *Kalmia* compounds could affect the survival of fungal inoculum in soil or the fungal symbionts in black spruce roots. Robinson (1972) proposed a similar hypothesis in explaining the growth inhibition of Sitka spruce [*Picea sitchensis* (Bong.) Carriere] seedlings in the presence of another heath-forming ericaceous shrub, *C. vulgaris*. Response of ECM to ericaceous compounds can be negative, positive, or neutral depending on the species and strains of the ECM (Mallik and Zhu, 1995). In this study, we found that certain black spruce ECM (particularly *P. involutus*) can respond positively to *Kalmia* phenolics with increased biomass. This, in turn, enhances seedling growth under laboratory and greenhouse conditions. Given the fact that biophysical conditions in the field can be very different from those in the controlled laboratory and greenhouse, the logical next step of this research is to test if black spruce seedlings preinoculated with *P. involutus* out planted in *Kalmia* dominated sites can overcome the growth inhibition by maintaining the symbiotic relationship.

We can draw several conclusions from this study. First, our results prove that certain ECM fungi can not only offer protection to host plants against phenolic allelochemicals released from neighboring plants, but they can also use them as a carbon source. This, in turn, can benefit the host plant. Our findings have important ecological significance in the sense that they point to a mechanism by which ectomycorrhizal fungal species can control species interactions in higher plants by changing the rhizosphere chemistry. Second, the ability of ECM fungi to degrade and detoxify phenolic allelochemicals is not only species-specific, but also specific to different strains of the same fungal species. Third, tree seedlings preinoculated with beneficial ECM fungi can degrade plant phenolics in peat/vermiculite mixture. This offers some hope toward overcoming phenolic-induced conifer growth inhibition in

ericaceous-dominated sites by outplanting tree seedlings preinoculated with carefully selected beneficial ECM fungi.

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Chemical Defenses of Cryptic and Aposematic Gastropterid Molluscs Feeding on their Host Sponge *Dysidea granulosa*

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Abstract Numerous opisthobranchs are known to sequester chemical defenses from their prey and use them for their own defense. Information on feeding biology is critical for understanding the ecology and evolution of molluscs, yet information on feeding biology is still scarce for many groups. Gastropterid molluscs are often found on sponges, but there is controversy as to whether they are true sponge feeders. On Guam, we found the gastropterids *Sagaminopteron nigropunctatum* and *S. psychedelicum* on the sponge *Dysidea granulosa*. They seem to rely on contrasting defense strategies as *S. psychedelicum* has vivid colors, consistent with the warning coloration found in many chemically defended opisthobranchs, whereas *S. nigropunctatum* is highly cryptic on the sponge. *S. nigropunctatum* is avoided by the pufferfish *Canthigaster solandri* in aquarium assays. We analyzed the secondary metabolites of the two species and found that both share polybrominated diphenyl ethers (BDEs) with their host sponge *D. granulosa*. *S. psychedelicum* and *S. nigropunctatum* sequester the major BDE in the sponge and accumulate it in the mantle at approximately the same concentration as in the sponge (4.03 and 2.37%, respectively), and concentrate it in their parapodia at over twice the sponge concentration (7.97 and 10.10%, respectively). We also detected trace amounts in the mucus secretion of *S. psychedelicum*, and quantified significant amounts in the mucus (1.84%) and egg masses (2.22%) of *S. nigropunctatum*. Despite contrasting color patterns displayed by the two gastropterid species, they seem to share a similar

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chemical defense strategy, i.e., they feed on *D. granulosa* and accumulate the major BDE of the sponge in their tissues.

Keywords Brominated diphenyl ethers (BDEs) · Chemical defense · *Dysidea granulosa* · Feeding specialists · *Sagaminopteron nigropunctatum* · *Sagaminopteron psychedelicum*

Introduction

Feeding biology plays a major role in the ecology and evolution of molluscs, particularly opisthobranchs (Kohn, 1983; Willan, 1984; Avila, 1995; Johnson and Willows, 1999). Many traits that drive the evolution of opisthobranchs, such as shell reduction or loss, have resulted from coevolution with diet organisms (Faulkner and Ghiselin, 1983; Cimino and Ghiselin, 1998, 1999; Cimino et al., 1999, 2001). Without shells, many opisthobranchs rely on other defense mechanisms. Camouflage or cryptic coloration is the most common strategy, with examples in a variety of organisms including opisthobranch mollusk. Since organisms must resemble their backgrounds (Endler, 1978), appropriate coloration is background-specific. Many opisthobranch molluscs are specialist predators, and cryptic species show evidence of a close association with their diets (Rudman, 1981; Morrow et al., 1992; Becerro et al., 2001, 2003). Aposematic or warning coloration is at the opposite extreme, and evolutionary theory postulates that aposematic coloration evolves in deterrent species because it is a more effective warning signal than the alternate cryptic coloration (Wallace, 1867; Poulton, 1890). In opisthobranch molluscs, warning coloration is based on the benefits provided by chemical defenses often sequestered from the opisthobranch food source (Faulkner and Ghiselin, 1983; Avila, 1995), and it seems associated with evolutionarily more derived, rather than more basal, taxa (Gosliner, 2001).

Despite the importance of feeding biology in the life history strategies of opisthobranchs, there is a lack of information on the feeding biology of many groups. Gastropterid molluscs are often found on sponges, which suggests they are sponge feeders. Despite the close association between gastropterids and sponges, it remains doubtful whether gastropterids actually feed on sponges since their radular teeth are similar to the closely related philinoid bubble shells, which feed on hard-shell prey (Hurst, 1965; Mikkelsen, 1996). Two sympatric gastropterid species of the genus *Sagaminopteron*, *S. nigropunctatum* and *S. psychedelicum*, show contrasting color patterns and are associated with the sponge *Dysidea granulosa* on which they are usually found. *S. nigropunctatum* displays cryptic coloration (Gosliner, 2001); it is well camouflaged with a pattern that is almost identical in color and texture to the sponge (Carlson and Hoff, 1973). In contrast, *S. psychedelicum* has a bright and remarkable color pattern that makes it obvious (Carlson and Hoff, 1974), which is consistent with an aposematic coloration (Gosliner, 2001). Both sagaminopteran species are associated with their host sponge and, in contrast to other gastropterids, neither is a spectacular swimmer, especially *S. nigropunctatum*, which seldom swims (Carlson and Hoff, 1973).

Many opisthobranchs feed on chemically defended prey (Faulkner, 1992; Avila, 1995). In a predator, the presence of secondary metabolites produced by its prey is considered evidence of a trophic relationship. Studies in chemical ecology show that

opisthobranchs sequester secondary metabolites from prey, accumulate, concentrate, and distribute them in their tissues, and use them for their own defense (Carté and Faulkner, 1983; Paul et al., 1990; Becerro et al., 2001, 2003). This strategy of sequestration, concentration, modification, and distribution of metabolites from the prey into the body of the predator is found widely in nudibranchs (Cimino and Ghiselin, 1999 and references therein), sacoglossans (Cimino and Ghiselin, 1998; Cimino et al., 1999; Becerro et al., 2001), notaspideans (Teeyapant et al., 1993; Ebel et al., 1999; Becerro et al., 2003), and sea hares (Johnson and Willows, 1999). *D. granulosa* is a chemically defended sponge that produces a number of polybrominated diphenyl ethers (Becerro and Paul, 2004). Given the close and consistent association of both gastropterid species with *D. granulosa* and the widespread occurrence of compound sequestration and biomagnification observed in specialist predators, we set forth the hypothesis that, if they are true specialist predators, both *S. nigropunctatum* and *S. psychedelicum* will share secondary chemistry with their host sponge *D. granulosa*. However, given the contrasting color patterns shown by the two species, they might differ in their defense strategies. Thus, we present two working hypotheses: (1) consistent with its warning coloration, *S. psychedelicum* will accumulate, concentrate, and distribute in its parapodia compounds sequestered from *D. granulosa*; and (2) the cryptic *S. nigropunctatum* will sequester sponge secondary metabolites in its digestive gland and will fail to concentrate and adequately distribute these metabolites in its tissues thus, relying on camouflage.

Methods and Materials

On Guam, *D. granulosa* (Bergquist 1965, n. sp., aff. *granulosa*; Kelly et al., 2003) (Family Dysideidae, Order Dictyoceratida) is a common shallow-water sponge mostly found on reef flats, where it can be abundant. *D. granulosa* is abundant up to a depth of 5–6 m in Gun Beach, a reef slope habitat with a series of 1- to 5-m-wide channels with vertical walls perpendicular to the shoreline and open to wave action and surge. On this location, *D. granulosa* has three polybrominated diphenyl ethers (BDEs) as major secondary metabolites (Becerro and Paul, 2004). On Gun Beach, the gastropterids *S. psychedelicum* and *S. nigropunctatum* are consistently found on *D. granulosa*, sometimes sharing the same specimen. Neither is highly abundant, and because of their small size (smaller than 0.5 cm in our study site), they are difficult to observe.

Chemical Analysis

We collected five specimens of *S. psychedelicum*, nine specimens of *S. nigropunctatum*, and 10 egg masses of *S. nigropunctatum*. We found no egg masses of *S. psychedelicum* at the time of collection. All samples were collected by scuba diving, and taken to the laboratory where they were frozen at -20°C and stored until analyzed and used in experiments.

Frozen gastropterids were thawed and rinsed with distilled water to remove any secretion released by the animals. Gastropterids were dissected to separate parapodia and “tail,” digestive gland, and the remainder of the body. Because of the small size and in order to have enough material for chemical quantification, the same body parts from all specimens were pooled in preweighed vials and freeze-

dried. Eggs were rinsed in distilled water and freeze-dried. Samples were then extracted with 1:1 (v/v) DCM/MeOH. This extraction procedure differs slightly from that used by Becerro and Paul (2004) to extract BDEs from sponge samples, since we used DCM/MeOH instead of DCM as the extraction solvent. However, BDEs are exhaustively extracted by both solvent mixtures, and we did not find more polar secondary metabolites in the sponges or the gastropterids. The crude extract was redissolved in DCM and filtered through glass wool and a 1-cm, 60–200 mesh silica gel pipette column in preparation for gas chromatography.

We used gas chromatography/mass spectrometry (GC-MS) to quantify BDEs in the gastropterid body parts. For quantification by GC-MS, we dissolved crude extracts in DCM (2 ml/mg) containing naphthalene (50 $\mu\text{g/ml}$) as internal standard (IS). Quantification was done with a Hewlett-Packard 5890 Series II GC fitted with an HP-5 capillary column (5% phenyl methyl crosslinked silicon siloxane, 30 m long, 0.25 mm i.d., 0.25 μm film thickness) and coupled to a Hewlett-Packard 5972 MS. Gas chromatography was performed following methods of Becerro and Paul (2004). One μl of extract solution at a concentration of 0.5 mg/ml was injected. We quantified the % yield of compounds by comparing the ratio “area of compound divided by area of IS” in the samples to a calibration curve calculated with known concentrations of pure compounds. To calculate the % yield of BDEs per sample dry mass, we multiplied the yield of BDEs in the extract by the yield of crude extract in the sample $\times 100$.

We compared the concentration of compound **2** [3,5 dibromo-2-(2',4'-dibromophenoxy)phenol] in gastropterid body parts to the concentrations found by Becerro and Paul (2004) in the sponge ectosome and choanosome. Because we pooled the same gastropterid body parts from the same species, we have a single concentration value for each body part, i.e., we obtained an average value from our replicates but we lack variance data. To test whether our single values differ statistically from the replicated ectosome and choanosome data ($N = 10$, respectively), we used a *t*-test for a single specimen compared with the mean of a sample (previous arcsin transformation to meet parametric assumptions) as described by Sokal and Rohlf (1995).

Ecological Experiments

We ran two sets of experiments to investigate the relationship between the gastropterids and the sponge, and to assess whether gastropterids are defended against generalist predators. All experiments were run with *S. nigropunctatum* because we found no *S. psychedelicum* at the time of collection. We collected pieces of *D. granulosa* hosting *S. nigropunctatum* at Gun Beach. To reduce the likelihood of the animals discharging secretion, we collected the opisthobranchs by breaking off pieces of sponge rather than removing the animal from the sponge. The gastropterids and sponges were placed in large plastic bags with seawater and transported to the University of Guam Marine Laboratory in a cooler. In the laboratory, sponges were cleaned of epifauna and cut into equal sized pieces of ectosome and choanosome. Ectosome refers to the outer layer of sponge (about 2 mm in thickness), whereas choanosome refers to the gray interior. The ectosome contains abundant cyanobacteria that makes this zone a greenish-purple color. There also are bacterial and chemical differences between these two sponge parts (Becerro and Paul 2004). In the field, gastropterids are found on the surface of the sponge (ectosome), and we tested whether gastropterids show a preference for the

ectosome over the inner choanosome. Paired ectosome and choanosome sponge pieces were placed in watch glasses with single gastropterids ($N = 17$) and left until the animal chose one of the sponge pieces up to a maximum of 1 hr. We then scored the number of gastropterids present. Animals were left in the bowl for an additional hour and scored again. We used a *binomial distribution* ($P = Q = 0.5$) to test for significant preferences between ectosome and choanosome sponge pieces (two-tailed P values reported) at the two time intervals.

To test whether *S. nigropunctatum* was defended against generalist predatory fish, the same specimens used in the previous choice experiment were then offered to the sharpnose pufferfish *Canthigaster solandri* (Richardson, 1845). The pufferfish were kept in individual flowthrough aquaria (10 l) and maintained on artificial commercial catfish food pellets. We recorded whether *S. nigropunctatum* was eaten, tasted and rejected, or ignored by the fish. Immediately after this trial, we offered the same fish a piece of equally sized skinned octopus tentacle. Since we tested the same individual fish twice (first for treatment and then control tests), we used a McNemar test to check whether *S. nigropunctatum* inhibited feeding of the pufferfish compared to control food (Sokal and Rohlf, 1995).

Results

The extract of *D. granulosa* from Gun Beach has three major BDEs. The compound 3,5 dibromo-2-(2',4'-dibromo-phenoxy)phenol (hereafter referred to compound **2**, as in Becerro and Paul 2004) is the major metabolite in the extract and corresponds to a % yield per dry mass of sponge of 2.78 ± 0.27 (mean \pm SE) in the ectosome and 3.21 ± 0.67 in the choanosome (Becerro and Paul 2004). Other BDEs closely related to **2** that are found in lower concentrations in the sponge were not detected in the gastropterids (see Becerro and Paul, 2004 for a more comprehensive analysis of the chemical variation of the sponge *D. granulosa*).

Both *S. psychedelicum* and *S. nigropunctatum* share secondary chemistry with the sponge *D. granulosa*. We detected **2** in all body parts from both species, including the egg mass of *S. nigropunctatum* (Fig. 1). However, concentration of **2** between body parts and species differed quantitatively (Fig. 1, note lack of variance in body parts due to replicate pooling). Concentration of **2** sequestered in the digestive gland may depend on the amount of food in the gut and the actual concentration of compounds in the material ingested. *S. nigropunctatum* concentrates **2** in the parapodia or mantle's edge up to an average concentration of 10.10% per dry mass, which is a significant increase as compared to the concentration found in the sponge ectosome and choanosome ($P < 0.001$ and $P = 0.019$, respectively). *S. psychedelicum* also concentrates **2** in the parapodia up to an average concentration of 7.97%, which is a significant increase as compared to the ectosome ($P = 0.001$) and almost as much as the choanosome ($P = 0.055$). The body tissues of both gastropterid species and the egg masses and secretion of *S. nigropunctatum* had concentrations of **2** similar to those found in either the sponge ectosome or choanosome ($P > 0.05$ for all comparisons). Compound **2** was also present in the secretion of *S. psychedelicum* but below the quantification threshold.

In laboratory choice experiments, *S. nigropunctatum* significantly selected the outer ectosome of *D. granulosa* over the interior choanosome. At the initial choice, 12 out of 17 molluscs selected the ectosome over the choanosome (binomial test,

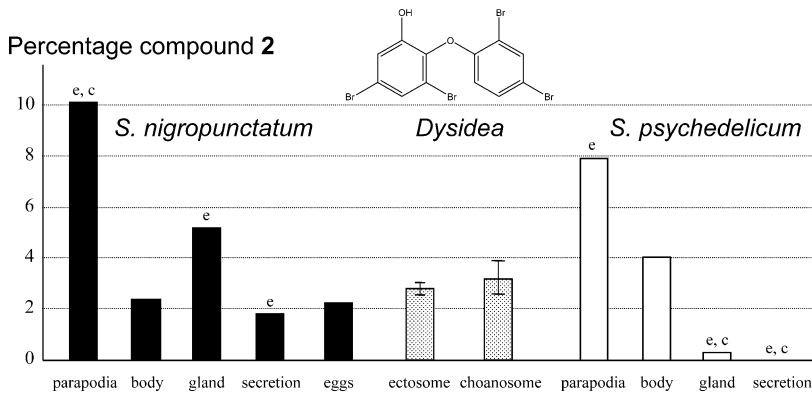


Fig. 1 Concentration (as % dry mass) of compound 2 (3,5-dibromo-2-(2',4'-dibromo-phenoxy)phenol) found in several tissues of the gastropterid *Sagaminopteron nigropunctatum* and *S. psychedelicum*. Note lack of error bars in gastropterid body parts due to replicate pooling. Mean (± 1 SE) percent of compound 2 in the ectosome and choanosome ($N = 10$) of the sponge *Dysidea granulosa* is shown for comparison. Sponge data from Becerro and Paul (2004). Letters “e” or “c” above bars denote significant differences between the bar value and the ectosome or choanosome data, respectively ($P < 0.05$)

$P = 0.047$). After 1 hr, three of the five specimens that chose pieces of choanosome had moved away and were observed on the ectosome ($N = 1$) or crawling on the aquarium ($N = 2$) (binomial test, $P = 0.018$). Individuals that chose a piece of ectosome remained on it until the end of the experiment.

In feeding assay experiments, *S. nigropunctatum* deterred feeding of the pufferfish *C. solandri* (McNemar $G = 4.159$, $P < 0.05$); eight out of 10 pufferfish tasted and rejected the gastropterid in the laboratory, three of which consumed the control food. No fish fed on the gastropterid and rejected control food.

Discussion

Cephalaspideans are a polyphyletic group of opisthobranch molluscs that consume a variety of diets such as polychaetes, molluscs, urchins, or foraminifera (Mikkelsen, 1996). There is some controversy about gastropterid feeding habits and whether or not they are true sponge feeders, with indirect evidence either supporting or casting doubts on this hypothesis (see below). The hypothesis that gastropterid are sponge feeders is mostly based on the close association between gastropterids and sponges, including the close association between *S. psychedelicum* and *S. nigropunctatum* and their host sponge *D. granulosa* investigated in this study (*Dysidea* cf. *reticulata* in Carlson and Hoff, 1973, 1974). Yet, a close association may not necessarily involve a trophic relationship. Nudibranch species of the genus *Trapania* are associated with sponges, which were assumed to be their food source. However, there is some evidence that *Trapania* species seem to feed on entoprocts that grow on top of the sponges rather than feeding on the sponges themselves (<http://www.seaslugforum.net/display.cfm?id=3767>).

Anatomical data argue against *Sagaminopteron* species as sponge feeders. Gastropterid radular teeth are similar to those of their relatives, none of which

feed on sponges but instead on hard-shell prey such as the polychaete *Pectinaria*, small clams, snails, sea urchins, and foraminiferans (Hurst, 1965; Burn and Bell, 1974a,b; Shonman and Nybakken, 1978). Accordingly, it is reasonable to hypothesize that *S. nigropunctatum* and *S. psychedelicum* feed on food sources they encounter while crawling on the sponge surface. For example, aeolid nudibranchs typically feed on cnidarians, but species of the genus *Calma* have a modified radular structure (a species-level character) to feed efficiently on fish egg masses (Calado and Ugorri, 2002). Alternatively, gastropterids could feed on sponges without evolving a modified radula. Sacoglossans are considered highly specialized herbivores that feed on green algae (Williams and Walker, 1999), but some species of the genera *Favorinus*, *Stiliger*, and *Olea* are oophagous rather than herbivorous (Thompson and Brown, 1984)—yet they show no radular adaptations that reflect their change of diet. Likewise, polyceratid nudibranchs typically feed on bryozoans, but species of the genera *Roboastra* and *Nembrotha* feed on nudibranchs and ascidians, respectively (Carté and Faulkner, 1986; Paul et al., 1990; Megina and Cervera, 2003), and show no radular modifications to do so.

Our study shows that *S. nigropunctatum*, *S. psychedelicum*, and their host sponge *D. granulosa* share secondary chemistry, which supports the hypothesis that gastropterids are sponge feeders. Pawlik et al. (1988) showed that the Spanish dancer nudibranch *Hexabranchus sanguineus* sequesters its chemical defense from its food sponge *Halichondria* sp., and distributes and concentrates the chemical defenses in the mantle, mucus secretion, and the egg masses. The gastropterids *S. psychedelicum* and *S. nigropunctatum* seem to share the same strategy, even though they are a fraction of the size. Both species sequester, distribute, and accumulate sponge BDEs in their parapodia, a strategy similar to that found not only in *H. sanguineus* but in many other opisthobranch molluscs that feed on chemically defended prey (Pawlik et al., 1988; Faulkner, 1992; Avila, 1995; Avila and Paul, 1997). Thus, compound **2** (3,5-dibromo-2-(2',4'-dibromo-phenoxy)phenol), the major BDE in *D. granulosa*, is present in the mantle of the gastropterids at over twice the concentration found in the sponge, and additionally is present in the digestive glands. When disturbed, both gastropterids discharge a mucus secretion that include **2**, which suggests BDEs contribute to predator deterrence. Also, *S. nigropunctatum* transfers **2** to egg masses, where it could help prevent predation or fouling by bacteria, fungi, or other benthic organisms (Matsunaga et al., 1986). BDEs have a variety of biological activities (Sharma and Vig, 1972; Fu et al., 1995; Handayani et al., 1997) including defense against predators (Duffy and Paul, 1992; Pennings et al., 1994). Compound **2** deters a variety of predators including pufferfish *C. solandri*, xanthid crabs *Leptodius* spp., and the sea hare *Stylocheilus longicauda* (Duffy and Paul, 1992; Pennings and Paul, 1993; Pennings et al., 1994) even at concentrations below those found here. The pufferfish *C. solandri* tasted and rejected specimens of *S. nigropunctatum* in the laboratory suggesting that it is defended against them.

Coral reefs are among the ecosystems with the highest levels of predation (Carpenter, 1986) including generalist fish predators that use visual cues to obtain food (Lowe-McConnell, 1987). *S. nigropunctatum* is highly cryptic on *D. granulosa* since the gastropterid mimics both the color and texture of the sponge. Egg masses and some orange parts on the head shield and siphon of *S. nigropunctatum* are the only indications of its presence. *S. nigropunctatum* also mimics *D. granulosa* chemically by incorporating the sponge major metabolite in its tissues. Consequently, predators may overlook *S. nigropunctatum* in the field as a result of visual and

chemical camouflage. When cryptic coloration fails, chemical defenses may help the gastropterid overcome predators.

In contrast, *S. psychedelicum* has a conspicuous color pattern, which suggests a true aposematic coloration (Edmunds, 1991; Gosliner, 2001). Being conspicuously advertised is just one of the four criteria needed to fit the definition of aposematic coloration (Edmunds, 1991). It is also necessary to know whether or not (1) the organism deters predators, (2) predators avoid attacking a potential prey because of its colors, and (3) the aposematic color pattern is more effective than being cryptic (Edmunds, 1991). Although not tested, it is reasonable to think that *S. psychedelicum* is also chemically defended against predators, because it shares the same chemical as *S. nigropunctatum* at concentrations above those known to deter potential consumers. Despite the high numbers of generalist predators sharing habitat with the gastropterid, we have seen no sign of predation on this species in the field. Whether or not the striking color pattern of *S. psychedelicum* is more effective than the alternative cryptic coloration found in *S. nigropunctatum* is unknown, but these gastropterids may be an excellent system to understand better the role that color patterns and chemistry play in predation.

Our data also show that *S. nigropunctatum* significantly prefers the ectosome of *D. granulosa* over the interior choanosome. Some species of the genus *Dysidea* are known to contain large number of cyanobacteria. The cyanobacteria *Oscillatoria spongeliae* may represent up to 50% of the tissue volume of *D. herbacea* and is thought to be responsible for the production of BDEs in the sponge (Unson et al., 1994; Faulkner et al., 2000). *D. herbacea* and *D. granulosa* have genetically distinct populations of *O. spongeliae* (Thacker and Starnes, 2003), which are restricted to the ectosome of *D. granulosa* (Becerro and Paul, 2004). Cyanobacteria might also be responsible for the production of the BDEs in *D. granulosa* (although see Elyakov et al., 1991; Voinov et al., 1991). Whether they are behind the preference of *S. nigropunctatum* for the ectosome as described for the opisthobranch *Tylodina perversa* (Becerro et al., 2003) is unknown. The possibility that other chemical or structural differences between the ectosome and choanosome of the sponge or other microorganisms associated with the sponge may be responsible for the selection by the gastropterid cannot be completely ruled out. The use of standard methods in chemical ecology along with appropriate experimentation may provide new light on the feeding biology of opisthobranch molluscs and help us understand the role that color patterns and chemical defenses play in their biology, ecology, and evolution.

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Predator-Labeling Effect on Byssus Production in Marine Mussels *Perna viridis* (L.) and *Brachidontes variabilis* (Krauss)

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Abstract Mussels *Perna viridis* and *Brachidontes variabilis* were exposed to chemical cues from the predatory crab *Thalamita danae* maintained on different diets, and byssal thread production of the mussels was studied. *P. viridis* produced the highest number as well as the thickest and longest byssal threads when they were exposed to crabs maintained on a diet of *P. viridis* as compared with those exposed to crabs maintained on a diet of the top shell *Monodonta labio*, the rock oyster *Saccostrea cucullata*, or crabs that were starved. For *B. variabilis*, results were similar, in that a diet containing *B. variabilis* elicited the greatest response as compared with other treatments. This indicates that the mussels were able to discriminate chemical cues released from predators maintained on different diets, and respond accordingly to the level of predation risk. By increasing the strength of byssal attachment as a defensive trait, the chance of being dislodged and consumed by crabs is reduced. As energy cost involved in the induction of an antipredatory response is considerable, this defensive trait seems to be an advantage to the mussels in enhancing efficiency. The short response time in byssal thread production allows the mussels to increase resistance against predation by crabs at the time when predation pressure is the highest in a tidal cycle.

Keywords Mussels · Chemical cues · Byssus production · Predator-labeling effect · *Thalamita* · Crabs

Introduction

Under predation risks, prey may be induced to develop physiological, morphological, or behavioral antipredatory responses so as to increase resistance to predation

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(Vermeij, 1987). Such responses are widely documented in various kinds of aquatic invertebrates, including protozoans (Wicklow, 1988), cnidarians (Grosberg, 1988), flatworms (Wisenden and Millard, 2001), gastropods (Appleton and Palmer, 1988; Trussell, 1996), bivalves (Leonard et al., 1999; Cheung et al., 2004a,b), sea urchins (Hagen et al., 2002), and ascidians (Pisut and Pawlik, 2002). The induction of an antipredatory response, however, may incur a cost in energy reserve, which could otherwise be utilized for other functions such as foraging and reproduction (Sih, 1980). Therefore, a capability to identify reliable information cues associated with predation risk is crucial to the induction of antipredatory responses, which in turn reduces the risk of injury or mortality and optimizes energy utilization. These informational cues can be visual, tactile, or chemical.

Chemical cues involved in the assessment of predation risks by aquatic animals have received increasing attention in recent years. These cues may originate and be released from predators *per se* (see review by Kats and Dill, 1998) or from prey animals when they detect or are attacked by predators (see review by Chivers and Smith, 1998). It is maladaptive, however, for the predator to release cues that could be identified by the prey, as this would cause the prey to elicit antipredatory responses (Hagen et al., 2002). An increasing number of findings from recent research have indicated that antipredatory responses are elicited by cues released from consumed conspecific or heterospecific prey through the predator (predator-labeling effect), but not from the predator *per se*. The predator may thus be undetectable by the prey when the diet of the predator does not include conspecific or heterospecific prey. Such predator-labeling effects occur in a number of aquatic animals including sea anemones (Howe and Harris, 1978), gastropods (Jacobsen and Stabell, 2004), sea urchins (Hagen et al., 2002), insects (Chivers et al., 1996), amphibians (Wilson and Lefcort, 1993), and fish (Mathis and Smith, 1993).

Antipredatory responses have been reported for mussels exposed to chemical cues of predators and damaged conspecifics. These responses include increase in shell thickness in the presence of the scent of crabs and whelks (Leonard et al., 1999; Smith and Jennings, 2000; Cheung et al., 2004a), size of the adductor muscle in the presence of the scent of starfish (Reimer and Harms, 2001), and the number of byssal threads and the strength of byssal attachment in the presence of the scent of crabs and starfish (Côtè, 1995; Leonard et al., 1999; Reimer and Harms, 2001; Cheung et al., 2004b).

Mussels form the major diet of crabs (Seed, 1993). To consume mussels, crabs have to cut the byssal threads before dislodging the mussels from their surroundings and cracking them open (Elner, 1978). An increase in the strength of byssal attachment, therefore, may serve as an effective defensive tactic against crab predation. In a laboratory experiment, Côtè (1995) showed that *Mytilus edulis* produced more and thicker byssal threads upon exposure to the scent of crabs. Leonard et al. (1999) transplanted the same species from a site with low crab predation pressure to another where crab predation was dominant and found that mussels produced 72% more byssal threads and the attachment strength was increased by 87%.

Mussels *Perna viridis* and *Brachindontes variabilis* are widely distributed throughout the Indo-Pacific, typically occurring on protected shores (Lee and Morton, 1985). Although both mussels are commonly found in sheltered harbors in Hong Kong, their distribution is different. *P. viridis* ranges from the lower intertidal zone downwards to depths of around 10 m, whereas *B. variabilis* occurs principally

in the midhigh zone, often nestling in crevices or among the encrusting shells of the rock oyster *Saccostrea cucullata*. Both mussel species form the major diet of the swimming crab *Thalamita danae*, which moves into the intertidal zone with each flood tide to feed rapaciously on small mussels (<15 mm shell length). In an analysis of the stomach contents of *T. danae*, more than 14 prey species were found, with *P. viridis* and *B. variabilis* being the most common prey (53.9% and 42.3% of the stomachs contained *P. viridis* and *B. variabilis*, respectively). Other prey items included the rock oyster *S. cucullata* and top shell *Monodonta labio*, which were present in 15.4% and 11.5% of the stomachs, respectively (Seed, 1990).

Our previous study showed that *P. viridis* is responsive to chemical cues of damaged conspecifics and predators, including the swimming crab *T. danae* and muricid gastropod *Thais clavigera*. *P. viridis* produced more byssal threads, which were also longer and thicker compared to the control mussels without being exposed to these cues (Cheung et al., 2004b). The design of that experiment, however, could not differentiate whether cues from the predators *per se* or cues from consumed conspecific and heterospecific prey through the predator; i.e., a predator-labeling effect induced the byssus production. To address this question, the present study thus used cues generated from the predator *T. danae* feeding on a diet of mussels or other prey items, and compared their effects on byssus production in *P. viridis* and *B. variabilis* in an attempt to test the presence of a predator-labeling effect in mussels. This is the first time that an antipredatory response has been reported for *B. variabilis*.

Methods and Materials

Perna viridis (shell length: 10–15 mm) were collected from a pier at Sham Pui Chau, and *B. variabilis* (shell length: 10–15 mm) were collected from a boulder shore at Ting Kok. Both sites are located in the inner Tolo Harbour, Hong Kong (22°20'N; 114°10'E), and are 5 km apart; both sites experience similar hydrography. Although these two mussels occurred at both sites, their numbers were different, with a high density of *P. viridis* found at Sham Pui Chau, and *B. variabilis* at Ting Kok. Upon return to the laboratory, the mussels were cleaned of epibionts and detached carefully from clumps by cutting the byssal threads with scissors (avoiding damage to the pedal apparatus, which could impair byssal thread secretion). Each species was maintained in separate fiber glass tanks (500 l) equipped with a filtering system and air supply. Mussels were fed with the green alga *Dunaliella tertiolecta* once every 2 d. Seawater at a salinity of 32‰ was maintained by a thermostatic heater at 28°C, which is the average summer seawater temperature in Hong Kong (Environmental Protection Department, 2004). Mussels were allowed to acclimate to laboratory conditions for 1 wk prior to experimentation. Intermolt male crabs *T. danae* (carapace width: 60–70 mm) used to induce byssal thread production in mussels were collected from the field and acclimated for 1 wk in a 500-l fiber glass tank under the same laboratory conditions as the mussels. Crabs were fed with shrimp meat once every 2 d. Only males were used to avoid potential bias that might be introduced through sexual differences in predatory morphology. Other than the two mussel species, prey used to feed the crabs in the experiment also included the rock oyster *S. cucullata* and top shell *M. labio* collected from Sham Pui Chau. All prey types, including mussels, were frozen after transportation to the laboratory.

Experimental Set-up

Four treatments were prepared for each mussel species, and four aquaria were assigned for each treatment as replicates. An individual of *T. danae* was put into each aquarium as a stimulus source, and each aquarium also contained eight experimental mussels (shell length: 10–15 mm). Each experimental mussel was kept in a separate perforated plastic vial (4 cm diam, 6 cm high) and identified by a numbered plastic tag on the shell. Shell length of each individual was measured by vernier calipers to the nearest 0.1 mm. To prevent physical contact between the mussels and crab, but allowing scent from the crab to enter the vials, the crab in the aquarium was separated from the vials by a plastic mesh.

For the study of byssal thread production in *P. viridis*, *T. danae* in the first treatment (Treatment PV) were fed on *P. viridis*, whereas those in the second and third treatments were fed on the rock oyster *S. cucullata* (Treatment SC) and top shell *M. labio* (Treatment ML), respectively. *T. danae* in the fourth treatment were starved and served as controls (Treatment C). Treatment groups in *B. variabilis* were similar except that *P. viridis* offered as food to crabs in the treatment PV was replaced by *B. variabilis* and labeled as Treatment BV. All prey items were defrosted before being fed to crabs.

Except for the controls, crabs in the other three treatments were offered either one of the three prey types once every 2 d over 1 wk before the start of the experiment. The amount of prey offered was standardized to 10 g wet weight per meal. To avoid chemical cues released from the prey that might affect the results, crabs were taken out and fed in another aquarium and were put back into the experimental aquaria after feeding. The same procedure was applied to the starved crabs (Treatment C), so as to standardize the effect of manipulation. No food was offered to the crabs 24 hr before commencement of the experiment nor throughout the 48-hr experimental period.

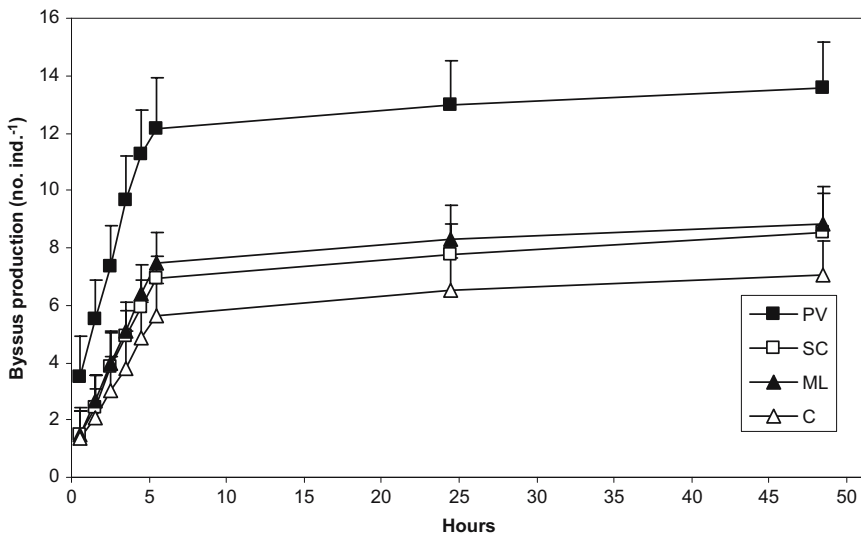


Fig. 1 Cumulative production of byssal threads (\pm SD) of *Perna viridis* as a function of time. PV: *Perna viridis* diet; SC: *Saccostrea cucullata* diet; ML: *Monodonta labio* diet; C: control

Byssus Production

The method developed by Côté (1995) was adopted with modifications. The number of byssal threads produced by each mussel was counted every 30 min for 6 hr. Further counts were taken 24 and 48 hr after the onset of the experiment. During the experiment, some mussels shed their byssal threads to release themselves and move to other attachment sites. The number of shed byssal threads was noted but not included in the byssal counts, as only the number of functional byssal threads was relevant to this study.

At the end of the experiment, all experimental mussels were detached from the vials by cutting the threads as near as possible to the point of insertion between the valves. Lengths and diameters of 5 threads were measured for each of 4 mussels chosen randomly from each aquarium. As there were 4 aquaria assigned to each

Table 1 Comparisons of total number of byssal threads produced after 48 hr and the length and diameter of individual byssal threads in *Perna viridis* as tested by nested ANOVA followed by multiple comparison procedure

	Mean \pm SD ^a	Nested ANOVA				Multiple comparisons*
		Replicate effect		Treatment effect		
		F	P	F	P	
<i>No. of byssal threads ind⁻¹</i>						
PV	13.59 \pm 1.56	1.03	0.43	137.79	<0.001	PV ML SC C
SC	8.53 \pm 1.34					
ML	8.81 \pm 1.33					
C	7.03 \pm 1.23					
<i>Mean length of byssal threads (mm)</i>						
PV	10.93 \pm 1.19	0.65	0.80	143.25	<0.001	PV ML SC C
SC	7.88 \pm 1.28					
ML	8.04 \pm 1.27					
C	7.45 \pm 0.94					
<i>Mean diameter of byssal threads (mm)</i>						
PV	0.171 \pm 0.051	1.19	0.29	22.43	<0.001	PV SC ML C
SC	0.138 \pm 0.049					
ML	0.131 \pm 0.047					
C	0.114 \pm 0.035					
<i>Cumulative byssal thread length (mm ind⁻¹)</i>						
PV	148.62 \pm 18.14	2.06	<0.05	404.33	<0.001	PV ML SC C
SC	65.28 \pm 11.14					
ML	70.89 \pm 10.83					
C	52.35 \pm 9.17					
<i>Cumulative byssal thread volume (mm³ ind⁻¹)</i>						
PV	345.23 \pm 71.10	23.50	<0.001	1108.65	<0.001	PV SC ML C
SC	101.34 \pm 27.85					
ML	96.82 \pm 21.86					
C	53.50 \pm 11.25					

PV, *Perna viridis* diet; SC, *Saccostrea cucullata* diet; ML, *Monodonta labio* diet; C, control.

^a Mean and SD are calculated using pooled data from all the replicates of the four aquaria.

*Treatment groups not underlined by the same lines are significantly different ($P < 0.05$).

treatment, 80 byssal threads were measured for each treatment. Byssal thread length was measured by using vernier calipers to the nearest 0.1 mm, and diameter was measured under a dissecting microscope equipped with an ocular micrometer at 1–1.5 mm from the adhesive disk. The volume of the byssal threads was computed from the length and diameter of the threads, assuming that the byssal thread was cylindrical. The above procedure was used for both studied species.

Statistical Analyses

Data obtained for mussels in an aquarium were pooled, and individual aquaria of the same treatment were considered as replicates (i.e., $N = 4$). Treatment and replicate effects on byssus production, and the length and diameter of individual byssal threads were analyzed with nested ANOVA followed by the Tukey multiple comparison procedure, with individual aquaria assigned as a random factor nested within the four treatments. Normality of the data was checked by using the Kolmogorov–Smirnov test, and homogeneity of variances by Bartlett's test. All statistical tests were performed by using the statistical software Minitab Release 14.

Results

Inducible Byssal Thread Production in *P. viridis*

All mussels steadily secreted byssal threads in the perforated plastic vials within 48 hr (Fig. 1). The mean number of threads produced at the end of the experiment varied from 7.03 (C) to 13.59 ind⁻¹ (PV) (Table 1). PV produced the highest

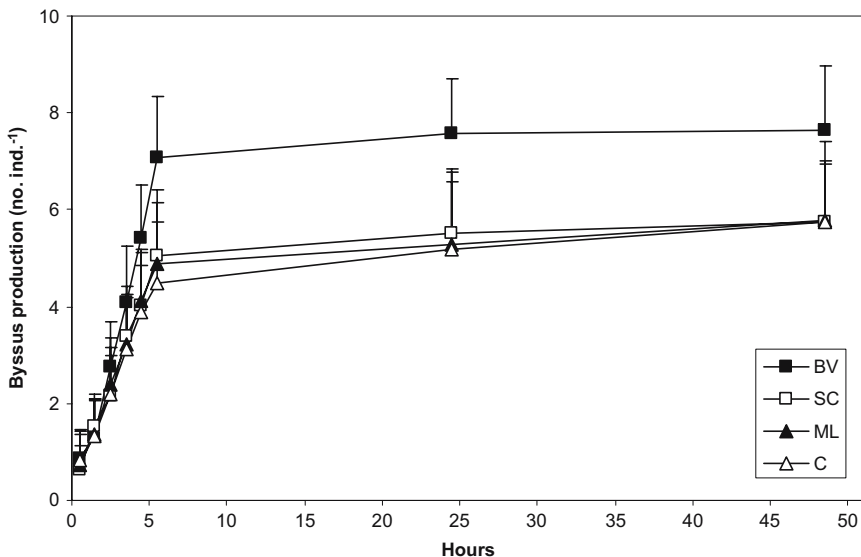


Fig. 2 Cumulative production of byssal threads (\pm SD) of *B. variabilis* as a function of time. BV: *Brachidontes variabilis* diet; SC: *Saccostrea cucullata* diet; ML: *Monodonta labio* diet; C: control

number of threads and was different from the other three treatments ($P < 0.001$), whereas ML and SC also produced more byssal threads than the control (C).

The mean length of threads ranged from 7.45 mm (C) to 10.93 mm (PV), in which PV produced significantly longer threads ($P < 0.001$) than the other three treatments (Table 1). Although the mean byssal length of ML was higher than that of C ($P < 0.05$), no significant difference was found between SC and C.

The mean diameter of threads varied from 0.114 mm (C) to 0.171 mm (PV), with PV producing thicker threads ($P < 0.001$) than the other three treatments (Table 1). The mean diameter of threads of SC was higher than that of C ($P < 0.05$); however, no significant difference was found between ML and C.

Cumulative byssal thread length (no. of byssal threads \times mean length of a thread) and cumulative thread volume (no. of byssal threads \times mean volume of a single thread) were highest for PV and significantly different from the other three

Table 2 Comparisons of total number of byssal threads produced after 48 hr and the length and diameter of individual byssal threads in *Brachidontes variabilis* as tested by nested ANOVA followed by multiple comparison procedure

	Mean \pm SD ^a	Nested ANOVA				Multiple comparisons*
		Replicate effect		Treatment effect		
		F	P	F	P	
<i>No. of byssal threads ind⁻¹</i>						
BV	7.63 \pm 1.34	1.13	0.34	15.17	<0.001	BV ML C SC
SC	5.69 \pm 1.23					
ML	5.78 \pm 1.24					
C	5.75 \pm 1.67					
<i>Mean length of byssal threads (mm)</i>						
BV	8.47 \pm 1.46	2.26	<0.05	87.41	<0.001	BV C SC ML
SC	5.34 \pm 1.32					
ML	5.28 \pm 1.52					
C	5.64 \pm 1.69					
<i>Mean diameter of byssal threads (mm)</i>						
BV	0.114 \pm 0.035	0.94	0.51	4.34	<0.05	BV C SC ML
SC	0.104 \pm 0.019					
ML	0.101 \pm 0.011					
C	0.105 \pm 0.022					
<i>Cumulative byssal thread length (mm ind⁻¹)</i>						
BV	64.53 \pm 11.47	1.26	0.25	115.18	<0.001	BV C SC ML
SC	30.41 \pm 7.83					
ML	30.28 \pm 6.15					
C	32.46 \pm 9.48					
<i>Cumulative byssal thread volume (mm³ind⁻¹)</i>						
BV	65.99 \pm 14.81	0.88	0.57	23.38	<0.001	BV C SC ML
SC	25.67 \pm 6.42					
ML	24.41 \pm 5.25					
C	27.99 \pm 7.97					

BV, *Brachidontes variabilis* diet; SC, *Saccostrea cucullata* diet; ML, *Monodonta labio* diet; C, control.

^aMean and SD are calculated using pooled data from all the replicates of the four aquaria.

*Treatment groups not underlined by the same lines are significantly different ($P < 0.05$).

treatments ($P < 0.001$). The values for SC and ML were also significantly higher than those for C ($P < 0.05$).

Inducible Byssal Thread Production in *B. variabilis*

Byssal thread production increased with time for all treatment groups within 48 hr (Fig. 2). The rate of thread production among different treatments was similar in the first 2 hr, but the difference between BV and other treatment groups increased with time. The mean number of threads produced at the end of the experiment varied from 5.69 to 7.63 ind⁻¹, with more threads being produced by BV than the other treatments ($P < 0.001$) as tested by nested ANOVA (Table 2).

The mean length of threads ranged from 5.28 mm (ML) to 8.47 mm (BV), and the mean diameter of threads varied from 0.101 mm (ML) to 0.114 mm (BV) (Table 2), in which BV produced longer ($P < 0.001$) and thicker ($P < 0.05$) threads than the other treatments.

Cumulative byssal thread length (no. of threads \times mean length of a thread) and cumulative thread volume (no. of threads \times mean volume of a single byssus) were also highest for BV and significantly different from the other treatments ($P < 0.001$).

Discussion

Perna viridis exposed to chemical cues from crabs maintained on a *P. viridis* diet produced more byssal threads than those exposed to chemical cues from crabs maintained on other prey diets or from starved crabs. The threads produced by the former group were also the longest, thickest, and of the highest volume among all treatments. Chemical cues produced by starved crabs elicited weaker response in *P. viridis* than those produced by crabs maintained on the rock oyster or top shell diet. Similar responses were observed in *B. variabilis* with greatest response being elicited by crabs maintained on a *B. variabilis* diet. However, no significant difference was observed in thread production between *B. variabilis* exposed to chemical cues from crabs maintained on the rock oyster or top shell diet, and those exposed to starved crabs. Since control crabs were not feeding during the experiment, differences in the degree of the response among treatments indicated that the crabs were labeled by their diets and could be differentiated by the mussels. To our knowledge, this is the first time the presence of a diet-related, predator-labeling effect on byssal thread production in mussels has been reported. As crabs are able to cut byssal threads by using their claws and dislodge individuals from a mussel bed, mussels with firmer attachment are less likely to be removed, leading to less predation by crabs, and hence, reduction in mortality (Lin, 1991; Hughes and Seed, 1995). As tensile stress increases with byssal thread length (Price, 1981), longer threads also help increase the resistance of the mussels being dislodged from the substratum. Predator-labeling effects on antipredatory responses have been reported in a number of aquatic animals (Howe and Harris, 1978; Mathis and Smith, 1993; Wilson and Lefcort, 1993; Chivers et al., 1996; Hagen et al., 2002; Jacobsen and Stabell, 2004). For example, avoidance response in the gastropod *Tegula funebralis* was more pronounced when they were exposed to crabs previously maintained on a *Tegula* diet than in crabs maintained on other diets (Jacobsen and Stabell, 2004). A stronger behavioral response was also elicited in the green sea urchin *Strongylocentrotus droebachiensis*

when animals were exposed to a sea urchin diet with seawater preconditioned by the predator (the Atlantic wolffish *Anarhichas lupus*) as compared to urchins maintained on a mussel diet or without food (Hagen et al., 2002). As suggested by Jacobsen and Stabell (2004), the chemical labels released by crustaceans may be emitted through excretion of urine, which serves the function of compensating short-term internal volume changes due to ingestion (Mantel and Farmer, 1983; Cheng and Chang, 1991). Elevated urine production has been observed in crabs exposed to prey extract, indicating that urine production is also associated with triggering of feeding responses (Jacobsen and Stabell, 2004).

Energy cost involved in the induction of antipredatory responses can be considerable (Covich et al., 1994). Rawlings (1994) found that the rocky shore gastropod *Nucella emarginata* showed dramatic reduction in reproductive activity due to heightened metabolic rate induced by exposure of snails to increased predation risks. Byssal threads constitute a significant component of total production by mussels, accounting for 3–10% of annual production in *P. viridis* (Shafee, 1979; Cheung, 1991) and up to 15% of total body energy in the ribbed mussel *Aulacomya ater* (Griffiths and King, 1979). After exposure to the scent of predators and damaged conspecifics for 48 hr, *P. viridis* has been shown to produce between 1.3 and 7.6 times more byssal threads as compared with controls (Cheung et al., 2004b). With the ability to discriminate between levels of predation risk via chemical labels, prey can respond according to the level of the risk and, hence, maximize the efficiency of energy utilization.

Crabs maintained on either one of the diets elicited a greater response in *P. viridis* than starved crabs. There are two possible explanations. The first is the addition effect of different sources of chemical cues. There were two sources of cues released from crabs maintained on a diet, one from the prey consumed and another from the crabs *per se* without considering that the prey cues may be chemically altered during passage through the predator (Jacobsen and Stabell, 2004). In contrast, only chemical cues were released from starved crabs. Second, such differences might be attributed to *P. viridis* detecting an elevated risk associated with the consumption of heterospecifics by the crabs. The design of the experiment, however, did not allow us to judge which explanation is more feasible because all prey types used form part of the diet of crabs in the field, although the preference varies with species. Nevertheless, the results for *B. variabilis* indicate that the addition effect of chemical cues may not be significant, at least in this species, because the same degree of response in byssal thread production was obtained for the starved crab group as for treatments with the top shell or rock oyster diet. An analysis of the stomach contents of a natural population of *T. danae* showed that 53.9% and 42.3% of the stomachs contained *P. viridis* and *B. variabilis*, respectively. In contrast, only 15.4% and 11.5% contained rock oysters and top shells, respectively (Seed, 1990). The predation risk associated with chemical cues from these less-preferred prey, therefore, is much lower compared to chemical cues from conspecifics.

The present study demonstrates that byssal thread production as an antipredatory response is most reactive in the first 5 hr of exposure to predator cues, with the response slowing after this period. This is consistent with other studies on mussels including *M. edulis* (Côté, 1995) and *P. viridis* (Cheung et al., 2004b). Such a time-dependent response, however, can not be correlated with the release rate of chemical cues associated with diet, which may decrease with time as digestion of

prey is completed. The same observation was found for mussels receiving chemical cues from starved crabs. Additionally, the mussels from all treatments were maintained in a closed system in which the crabs and seawater were not renewed during the experiment. Chemical cues from crabs are thus expected to accumulate throughout the experiment instead of deteriorating with time, unless degradation is faster than accumulation of cues.

Juveniles (<15 mm) of *P. viridis* and *B. variabilis* are the prey most preferred by *T. danae* on Hong Kong sheltered rocky shores. These crabs live in subtidal waters and move with each flood tide into the intertidal zone to feed (Seed, 1990). More and thicker byssal threads increase the time and difficulty for crabs to dislodge the mussels from the surrounding bed before they can be cracked and opened (Elner, 1978). As feeding time of crabs is constrained by duration of the flood tide, which only lasts for several hours in locations with semidiurnal tidal conditions such as Hong Kong (Morton and Morton, 1983), a short response time to chemical cues from predators is critical to the survival of the mussels. It allows an increase in the resistance to predation at a time when predation risk is the highest. This is adaptive for survival in terms of energy conservation because, when predation risk becomes less threatening when the tide recedes, species can divert less energy to byssus production so that more energy is conserved for other life processes such as reproduction.

In summary, mussels were able to discriminate chemical cues released from predators maintained on different diets and respond accordingly to the level of predation risk. Those maintained on a diet of conspecific mussels produced the largest number of byssal threads that were also the longest and thickest. The ability to discriminate between levels of predation risk by diet-dependent chemical cues from predators allows mussels to respond according to the level of the risk and, hence, to maximize efficiency of energy utilization. The short response time in byssal thread production enhances the mussel's ability to react appropriately against predation when the intensity changes with the tides.

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Consumption and Metabolism of 1,2-Dimethoxy-4-(3-Fluoro-2-Propenyl)Benzene, a Fluorine Analog of Methyl Eugenol, in the Oriental Fruit Fly *Bactrocera dorsalis* (Hendel)

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Abstract Methyl eugenol (ME) is a natural phenylpropanoid highly attractive to oriental fruit fly *Bactrocera dorsalis* (Hendel) males. The flies eagerly feed on ME and produce hydroxylated metabolites with both pheromonal and allomonal functions. Side-chain metabolic activation of ME has long been recognized as a primary reason for hepatocarcinogenicity of this compound on rodents. In an attempt to develop a safer alternative to ME for fruit fly management, we developed a fluorine analog 1,2-dimethoxy-4-(3-fluoro-2-propenyl)benzene (I), which, in earlier field tests, was as active to the oriental fruit fly as ME. Now we report that *B. dorsalis* males are not only attracted to, but also eagerly consume (up to ~1 mg/insect) compound I, thus recognizing this fluorinated benzene as a close kin of the natural ME. The flies metabolized the fluorine analog I in a similar fashion producing mostly two hydroxylated products, 2-(3-fluoro-2-propenyl)-4,5-dimethoxyphenol (II) and (*E*)-coniferyl alcohol (III), which they stored in rectal glands. However, the introduction of the fluorine atom at the terminal carbon atom of the double bond favors the ring hydroxylation over a side-chain metabolic oxidation pathway, by which coniferyl alcohol is produced. It also appears that fluorination overall impedes the metabolism: at high feed rate (10 μ l per 10 males), the flies consumed in total more fluorine analog I than ME but were unable to metabolize it as efficiently as ME.

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Introduction

The oriental fruit fly *Bactrocera dorsalis* (Hendel) is a wide-spread pest of a broad range of tropical, subtropical, and temperate host plants (US Department of Agriculture, 1983; White and Elson-Harris, 1992). Males of *B. dorsalis* are strongly attracted to and compulsively fed on methyl eugenol (ME) (Steiner, 1952), a commonly occurring plant phenylpropanoid (Metcalf, 1990). ME is also a common component of spices and is being used as food-flavoring agent at low concentrations (Hall and Oser, 1965). The use of ME in traps and killing stations assures successful detection, control, and eradication of 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 an ecological significance in the fly's communication system. It has been demonstrated that mature males after feeding on ME produced metabolites, mostly 2-(2-propenyl)-4,5-dimethoxyphenol (IV) and (*E*)-coniferyl alcohol (III), that display pheromonal and allomonal properties (Nishida et al., 1988a,b; Tan and Nishida, 1996). In addition, ME-acquired males have an earlier onset of courtship and are sexually more competitive than ME-deprived males (Tan and Nishida, 1996, 1998; Shelly, 2000).

Posing a potential problem to the continued use of ME in area-wide pest management programs are reports indicating that it causes hepatic tumors in mice (Miller et al., 1983), induces intrachromosomal recombination in a yeast assay (Schiestl et al., 1989), and elicits an abnormal response in a bacterial DNA repair test (Sekizawa and Shibamoto, 1982). The National Toxicology Program (NTP), US Department of Health and Human Services, evaluated ME and found that given orally to rats, it induced liver and stomach tumors in both sexes and kidney, mammary gland, and skin tumors in males (NTP, 1998). Recently, NTP listed ME as reasonably anticipated to become a human carcinogen based on sufficient evidence of its carcinogenicity in experimental animals (NTP, 2002). In 2001, the Expert Panel of the Flavor and Extract Manufacturers' Association performed a comprehensive review of all data relevant to safety evaluation of ME and concluded that daily intake of threshold doses that were carcinogenic in rodents was much higher than anticipated in human dietary intake. Hence, its potential to induce hepatotoxicity and carcinogenicity through dietary intake was expected to be negligible (Smith et al., 2002).

Since early warnings about the carcinogenicity of ME, a number of compounds have been evaluated as attractants for *B. dorsalis* (Mitchell et al., 1985; Khrimian et al., 1993, 1994; Liquido et al., 1998). In 1994, we discovered a fluorine analog, (*E*)-1,2-dimethoxy-4-(3-fluoro-2-propenyl)benzene (*I-E*), which in short field tests was as attractive as and more persistent than ME and, remarkably, was about twice as active as the *Z* isomer (Khrimian et al., 1994; Liquido et al., 1998). Metabolic activation of ME and production of 1'-hydroxy metabolite through enzymatic side-chain hydroxylation has been demonstrated as primarily responsible for hepatotox-

icity and carcinogenicity of ME (Smith et al., 2002). Earlier, we speculated that introduction of fluorine into the allylic group of ME might enhance its metabolic stability (Khrimian et al., 1994). Whether or not that was the case, the fluorine analog I showed reduced toxicity and reduced recombinogenicity in the yeast deletion assay compared to ME (Brennan et al., 1996). From the time NTP updated the list of carcinogens (NTP, 2002), we started to more vigorously pursue studies on fluoroanalog I as an alternative to ME for detection and control of oriental fruit fly. Here, we report on feeding behavior and metabolism of 1,2-dimethoxy-4-(3-fluoro-2-propenyl)benzene in *B. dorsalis* as well as its new, economical synthesis.

Methods and Materials

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, HI, USA. 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 in a room at 25–26°C, 50–70% rh, and a 12:12-hr light/dark cycle.

Chemicals

All reagents and solvents were purchased from Aldrich Chemical Co. unless otherwise specified. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl. Methylene chloride was distilled from calcium hydride. Coniferyl alcohol was prepared from ferulic acid (Quideau and Ralph, 1992), 2-(2-propenyl)-4,5-dimethoxyphenol (IV) was synthesized from 3,4-dimethoxyphenol (Benbow and Katoch-Rouse, 2001), and 3,4-dimethoxyphenylacetaldehyde was made from veratraldehyde (Ban and Oishi, 1958). Specific caution is necessary because osmium tetroxide is highly toxic and benzene is a carcinogen.

1,2-Dimethoxy-4-(3,3-difluoro-2-propenyl)benzene (V)

A 1-l three-neck flask equipped with a mechanical stirrer, dropping funnel, thermometer, and N₂ inlet was flashed with N₂ and loaded with hexamethylphosphorous triamide ([(CH₃)₂N]₃P, 70 ml, 0.387 mol). A solution dibromodifluoromethane (18 ml, 0.197 mol) in dry THF (350 ml) was added dropwise at 0°C under N₂ atmosphere. A thick white paste was formed close to the end of addition. The mixture was warmed to room temperature (RT) and stirred for another 30 min. A solution of 3,4-dimethoxyphenylacetaldehyde (17.4 g, 0.097 mol) in dry THF (80 ml) was added slowly at 25°C, whereupon the temperature was maintained at 25–30°C by cooling in a water bath. The yellowish suspension was stirred 1 hr at RT (or until TLC showed the completion of reaction), poured into the ice water (~600 ml), and extracted with hexane/ether, 1:1 (4 × 200 ml). The organic layer was washed with water (2 × 100 ml), dried (Na₂SO₄), and evaporated. The remainder was distilled in vacuum to furnish 18.03 g (87%) V of 98% purity. ¹H NMR: 3.27 (br. d, J = 8.0 Hz,

CH₂), 3.85 and 3.87 (both s, CH₃O), 4.37 (dtd, $J_{\text{HF-trans}} = 25.0$, $J_{\text{HF-cis}} = 2.0$ Hz, CH₂CH=C), 6.65–6.85 (m, arom, 3H). GC-MS (EI): 214 (M⁺, 100%), 199 (12), 183 (31), 77 (19). Anal. found: C 61.54; H 5.61. C₁₁H₁₂F₂O₂ requires C 61.67 and H 5.66.

1,2-Dimethoxy-4-(3-fluoro-2-propenyl)benzene (I)

A solution of Red-Al[®] (48 ml, 70% w/w in toluene, $d = 1.036$, 3.4 M) was added slowly to a solution of V (17.00 g, 0.08 mol) in dry benzene (350 ml) at 0–5°C under N₂. Reaction mixture was warmed to 25°C and stirred for 20 hr or until GC indicated the completion of reduction. The mixture was poured into ice water and acidified with concentrated HCl. The organic layer was separated, and the aqueous was extracted with ether/hexane, 1:1 (2 × 100 ml). Combined organic extracts were washed with brine, dried, and distilled to give 14.50 g (92%) I (99% pure, *E/Z* 88:12); b.p. 55–56°C/0.05 mm Hg. ¹H NMR and MS spectra were identical to those described earlier (Khrimian et al., 1994).

2-(*tert*-Butyldimethylsilyloxy)-4,5-dimethoxyphenylacetaldehyde (VI)

Triethylamine (2.96 g, 29.25 mmol) followed by *tert*-butyldimethylsilyl triflate (6.19 g, 23.4 mmol) was added to a solution of IV (3.78 g, 19.50 mmol) in CH₂Cl₂ (40 ml) at 0°C. The mixture was allowed to warm to RT and was diluted after 2 hr with ether (150 ml) and washed with brine (3 × 30 ml). The organic layer was dried (Na₂SO₄), filtered, concentrated *in vacuo*, and purified by flash chromatography on SiO₂ (hexanes/ethyl acetate, 20/1 to 5/1) to yield a silyl ether of I (5.34 g, 89% yield, 99% pure). GC-MS (EI): 308 (M⁺, 93%), 251 (100), 223 (76), 220 (27), 177 (20), 73 (68). A solution of OsO₄ (508 mg, 2 mmol) in acetone (40 ml) was added to a solution of the silyl ether (3.08 g, 10 mmol) in ether (200 ml). The resulting dark-brown solution was stirred for 10 min prior to addition of water (200 ml). Finely powdered NaIO₄ (21.39 g, 0.1 mol) was added in five portions over a period of 5 hr. The mixture was stirred for additional 3 hr, diluted with ether (100 ml), and the layers were separated. The organic phase was washed with brine, dried (Na₂SO₄), and purified by flash chromatography on SiO₂ (hexane/ethyl acetate, 10:3) to yield VI as a brown oil (2.50 g, 81%) of 99% purity. GC-MS (EI): 310 (M⁺, 25%), 281 (21), 253 (100), 238 (37), 222 (74), 209 (16), 195 (30), 75 (49), 73 (75).

1-(*tert*-Butyldimethylsilyloxy)-4,5-dimethoxy-2-(3,3-difluoro-2-propenyl)benzene (VII)

CF₂Br₂ (1.52 ml, 16.65 mmol) was added to a solution of hexamethylphosphorous triamide (6.0 ml) in THF (30 ml) at 0°C. The mixture was stirred at RT for 1 hr, and a solution of aldehyde VI (2.53 g, 8.20 mmol) in THF (10 ml) was added. After stirring for 1.5 hr and workup described for the compound V, the crude mixture was chromatographed (hexane/ethyl acetate, 8:1) to furnish benzene VII (1.81 g, 64%) of 99% purity. ¹H NMR: 0.21 [s, 6H, Si(CH₃)₂], 1.00 (s, 9H), 3.21 (br. d, 2H, $J = 8.0$ Hz, CH₂), 3.80 and 3.82 (both s, CH₃O), 4.33 (dtd, $J_{\text{HF-trans}} = 25.0$, $J_{\text{HF-cis}} = 2.0$ Hz, CH₂CH=C), 6.38 (s, 1H, arom), 6.62 (s, 1H, arom). GC-MS (EI): 344 (M⁺, 51%), 243 (100), 77 (18), 73 (20). Anal. found: C 59.22; H 7.88. C₁₇H₂₂F₂O₃Si requires C 59.27 and H 7.61.

2-(3-Fluoro-2-propenyl)-4,5-dimethoxyphenol (II)

A solution of difluorobenzene **5** (1.758 g, 5.11 mmol) and Red-Al[®] (4.46 ml of 3.33 M in toluene, 14.85 mmol) in dry benzene (20 ml) was refluxed, and the reaction progress was monitored by GC. After about 48 hr, the mixture was taken into ice water, acidified with 20% HCl, and extracted with ether/hexane, 1:1. Combined organic extracts were washed with brine, dried, and concentrated. The remainder was treated at 0°C with a THF solution of tetrabutylammonium fluoride (3.0 ml, 1 M) and gradually warmed to RT to complete the removal of TBDMS protecting group. Water was added, and the products were extracted with ether, dried, and concentrated. Flash chromatography on SiO₂ with hexane/ethyl acetate, 1:1, afforded phenol II (0.960 g, 89%) with 87:13 ratio of *E* and *Z* isomers. M.p. 68–69°C. ¹H NMR: 3.18 (br. d, 2H, *J* = 7.2 Hz, CH₂-*E*), 3.37 (br. d, 2H, *J* = 7.2 Hz, CH₂-*Z*), 3.80 and 3.81 (both s, CH₃O), 4.70 (OH), 4.92 (dtd, *J*_{HF-trans} = 46.0, *J*_{HH-cis} = 4.2 Hz, CH₂CH-*Z*), 5.53 (ddt, *J*_{HF-cis} = 18.2, *J*_{HH-trans} = 11.0 Hz, CH₂CH-*E*), 6.41 and 6.61 (both s, arom, II-*E*), 6.43 and 6.64 (both s, arom, II-*Z*), 6.56 (br. dd, *J*_{HF} = 85.5, *J*_{HH-trans} = 11.0 Hz, CFH-*E*). GC-MS (EI) II-*E*: 212 (M⁺, 100%), 197 (21), 181 (9), 69 (27), 59 (19). *Z* isomer had almost identical mass spectrum. Anal. found: C 62.26; H 6.36. C₁₁H₁₃FO₃ requires C 62.26 and H 6.17.

Analytical Procedures

¹H NMR spectra were recorded at 300 MHz in CDCl₃ with TMS as an internal standard on a Bruker QE-300 spectrometer. Chemical shifts are reported in δ units. GC analyses were performed on a Shimadzu 17A gas chromatograph equipped with a flame ionization detector, an autosampler AOC-20s, and autoinjector AOC-20i. Hydrogen was used as carrier gas at 1.2 ml/min. RTX-1701 column (60 m × 0.25 mm, Restek Corporation) was used for routine analyses of synthesized and reference chemicals. HP-5 column (30 m × 0.25 mm × 0.25 μm film) was used for quantitative analyses of fly extracts in a splitless mode. 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 (70 eV) were obtained with an Agilent Technologies 5973 mass selective detector interfaced with 6890 N GC system equipped with a 30 m × 0.25 mm i.d. × 0.25 μm film HP-5MS column. Helium was used as the carrier gas at 1 ml/min. TLC analyses were conducted on Whatman AL SIL G/UV plates using hexane/ethyl acetate as a mobile phase. For visualization of spots, 20% ethanol solution of phosphomolybdic acid and/or UV light was used. Elemental analyses were conducted by Galbraith Laboratories (Knoxville, TN, USA).

Bioassay

In test no. 1, 50 laboratory-reared mature oriental fruit-fly males were evenly placed in five ~2-lb plastic containers (10/container) equipped with water inserts and screened covers. Flies in two containers were offered 10 μl ME (2 × 5 μl) on a slip of filter paper (25 × 5 mm) taped to a blank water pot. Twenty flies in two other cages were offered the fluorine analog I (10 μl/cage), and 10 males in the last cage were presented with a filter paper containing 10 μl water. The behavioral observations

(landing and feeding on the source) were recorded, and filter papers were removed after about 20 min. Water containers were replaced, and the flies were left overnight with a sugar cube on the cover. After 18 hr, the rectal glands of 10 flies each fed with ME and I as well as 10 control flies were dissected and placed in conical plastic vials with 20 μ l ethanol. The glands were crushed with a wire rod, sealed with parafilm, and centrifuged at \sim 4000 rpm for 10 min. Six hours later (or 24 hr into the test), the remaining 10 flies fed with ME and I were dissected. Three flies fed with fluorine analog I were dead at the time of second dissection. All 20 extracts were analyzed by GC-MS by splitless injection of 1- μ l solutions. The abdomens of 17 flies fed with I that were alive after 24 hr were separated from other body parts, placed in 20 μ l ethanol, crashed, and analyzed by TLC using hexane/ethyl acetate, 1:1. Then, the extracts were evaporated and flash-chromatographed on a short SiO₂ column (30 \times 5 mm) with hexane/ethyl acetate, 3:1. The fractions containing unmetabolized fluorine analog I were further combined, concentrated to ca. 1 ml, and quantified by GC using external standards of I. The remaining body parts (e.g., thorax, head, etc.) of 17 flies fed with I were combined, crashed, placed in ethanol (\sim 1 ml), and analyzed by TLC. Only contents of rectal glands of ME-fed flies were analyzed by GC-MS analogous to fluorine analog I-fed flies. A standard solution of coniferyl alcohol (20 ng/ μ l) was used to estimate the quantities of metabolites.

Test no. 2 was conducted analogously to test no. 1 with 10 flies presented with 10 μ l of ME, another 10 flies offered 10 μ l of fluorine analog I, and 10 flies offered water as control. The presentation time was 40 min. After 24 hr, rectal glands were dissected, placed in 100 μ l EtOH, treated as described above, and analyzed by GC with external standards of III, IV, and II. The other body parts for each fly, including abdomens, were individually placed in 20 μ l EtOH, analyzed by TLC, and then separately chromatographed on SiO₂ as described in test no. 1. The fractions containing starting ME and I were analyzed by GC using external standards.

In test no. 3, 10 flies were presented with 1.0 μ l (10 μ l of 10% v/v hexane solution) of ME and I, and 10 flies were offered water as a control. After 24 hr, rectal gland extracts were obtained in 220 μ l EtOH and analyzed by GC using standards of III, IV, and II. Abdomens were separated from other body parts, extracted with 200 μ l ether, evaporated to \sim 50 μ l, and analyzed by TLC. The abdomen extracts from FA-fed flies were further analyzed by GC. The other body parts were taken into 200 μ l ether, evaporated to about 50 μ l, and analyzed by TLC.

Data Analysis

Mean ratios were analyzed by using PROC GLM followed by a Tukey's 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, Cary, NC, USA).

Results

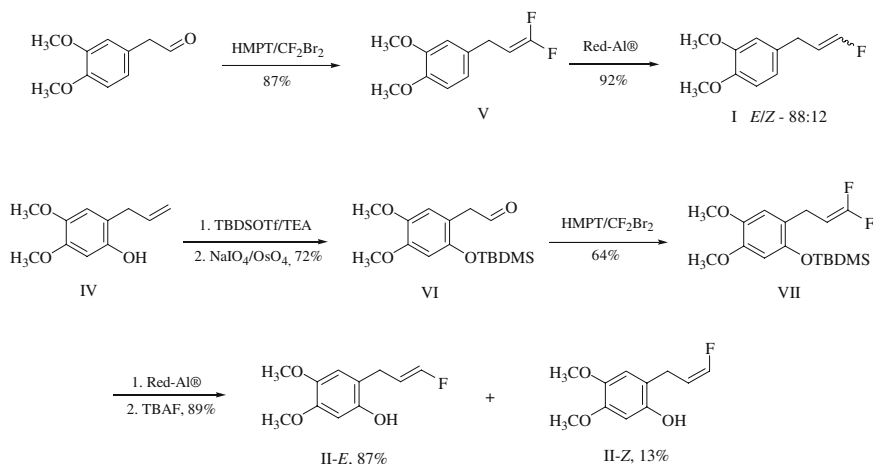
Synthesis

Earlier, we synthesized both *E* and *Z* isomers of 1,2-dimethoxy-4-(3-fluoro-2-propenyl)benzene (I) utilizing Wittig reactions of 3,4-dimethoxyphenylacetaldehyde

with stabilized ylides (Khrimian et al., 1994). Although the methods proved satisfactory for small syntheses, we envisioned difficulties in scaling up, especially in the case of *I-E* isomer, the synthesis of which required expensive reagents and multiple chromatographic purifications. Now we report on a more efficient two-step synthesis of **I** in a total 80% yield from 3,4-dimethoxyphenylacetaldehyde (Scheme 1). Our new synthesis utilized a straightforward Wittig difluoroolefination with a subsequent selective reduction of intermediate difluoroolefin (Hayashi et al., 1979). Notably, both intermediate and final products were purified via distillation that renders the method amenable for scale-up. We obtained about the same ratio of *I-E* and *I-Z*, 88:12, as in our previous approach and plan to continue field studies with this material, which is now being manufactured by a contractor. The same synthetic strategy was utilized to make the principal metabolite **II** that is formed by feeding *B. dorsalis* males with fluorobenzene **I**. Synthetic allylphenol **IV**, which happened to be a key metabolite of oxidative biotransformation of ME in *B. dorsalis*, served as a starting material in the second synthesis (Scheme 1). A TBDMS protection of phenol **IV** with subsequent oxidative cleavage of the double bond following Benbow and Katoch-Rouse, 2001 furnished aldehyde **VI** in a 72% isolated yield. Difluoroolefination of **VI** with HPMT/CBr₂F₂ followed by selective reduction of **VII** with Red-Al[®] gave the mixture of *II-E* and *II-Z* in 87:13 ratio. GC retention times of the geometric isomers and respective mass spectra matched those isolated from *B. dorsalis*.

Feeding Behavior and Metabolism

In test no. 1, we noticed that eight to nine *B. dorsalis* males touched the filter paper impregnated with 10 μ l of **I** and started feeding on it. In case of ME, the number of flies that contacted the source was five to six, and no flies landed on filter paper treated with water. After about 20 min, the flies left the source in both treatments, and feeding was discontinued. By the time of dissection of first 10 individuals (18 hr), all flies in both treatments and in control were healthy, but



Scheme 1 Syntheses of the fluorine analog **I** and the principal metabolite **II**

after another 6 hr, three flies fed with fluorine analog I died. GC-MS analysis of the rectal glands of all 20 *B. dorsalis* males fed with I (including dead flies) showed three main peaks, one of which was easily identified as (*E*)-coniferyl alcohol (III) matching with the standard by retention time (13.46 min) and mass spectrum. The other two (RT 12.70 and 12.97 min) had almost identical mass spectra with a strong ion at 212 *m/z* that could have been attributed to a molecular ion of *E* and *Z* fluoroolefinic phenols because of similarities with the mass spectrum of IV (Nishida

Table 1 Amounts of metabolites and unmetabolized methyl eugenol and fluorine analog I in individual oriental fruit-fly species from test no. 2^a

Methyl eugenol (ME)				
Insect no.	Metabolites ^b (μg)		IV/III (mol/mol)	Unmetabolized, ME ^c (μg)
	IV	III		
1	9.6	9.3	1.0	235.0
2	9.7	10.2	0.9	99.0
3	3.0	5.6	0.5	86.8
4	10.5	13.8	0.7	25.5
5	26.3	24.8	1.0	253
6	1.3	1.1	1.1	52.6
7	19.5	26.7	0.7	801.5
8	9.6	12.0	0.7	133.2
9	15.9	19.8	0.7	268.0
10	9.9	11.7	0.8	128.7
Total		250.3		2083
Mean ± SEM	11.5 ± 2.3	13.5 ± 2.5	0.8 ± 0.1 b ^d	208 ± 71
Fluorine analog I				
Insect no.	Metabolites ^b (μg)		II/III (mol/mol)	Unmetabolized, I ^c (μg)
	II (<i>E</i> + <i>Z</i>)	III		
1	5.5	1.3	3.6	482
2	5.3	0.6	7.6	1.4
3	17.6	2.1	7.1	1014
4	5.3	0.8	5.7	n/d
5	9.4	1.3	6.2	681
6	6.9	1.3	4.5	28.3
7	3.7	0.4	7.9	30.5
8	10.7	1.6	5.7	863
9	18.6	2.4	6.6	1082
10	4.1	0.6	5.8	587
Total		99.5		4769.2
Mean ± SEM	8.7 ± 1.7	1.2 ± 0.2	6.1 ± 0.4 a ^d	529 ± 142

^aTen flies were presented with 10 μl ME and analog I.

^bFound in rectal glands.

^cTotal in other body parts.

^dMeans followed by different letters are significantly different at the 0.05 level, PROC GLM, Tukey's test (SAS Institute, Version 8.2).

et al., 1988a,b). Having synthesized the targeted products, we identified these peaks as II-E and II-Z. In addition to three main peaks, there were some minor products in these rectal gland extracts but no starting I. However, unmetabolized I was found in fairly large quantities in the abdomen of the flies. In the total 17 males dissected, we found 2948 µg starting fluorinated benzene I (or in average 173 µg per fly) indicating an intense feeding. Finally, there were no phenylpropanoids found in other body parts of the flies fed with fluorine analog I. All 20 flies exposed to ME accumulated phenol IV and coniferyl alcohol III in their rectal glands as expected (Nishida et al., 1988a,b), in some cases in fairly large quantities (15–20 µg). There were no phenylpropanoids found in control flies.

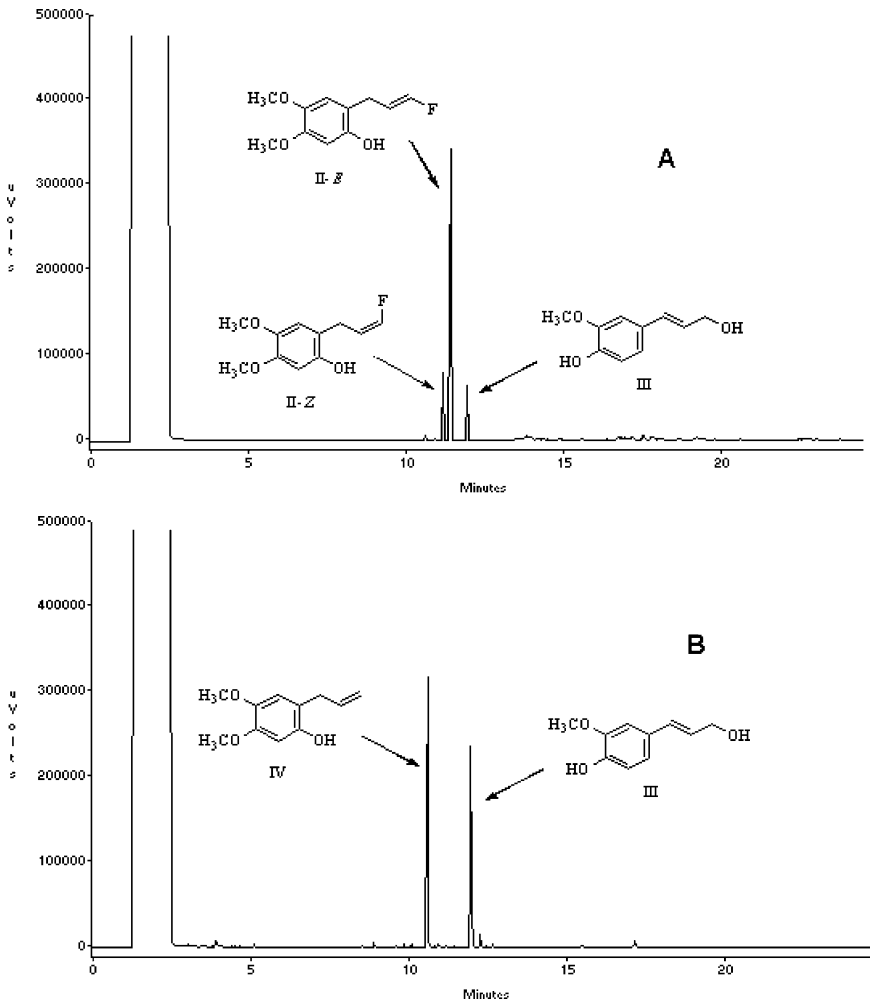


Fig. 1 Gas chromatograms (HP-5, 30 m × 0.25 mm × 0.25 µm, FID) of gland extracts of individual oriental fruit fly males fed with: (A) fluorine analog I (Table 1, species no. 3, II + III = 19.7 µg; II/III = 7.1, mol/mol) and (B) methyl eugenol (Table 1, species no. 1, IV + III = 18.9 µg; IV/III = 1, mol/mol)

In test no. 2, we repeated the experiment with 10 μl of each ME and benzene I and ran the feeding for 40 min. In both treatments, five to six flies initially landed on filter papers; then after 15–20 min, all flew away. In the last 15 min, some flies returned to feeding sources, but we had no knowledge whether all flies touched the filter papers. Dissection of rectal glands was conducted after 24 hr. Quantitative analyses of metabolites as well as unmetabolized ME and fluorine analog I in individual flies are presented in Table 1. With the exception of two species (nos. 2 and 4) that apparently did not touch the source, the rest of fluorine analog-fed *B. dorsalis* males contained large amounts of unmetabolized material in their abdomens, ranging from 28 μg up to ~ 1 mg. Remarkably, a total of 10 flies consumed the fluorinated benzene I (4769.2 ± 99.5 μg) in more than twice the amount of ME (2083.3 ± 250.3 μg), but produced about half the amount of metabolites (99.5 vs. 250.3). The other striking difference was the ratio of metabolites derived from ME and fluorinated benzene I. The average molar ratio of phenol IV and coniferyl alcohol III (Table 1) was 0.8 (± 0.1 SEM), whereas the average ratio II/III was 6.1 (± 0.4 SEM). Gas chromatograms of the rectal gland extracts of two males (one fed with ME and another with benzene I) that produced about the same total amounts of metabolites are presented in Figure 1.

In test no. 3 with 1.0 μl of ME and fluorine analog I, with the exception of one fly fed with analog I, there were no unmetabolized starting materials after 24 hr. The amounts and ratios of metabolites are presented in Table 2. Analogous to the previous test, a total of 10 flies consumed nearly twice as much the fluorine analog I as ME (9.10 vs. 4.58 μg). But in this low-dose consumption, both test compounds

Table 2 Amounts of metabolites in individual oriental fruit-fly males fed with methyl eugenol and fluorine analog I from test no. 3^a

Insect no.	Methyl eugenol (ME) metabolites ^b			Fluorine analog I metabolites ^b		
	IV (ng)	III (ng)	IV/III (mol/mol)	II (<i>E</i> + <i>Z</i>) (ng)	III (ng)	II/III (mol/mol)
1	126	20	5.8	326	36	7.7
2	213	55	3.6	780	60	11.0
3	125	37	3.1	600	39	13.0
4	371	313	1.1	419	39	9.1
5	194	33	5.5	1135 ^c	191	5.0
6	248	174	1.3	486	43	9.6
7	549	628	0.8	418	50	7.1
8	64	20	3.0	613	45	11.6
9	734	482	1.4	n/d	n/d	
10	117	77	1.4	1851	148	10.
Total		4580			9103	
Mean \pm SEM	274 \pm 68	183 \pm 69	2.7 \pm 0.6 b ^d	736 \pm 161	72 \pm 18	9.4 \pm 0.8 a ^d

^a Ten flies were offered 1.0 μl of ME and I.

^b Found in rectal glands. With the exception of one fly fed with I (line no. 5), no unmetabolized ME or analog I was found.

^c 1893 ng of I was found in the abdomen.

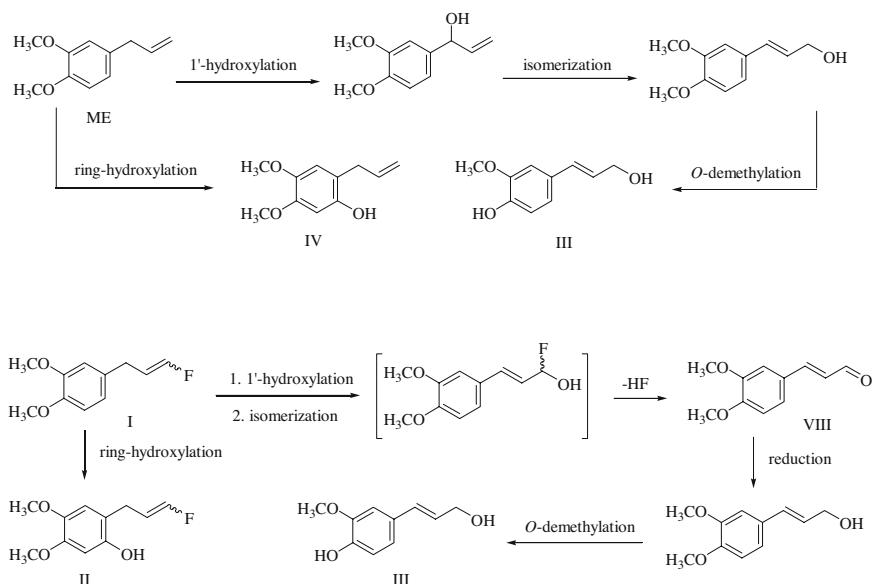
^d Means followed by different letters are significantly different at the 0.05 level, PROC GLM, Tukey's test (SAS Institute, Version 8.2).

were almost completely metabolized. Again, the mean ratio IV/III was significantly lower than the mean ratio II/III.

Discussion

Formation of coniferyl alcohol III as one of two main metabolites produced by *B. dorsalis* males after feeding on fluorine analog I indicated the occurrence of a side-chain enzymatic hydroxylation, analogously to a biotransformation of ME in the oriental fruit fly. Although mechanistic aspects of ME metabolism in *B. dorsalis* have not been articulated, there is ample information in literature on metabolism of ME in rodents (Solheim and Scheline, 1976; Smith et al., 2002) and even humans (Smith et al., 2002 and references therein). In rats, dimethoxyphenol IV was reportedly one of the major metabolites, but coniferyl alcohol III had not been found (Solheim and Scheline, 1976). In a recent review, Smith et al. (2002) outlined three major metabolic pathways of ME and related phenylpropanoids in mammals: *O*-demethylation to phenols, epoxidation of the double bond followed by hydration, and 1'-hydroxylation to form a benzylic alcohol, which often undergoes allylic rearrangement to a more stable primary alcohol. There was no mention of coniferyl alcohol even as a by-product. Surprisingly, ring hydroxylation leading to phenol IV had been overlooked. At any rate, it appears that coniferyl alcohol is a unique metabolite of ME in *B. dorsalis* and related complex sibling species (Wee et al., 2002), destined to perform a significant pheromonal and allomonal role in fruit-fly biology and ecology (Nishida et al., 1988a,b; Tan and Nishida, 1998, 1996; Wee and Tan, 2001). Mechanistically, the formation of III from ME in *B. dorsalis* could be rationalized as a combination of two major pathways: 1'-hydroxylation with further allylic isomerization and *O*-demethylation in whichever order (Scheme 2). In fact, one product in the 1'-hydroxylation pathway, *cis*-3,4-dimethoxycinnamyl alcohol, was isolated by Nishida et al. (1988b). From this standpoint, metabolism of fluorinated analog I in *B. dorsalis* would lead to a cinnamic aldehyde intermediate VIII (hypothetically through elimination of HF from a 1-fluorocinnamic alcohol) followed by a reduction and further demethylation to coniferyl alcohol III (Scheme 2). The proposed scheme explains the loss of fluorine during metabolic transformation of I to III. However, the aldehyde VIII or its *O*-demethylated analog is yet to be found in the *B. dorsalis* rectal gland extracts. An alternative route includes an enzymatic defluorination of I to form ME, which then follows 1'-hydroxylation–isomerization pathway described above leading to III. Further research will focus on finding evidence supporting either of these hypotheses.

Whether or not the death of three flies in test no. 1 after feeding on fluorine analog I could be associated with a partial metabolic loss of fluorine (in the form of HF or any other) is yet to be determined. In test no. 2 conducted with the same load of I, but even longer feeding, all flies seemed healthy. It seems also possible that feeding on large quantities of I and the inability to efficiently metabolize sicken *B. dorsalis* males, as the consumption of large amounts of even natural product ME reportedly caused the death in the oriental fruit fly (Steiner, 1952). More dose-response studies should be conducted to see whether the fitness of *B. dorsalis* males could be compromised by the intake of fluorine analog I.



Scheme 2 Suggested metabolic transformations of methyl eugenol and fluorine analog I in *B. dorsalis*

Clearly, *B. dorsalis* males perceive the analog I as strong as ME. In two experiments (test nos. 2 and 3), *B. dorsalis* males consumed even total larger quantities of I than the parent molecule. Although not all flies directly contacted the filter papers, they still consumed appreciable amounts of material through a vapor phase. Under our experimental setting, individual *B. dorsalis* males consumed up to 1 mg of I. The highest number for ME intake in *B. dorsalis* was reportedly 2.2 μl , and the average quantity was 700 μg (Wee et al., 2002). The fact that *B. dorsalis* males voraciously feed on fluorine analog I might have a practical implication because eradication programs, by and large, rely on reducing the populations of wild oriental fruit fly males by exposure of ME mixed with toxicants.

Two important observations seem interconnected. At high feed (test no. 2), 10 oriental fruit fly males metabolized after 24 hr only 2.0% of the fluorine analog I they consumed, whereas flies fed with ME metabolized 10.7% of their intake, indicating that incorporation of the fluorine atom overall impeded the metabolism. As a result, total amount of metabolites from ME was higher than from analog I. At low feed (test no. 3), the flies were still able to efficiently metabolize almost the entire intakes of the analog I (and not surprisingly, ME), which resulted in the reversal of metabolite quantities. However, in both test nos. 2 and 3 run at different doses, the fluorination unmistakably redirected the metabolism in favor of ring-hydroxylation pathway. Introduction of fluorine (as an isosteric replacement for hydrogen) in the biologically active substances including insect semiochemicals has long been pursued for altering volatility and lipophilicity, as probes for studying the insect communication mechanism, enhancing metabolic stability, and as enzyme inhibitors (for a recent review, see Pesenti and Viani, 2004). Whatever the mecha-

nism, the introduction of fluorine at the terminal atom of the double bond of ME appears to overall retard the oxidative biotransformation of this important plant phenylpropanoid in *B. dorsalis* and specifically influenced side-chain hydroxylation. It is intriguing whether the metabolism of fluorine analog I in mammals will follow the same path.

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Orientalional Behaviors and EAG Responses of Male Codling Moth After Exposure to Synthetic Sex Pheromone from Various Dispensers

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Abstract The effect of brief pheromone exposures on responses of codling moth (*Cydia pomonella* L.) males was tested by flight-tunnel and electroantennogram (EAG) studies. Males were preexposed to pheromone for up to 3 min as they sat in release cages or for shorter times (a few seconds to several min) upon initiating flights or orienting in plumes. Brief exposures to Isomate-C Plus dispensers nearly eliminated moth orientations to 0.1 mg codlemone [(*E,E*)-8,10-dodecadien-1-ol] and 0.1 mg three-component (codlemone/12OH/14OH, 100:20:5 ratio) lures 15 min later. However, there was no associated change in EAG responses between preexposed and control moths. Behavioral responses of Isomate-C Plus-exposed males were normal 24 hr following exposure. The reduced sexual responsiveness observed following exposure to Isomate dispensers appeared to be associated with an elevation of response threshold. Brief preexposure to 0.1 mg codlemone and three-component lures also reduced orientational behavior of males 15 min later, but to a lesser degree than when preexposed to Isomate-C Plus dispensers. Male behavior following preexposure to a 0.1 mg codlemone/pear ester [(*2E,4Z*)-2,4-decadienoate] lure (1:1 ratio) was no different from exposure to codlemone only. Orientational disruption in plots treated with 10 dispensers of Isomate-C Plus per tree was 88.3 and 95.9% for 1.0 and 0.1 mg codlemone lures, respectively. Some males did orient to 0.1 mg codlemone lures so we caution that flight-tunnel experiments on preexposure may overestimate the actual pheromone exposure dosage received by feral moths in treated orchards. Importantly, this work documents that a portion of feral males within a population has the capacity to overcome communicational disruption by high densities of Isomate-C Plus dispensers.

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Introduction

The codling moth, *Cydia pomonella* (L.), is a worldwide pest of pome fruit (Vickers and Rothschild, 1991). The identification of (*E,E*)-8,10-dodecadien-1-ol (codlemone) as the major pheromonal component (Roelofs et al., 1971) led to attempts to control it by mating disruption (Cardé et al., 1977; Mani et al., 1978; Moffit, 1978). Although a number of studies report successful mating disruption of codling moth and associated crop protection, failures have also been documented, and improvements are called for (Vickers and Rothschild, 1991; Minks, 1997).

A number of laboratory and field experiments have attempted to determine the dosage of pheromone required for effective codling moth mating disruption. A 65% reduction of mating was observed when females were caged with males in 1.2-l containers with 1.0 mg of codlemone (Fluri et al., 1974), whereas by exposing males and females to air moving over one or three 1.0 mg lures of codlemone, there was only a 38% mating reduction (Charmillot et al., 1976). More recently, Judd et al. (2005) established that exposures of male codling moths to ca. 35 µg codlemone/l of air in static air chambers for 10–30 min nearly eliminated response to otherwise attractive lures in a flight tunnel and reduced electroantennogram (EAG) responses. Normal behavioral responses returned after >4 hr and EAG sensitivity after >1 hr. However, caging male codling moths for 30–34 hr in an orchard treated with 1000 Isomate-C dispensers per hectare had no measurable impact on behavioral responses to pheromone in a flight tunnel (Judd et al., 2005). Similar results were obtained by Schmitz et al. (1997) and Rumbo and Vickers (1997) for the European grape moth, *Lobesia botrana* (Denis and Schiffermüller), and the Oriental fruit moth, *Grapholita molesta* (Busck), respectively. Judd et al. (2005) suggested that if male codling moths landed near Isomate-C Plus dispensers for several minutes, they might receive a sufficiently high dose of pheromone to reduce behavioral responses as seen in their preexposure laboratory experiment.

Thirteen minor compounds have also been identified from the sex pheromone gland of female codling moths (Witzgall et al., 2001); dodecanol (12OH) and tetradecanol (14OH) were quantitatively most significant, enhancing behavioral responses of males to codlemone (Einhorn et al., 1984; Arn et al., 1985). Consequently, these two compounds and codlemone are the active ingredients in Isomate-C and -C Plus polyethylene tube dispensers (Pacific Biocontrol Co., Vancouver, WA, USA), which are widely used for mating disruption of codling moth (Thomson et al., 1998).

Codling moth males have been captured in traps baited with Isomate-C dispensers (Barrett, 1995), and they land within a few centimeters of similar dispensers placed directly in untreated (Witzgall et al., 1999) and pheromone-treated (Stelinski et al., 2005a) orchards. Although attracted moths departed within <60 sec (Stelinski et al., 2005a), as seen with three other tortricids (Stelinski et al., 2004a), it is possible that such pheromone exposure contributes to mating disruption.

The purpose of this study was to quantify the effects of brief (seconds to 3 min) preexposures of male codling moths to various pheromone treatments in a flight

tunnel where moths had the capability of flying from release cages during the preexposure treatment. This context approximated the level of pheromone exposure males could receive during brief and close encounters with pheromone dispensers in the field (Stelinski et al., 2005a). In addition, a field experiment was conducted in which high densities (10/tree \approx 5000/ha) of Isomate-C Plus dispensers were deployed throughout tree canopies in an effort to maximize exposure of feral moths to pheromone. Captures of moths in traps baited with lure loadings assayed in the flight tunnel were used to relate flight-tunnel data to field responses of feral moths.

Methods and Materials

Insect Colonies

Codling moth males were drawn from 1- and 5-yr-old laboratory colonies established with pupae from untreated apple orchards in Michigan and North Carolina, USA, respectively. Moths from North Carolina were supplied by Benzon Research (Carlisle, PA, USA). Moths were reared at 24°C and 60% RH on pinto-bean-based diet (Shorey and Hale, 1965) under a 16:8 (L/D) photoperiod. Pupae were sorted by sex, and adults emerged in 1-l plastic cages containing 5% sucrose in plastic cups with cotton dental wicks protruding from their lids.

Chemicals and Release Devices

Polyethylene tube pheromone dispensers (Isomate-C Plus containing 205 mg of 53.0% codlemone, 29.7% 12OH, 6.0% 14OH, and 11.3% inert ingredients) were used to preexpose moths. The lures were red rubber septa (The West Company, Linville, PA, USA) loaded with 0.1, 1.0, or 10.0 mg of codlemone (Bedoukian Co, Danbury, CT, USA) that was >98% isomerically pure. Three-component red septa lures were loaded with 0.1 mg of codlemone, 12OH, and 14OH in a 100:20:5 ratio (El-Sayed et al., 1999). Codlemone + pear ester lures were loaded with 0.1 mg of each component in a 1:1 ratio into gray halobutyl septa (Trécé Inc, Adair, OK, USA). Pheromone blends were prepared in HPLC-grade hexane and stored at -18°C. All lures were aged in a fume hood for at least 48 hr prior to use in flight-tunnel assays, whereas Isomate dispensers were aged under the same conditions for 2 wk to allow dissipation of excess pheromone that might have built up on their surfaces during shipping and freezer storage. For EAG recordings, stimulus cartridges used to deliver pheromone to insect antennae were prepared according to Stelinski et al. (2003a,b) by using the codlemone described above.

Flight-Tunnel Assays

Behavioral assays were conducted in a Plexiglas flight tunnel (Stelinski et al., 2004b, 2005b). The flight-tunnel assay procedures were a slight modification of those described by Stelinski et al. (2005b). Male codling moths, 2–3 d-old, were collected 0.5 hr prior to the end of a 16 hr photophase and placed into cylindrical (8 cm long \times 8 cm diam.) release cages made from aluminum window screening. Each cage, containing two moths, was placed into the flight tunnel for 0.5 hr of acclimation prior

to assays. Subsequently, bioassays ran for a maximum of 1.5 hr. At the upwind end of the tunnel, pheromone dispensers (lures or Isomate dispensers) were placed 1 cm above a horizontal 7.5×12.5 cm yellow card (American Scholar, Bay Shore, NY, USA) attached to a horizontally clamped, 9-cm glass rod attached to a steel ring stand 25 cm above the tunnel floor. Release cages holding male moths were placed at the downwind end of the tunnel at a height matching that of the pheromone dispenser.

Males were allowed a maximum of 3 min to respond to an inserted pheromone dispenser, as this was the longest time feral moths remained in close proximity of Isomate-C Plus dispensers in treated orchards (Stelinski et al., 2005a). However, the majority (>80%) of preexposed males left release cages within 1 min and, thus, did not receive the full 3 min of exposure. Following preexposure, males were assayed to various lures described below. During assays following preexposure, males were allowed 3 min to respond. The behaviors recorded were wing fanning, non-anemotactic flight from the release cage, anemotactic flight in the pheromone plume without touching the release device, upwind anemotactic flight followed by landing on the platform and touching the release device. Also, the numbers of individuals with no detectable behavioral change were recorded.

Experiment 1

For preexposures, moths were released in plumes generated by a rubber septum loaded with 0.1 mg of codlemone or an Isomate dispenser and then, regardless of the behaviors exhibited during preconditioning, were subsequently assayed 15 min later to a 0.1 mg codlemone lure. Two types of controls were conducted. “Naive” moths had no prior exposure to pheromone or the flight tunnel prior to assay. “Control” moths were preexposed to clean air in the flight tunnel and otherwise handled identically to pheromone-exposed moths.

The experiment was conducted as a randomized complete block design; 47 replicate groups of two moths were assayed for each treatment. To avoid any possibility of pheromone contamination in control and naive moths, pheromone preexposed, air preexposed, and naive moths were assayed on different days. This protocol was used in all other flight-tunnel experiments.

Experiment 2

Males exposed to either 0.1 mg codlemone lures or Isomate-C Plus dispensers were assayed to 0.1 mg codlemone lures 24 hr after the preexposure treatment. Following preexposure, moths were kept in an environmental chamber under the temperature and light cycle conditions described above for the interval prior to testing. Thirty-seven replicate groups of two moths were assayed per treatment.

Experiments 3 and 4

In these assays, males were exposed to 0.1 mg three-component lures as preexposure treatments. We chose to assay the effect of preexposure to these three-component lures in addition to the codlemone-only lures given that Isomate-C

Plus dispensers also contain the 14 and 12 alcohols. In addition, these experiments tested the hypothesis that males preexposed to Isomate dispensers would respond differently to three-component vs. codlemone-only lures. Seventy-three and 61 replicate groups of two moths were assayed per treatment for the 15 min and 24 hr postexposure intervals, respectively.

Experiment 5

Here we tested the hypothesis that preexposure to Isomate-C Plus dispensers raises the response threshold of male codling moths to subsequent presentations of codlemone. Forty-eight, 34, and 39 replicate pairs of treated and control groups of two moths were assayed to 0.1, 1.0, or 10 mg lures, respectively, 15 min after preexposure to codlemone.

Experiment 6

Males were preexposed to a 1:1 mixture of codlemone + pear ester and subsequently assayed to 0.1 mg codlemone lures. Forty-one and 40 replicate groups of two moths were assayed per treatment for the 15-min and 24-hr postexposure intervals, respectively.

Experiment 7 (Electroantennograms)

The EAG system and test protocols were identical to those of Stelinski et al. (2003a,b). EAGs were conducted on male codling moths 15 min or 24 hr after a preexposure to a 0.1 mg codlemone lure, a 0.1 mg codlemone/pear ester lure (1:1 ratio), a 10.0 mg codlemone lure, or an Isomate-C Plus dispenser in the flight tunnel. EAGs performed on pheromone-exposed moths were alternated with assays of unexposed, naive moths. Ten moths were assayed per treatment for each preexposure treatment and time interval combination tested.

Experiment 8

Oriental disruption of male codling moth by high densities of Isomate-C Plus dispensers was measured in the field. They were hung 10 per tree such that all possible points within the canopy were <1.5 m from a dispenser. The density of dispensers used (5000/ha) was five times the recommended dose to ensure that feral males were exposed to very high levels of pheromone. The paired treatment and control plots of 16 trees (0.07 ha) were at least 15 m apart in a two-by-two design and the five replicates separated by at least 35 m. The treatments were applied at the onset of the second codling moth generation in Michigan, USA, and the experiment ran from 15 July through 30 August 2004. Each plot was monitored with two delta traps (LPD Scenturian Guardpost, Suterra, Bend, OR, USA); one had a 0.1 mg lure and the other a 1.0 mg lure. Traps were hung ca. 2–3 m above ground level in the upper third of the tree canopy in two of the four central trees of each plot. Traps were positioned as far away as possible from Isomate dispensers (at least 60 cm). Moths captured in traps were counted and removed twice weekly.

Statistical Analyses

The numbers of moths contacting lures, orienting without source contact, flying out of release cages without orienting, wing fanning without flight, or exhibiting no detectable behavioral change for each treatment were transformed [$\log(x + 1)$] and submitted to analysis of variance (ANOVA), followed by Tukey's test (SAS Institute, 2000) for separation of means. EAG data were subjected to ANOVA (SAS Institute, 2000). Captures of moths in traps in treated and control orchards were compared by using paired *t* tests. The significance level was $\alpha < 0.05$. Percent orientational disruption for the field experiment (experiment 8) was calculated as $1 - (\text{mean moth catch per trap in the Isomate-treated plot} / \text{mean moth catch per trap in the control plot}) \times 100$. Data in Tables 1, 2, 3, and 4 are presented as proportions of the total number of moths responding in the flight tunnel.

Results

Experiment 1

The responses of naive and control moths were virtually indistinguishable for each behavioral category (Table 1). After preexposure to Isomate-C Plus dispensers, significantly more males either remained in their release cage or exhibited nonoriented flight than the control; only 2 out of 74 moths oriented anemotactically to 0.1 mg lures and none contacted the source (Table 1). Significantly fewer males reached the source after preexposure to 0.1 mg lures, but there was no difference in the proportion orientating without source contact (Table 1).

Table 1 Behaviors of naive, control (air preexposed), and pheromone-exposed codling moths 15 and 24 hr after preexposure to 0.1 mg codlemone lures

	Proportion of males exhibiting				
	No behavioral change	Wing fanning only	Fly out without orientation	Orientation without source contact	Source contact
15 min postexposure					
Naive (no exposure)	0.00b	0.01a	0.13b	0.28a	0.56a
Control (clean air)	0.00b	0.03a	0.11b	0.33a	0.53a
Codlemone lure exposed	0.02b	0.00a	0.46a	0.30a	0.19b
Isomate-C Plus exposed	0.54a	0.06a	0.37a	0.02b	0.00b
24 hr postexposure					
Naive (no exposure)	0.02a	0.00a	0.14a	0.28b	0.56a
Control (clean air)	0.01a	0.20a	0.24a	0.27b	0.46a
Codlemone lure exposed	0.00a	0.00a	0.16a	0.58a	0.26b
Isomate-C Plus exposed	0.00a	0.00a	0.11a	0.36b	0.52a

Numbers in the same column followed by the same letter are not significantly different (ANOVA followed by Tukey's test, $\alpha < 0.05$).

Experiment 2

Twenty-four hours postexposure, there were no significant differences among responses of Isomate-dispenser-exposed, control, or naive moths to 0.1 mg codlemone lures (Table 1). In contrast, significantly more males preexposed to 0.1 mg lures oriented without source contact, although significantly fewer reached the source than in the other treatments (Table 1).

Experiment 3

Significantly fewer males preexposed to Isomate dispensers or three-component lures contacted 0.1 mg three-component lures 15 min postexposure (Table 2). In addition, significantly fewer Isomate-dispenser-exposed males oriented without source contact, whereas more exhibited no behavioral change than control moths (Table 2).

Experiment 4

After 24 hr, the only effect of preexposure was that significantly fewer Isomate-dispenser-exposed males flew out of release cages without orienting compared with the other treatments (Table 2).

Experiment 5

Significantly more Isomate-exposed moths remained in the release cage and exhibited nonoriented flight than control moths; consequently, fewer oriented to and contacted

Table 2 Behaviors of control (air preexposed) and pheromone-exposed codling moths in response to three-component (Codlemone/12OH/14OH, 100:20:5 Ratio) lures

	Proportion of males exhibiting				
	No behavioral change	Wing fanning only	Fly out without orientation	Orientation without source contact	Source contact
15 min postexposure					
Control (clean air exposed)	0.01b	0.00a	0.24b	0.13a	0.61a
3-Component lure exposed	0.04b	0.01a	0.47a	0.24a	0.24b
Isomate-C Plus exposed	0.47a	0.00a	0.47a	0.04b	0.02c
24 hr postexposure					
Control (clean air exposed)	0.01a	0.05a	0.16a	0.11a	0.66a
3-Component lure exposed	0.04a	0.00a	0.22a	0.16a	0.58a
Isomate-C Plus exposed	0.00a	0.00a	0.04b	0.18a	0.78a

Numbers in the same column followed by the same letter are not significantly different (ANOVA followed by Tukey’s test, $\alpha < 0.05$).

Table 3 Behaviors of air preexposed versus Isomate-C plus dispenser-exposed codling moths in response to 0.1, 1.0, OR 10.0 mg codlemone lures 15 min postexposure

	Proportion of males exhibiting				
	No behavioral change	Wing fanning only	Fly out without orientation	Orientation without source contact	Source contact
0.1 mg codlemone lure					
Clean air exposed	0.00b	0.02b	0.19b	0.23a	0.56a
Isomate-C Plus exposed	0.44a	0.12a	0.40a	0.04b	0.00a
1.0 mg codlemone lure					
Clean air exposed	0.26a	0.07a	0.37a	0.27b	0.03a
Isomate-C Plus exposed	0.00b	0.05a	0.30a	0.58a	0.05a
10.0 mg codlemone lure					
Clean air exposed	0.87a	0.01a	0.09b	0.03b	0.00a
Isomate-C Plus exposed	0.21b	0.08a	0.46a	0.29a	0.00a

Numbers in the same column followed by the same letter are not significantly different (ANOVA followed by Tukey's test, $\alpha < 0.05$).

the 0.1 mg lure (Table 3). In contrast, more preexposed males took flight and significantly more oriented to both 1.0 and 10.0 mg lures than controls. However, at both concentrations, there were no differences in the number reaching the source in treated and control treatments (Table 3). For the 10 mg lure, significantly more control males remained in the release cage than the preexposed ones (Table 3).

Experiment 6

At 15 min following preexposure to a 0.1 mg codlemone + pear ester lure, significantly more males remained in the release cage and significantly fewer contacted the lure than in the control (Table 4). However, responses were not different from controls 24 hr after preexposure (Table 4).

Experiment 7

Mean EAG responses of naive males were indistinguishable, and not statistically different, from those assayed either 15 min or 24 hr after flight-tunnel preexposure

Table 4 Behaviors of air preexposed versus 0.1 mg codlemone: pear ester lure (1:1 ratio) preexposed codling moths in response to 0.1 mg codlemone lures

	Proportion of males exhibiting				
	No behavioral change	Wing fanning only	Fly out without orientation	Orientation without source contact	Source contact
Clean air	0.06b	0.04a	0.32a	0.06a	0.59a
15 min postexposure	0.22a	0.04a	0.26a	0.19a	0.28b
24 hr postexposure	0.09b	0.06a	0.43a	0.09a	0.51a

Numbers in the same column followed by the same letter are not significantly different (ANOVA followed by Tukey's test, $\alpha < 0.05$).

to a 0.1 mg codlemone, 0.1 mg codlemone + pear ester, or 10 mg codlemone lure or an Isomate-C Plus dispenser (data not shown).

Experiment 8

Significantly more males were captured in traps baited with either 1.0 mg (99.1 ± 21.8 vs. 11.6 ± 4.2) or 0.1 mg (58.2 ± 13.3 vs. 2.4 ± 1.1) lures in control plots than those treated with 10 Isomate-C Plus dispensers per tree. Orientational disruption was 88.3 and 95.9% for the 1.0 and 0.1 mg lures, respectively.

Discussion

Brief (seconds to 3 min max) exposures of codling moth males to Isomate-C Plus dispensers in a flight tunnel drastically reduced the proportion of successful orientations to otherwise highly attractive codlemone or three-component (codlemone/14OH/12OH) lures. The effect appeared to be dose dependent; exposure to 0.1 mg lures with codlemone only or with codlemone and the 14 and 12 alcohols did not reduce behavioral responses of males 15 min later to the same degree as did exposure to Isomate dispensers, which release pheromone at ca. 20 $\mu\text{g/hr}$ (Knight, 1995). The effect is likely habituation, given that EAG responses to codlemone were normal in preexposed moths exhibiting reduced behavioral responsiveness. This habituation appears to be a classic threshold elevation (Mafra-Neto and Baker, 1996), as more preexposed moths oriented to elevated dosages of codlemone (1 and 10 mg) than did control moths (Table 3). Conversely, normally attractive dosages of codlemone (0.1 mg) elicited little behavioral response from moths exposed to Isomate dispensers. In field trials, more males are captured in pheromone-treated orchards using higher loadings of codlemone (10.0 mg) compared with lower loadings (1.0 mg), whereas the converse is true in untreated orchards (Charmillot, 1990; Barrett, 1995). Our flight-tunnel data support the hypothesis that pheromone pre-exposure in pheromone-treated orchards raises the behavioral response threshold of codling moth males to subsequent encounters with pheromone plumes (Vickers and Rothschild, 1991). However, the majority of preexposed moths resumed normal behavioral responsiveness within 24 hr of the initial preexposure in the current study. This suggests that a pheromone-induced elevation of response threshold could be reversed in feral males capable of moving to and remaining for some time in pheromone-free air.

An exception was that 24 hr following initial exposure, fewer moths preexposed to 0.1 mg codlemone contacted identical lures compared to those preexposed to Isomate-C Plus (Table 1). This difference is likely due to the type of pheromone preexposure received, as <2% of moths touched the Isomate source during preexposure, compared with >50% of those preexposed to 0.1 mg codlemone (Table 1). It is possible that codlemone was adsorbed onto the insects' cuticle during contact and, thus, exerted a longer-lasting effect. If true, then dispensers, such as hollow fibers (Moffitt and Westgard, 1984; Knight, 2003), that elicit direct source contacts could impact male responsiveness for longer periods than dispensers that do not.

Preexposure of caged male codling moths to an Isomate dispenser for 24 hr increased their behavioral responsiveness to the pear ester [(2*E*,4*Z*)-2,4-decadienoate] in flight-tunnel assays (Zhihua et al., 2005). In addition, captures of both male and female codling moths in pear-ester-baited traps were higher in pheromone-treated vs. untreated orchards (Zhihua et al., 2005). In the current investigation, brief preexposure of males to a low dose of codlemone + pear ester did not affect their subsequent behavioral responses any more than preexposure to codlemone alone. This does not rule out the possibility of habituation at higher dosages of pear ester.

Codling moth males exhibit a prolonged reduction of EAG responses following 10–30 min exposures to codlemone, lasting ca. 1–1.5 hr (Judd et al., 2005; Stelinski et al., 2005c). Similar “long-lasting” adaptation has been reported for other moths, including polyphemus moth, *Antheraea polyphemus* (Cramer) (Kaissling, 1986), the obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Stelinski et al., 2003a), and the Pandemis leafroller, *Pandemis pyrusana* Kearfott (Stelinski et al., 2005c). In cases where the airborne concentration of pheromone has been quantified (Stelinski et al., 2003b; Judd et al., 2005), micrograms per liter of air are required to induce long-lasting adaptation. In field trials, long-lasting antennal adaptation in male obliquebanded leafrollers following 24 hr of exposure to Isomate dispensers was seen if males were held 1–2 cm from dispensers but not if the distance was increased to 2 m. Given that long-lasting adaptation was not recorded following any of the preexposure treatments in the current study, males probably did not receive the $\approx 355 \mu\text{g min}^{-1} \text{l}^{-1}$ dose required to induce long-lasting adaptation (Judd et al., 2005). Stelinski et al. (2005c) questioned the importance of long-lasting adaptation as a contributing factor to mating disruption, as airborne concentrations of pheromone to induce the phenomenon are much higher than those recorded in pheromone-treated orchards (Koch et al., 1997, 2002).

Since exposure of codling moth males to Isomate-C Plus dispensers strongly reduced subsequent responsiveness to codlemone in the flight tunnel, we postulated that feral males, landing near such dispensers in the field would be effectively eliminated from an olfaction-based search for females on that night, given the short diel window of male and female sexual activity (Castroville and Cardé, 1979). However, some feral males were captured in traps baited with 0.1 mg lures despite having 10 Isomate-C Plus dispensers hung throughout tree canopies. This spotlights a vulnerability of polyethylene tube dispensers for pheromonal control of codling moth under high population densities. Given that delta traps limit the importance of close-range visual cues, it is possible that disruption of feral females may have been even lower.

The data from our field experiment suggest that some feral males may receive a pheromone exposure below that imparted by our flight-tunnel preexposure treatments. Thus, we suggest that disruption outcomes in the laboratory, where male moths have been carefully preexposed to microgram per liter dosages of pheromone for several minutes and subsequently shown to exhibit reduced sexual response, may need to be interpreted with caution. These exposures are likely higher than those received by feral males under disruption protocols in the field. Additionally, feral males may actively modulate their exposure by flying to pheromone-free air or to areas of lower pheromone concentrations. Alternatively, those males captured in traps with 0.1 mg codlemone lures in our field experiment

might represent a tolerant phenotype selected by years of pheromone application at this study site.

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Evidence for Four-Component Close-Range Sex Pheromone in the Parasitic Wasp *Glyptapanteles flavicoxis*

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Abstract Females of the parasitic wasp *Glyptapanteles flavicoxis* (Hymenoptera: Braconidae) deposit a close-range sex pheromone from their abdominal tip that attracts conspecific males and elicits wing-fanning behavior. In this study, we isolated the pheromone components and determined their role in the males' behavior. In coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses of the females' body extract, four components (below GC detection) elicited strong responses from male antennae. Monitored by GC-EAD, the components were separated by flash silica gel and high-performance liquid chromatography. Y-tube olfactometer experiments with one or more components revealed that all are necessary to elicit short-range attraction and wing-fanning responses by males. These components remained below detection threshold of the mass spectrometer (~10 pg) even when 4500 female equivalents were analyzed in a single injection, which attests to the potency of the pheromone and the insects' sensitivity to it.

Keywords *Glyptapanteles flavicoxis* · *Lymantria dispar* · Hymenoptera · Braconidae · Parasitoid · Close-range sex pheromone · Wing fanning

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Introduction

Sex pheromones in hymenopteran parasitic wasps are typically produced by females. They have been reported in seven families [Aphelinidae, Chalcididae, Cynipidae, Pteromalidae, Scelionidae, Braconidae, and Ichneumonidae (Kainoh, 1999)], but have been identified in only a few species, including *Itopectis conquisitor* (Robacker and Hendry, 1977), *Syndipnus rubiginosus* (Eller et al., 1984), *Macrocentrus grandii* (Swedenborg and Jones, 1992a,b), *Ascogaster reticulatus* (Kainoh et al., 1991), *Cardiochiles nigriceps* (Syvertsen et al., 1995), and *Ascogaster quadridentata* (DeLury et al., 1999).

In the Braconidae, sex pheromones have been reported in *Opius alloeus* (Boush and Baerwald, 1967), *Apanteles medicaginis* (Cole, 1970), *Apanteles medicaginis glomeratus* (Obara and Kitano, 1974), *Apanteles medicaginis melanoscelus* (Weseloh, 1976, 1980), *Cotesia rubecula* (Field and Keller, 1994), *Cotesia flavipes* (Kimani and Overholt, 1995), *Praon volucre* (Nazzi et al., 1996), and *Fopius arisanus* (Quimio and Walter, 2000). Most are long-range attractants.

Substrate-borne sex pheromones in parasitoids are rare. Female *Aphelinus asychis* (Hymenoptera: Aphelinidae) appear to have a trail pheromone, but do not exhibit specific trail-marking behavior (Fauvergue et al., 1995). In *Trichogramma brassicae* (Hymenoptera: Trichogrammatidae), a substrate-borne pheromone induces male searching in an area previously explored by females and attracts males from short distance (Pompanon et al., 1997). Female *As. reticulatus*, egg-larval parasitoids of the smaller tea tortrix, *Adoxophyes* sp., employ short-range pheromones that activate searching by males and increase the probability of mating (Kamano et al., 1989).

Some parasitic wasps have multiple-component pheromones. For example, male *M. grandii* are attracted to the female-produced components (*Z*)-4-tridecenal and (*Z,Z*)-9,13-heptacosadiene (Swedenborg and Jones, 1992a,b). The behavioral activity of both compounds is enhanced by (3*R*,5*S*,6*R*)-3,5-dimethyl-6-(methyl-ethyl)-3,4,5,6-tetrahydropyran-2-one as a third component that is biosynthesized in mandibular glands of both males and females (Swedenborg et al., 1993). In the ichneumonid *Eriborus terebrans*, the nonpolar pheromone component by itself is inactive, but when added to the polar component provokes the male's behavioral response (Shu and Jones, 1993).

Glyptapanteles flavicoxis (Hymenoptera: Braconidae) is a gregarious, koinobiont endoparasitoid of larval Indian gypsy moth, *Lymantria obfuscata* (Lepidoptera: Lymantriidae) (Marsh, 1979). In 1981, it was imported from India and released into North America as a potential biological control agent for larvae of the European gypsy moth, *Lymantria dispar* (Krause et al., 1990). Female *G. flavicoxis* press their abdominal tip to the substrate, apparently depositing pheromone that elicits wing fanning by males (J. Fuest, personal communication). This interpretation of the females' behavior is supported by reports of abdominal pheromone glands in other braconid females, including *Ap. glomeratus* (Tagawa, 1977), *Ap. melanoscelus* (Weseloh, 1980), *Ap. plutellae*, *Ap. liparidis*, *Ap. baoris*, *Ap. ruficrus*, and *Ap. kariyai* (Tagawa, 1983). Female *G. flavicoxis* also emit an airborne component (ethyl dodecanoate) that, by itself, is not effective in attracting conspecific males (J. Fuest, personal communication).

Our objectives were to investigate whether female *G. flavicoxis* use sex pheromone components, and, if so, to isolate them and determine their behavioral role.

Methods and Materials

Experimental Insects

The rearing colonies of experimental insects in the Global Forest Quarantine Facility at Simon Fraser University (SFU) were started and augmented with specimens obtained from the Beneficial Insects Introduction Research Laboratory, United States Department of Agriculture, Agricultural Research Service, Newark, Delaware. To facilitate mating in *G. flavicoxis*, 10 females and 30 males were placed in plastic mesh cages (10 × 10 × 6 cm) (Hu et al., 1986), and provisioned with cotton wicks (1 × 10 cm; Richmond Dental, Charlotte, NC, USA) soaked in sugar water solution. Oviposition cages (18 × 18 × 12 cm) contained 10–15 mated females, five *L. dispar* larvae (3–4 instar) (Fuester et al., 1987), and artificial diet for the larvae (Bell et al., 1981). After 1–2 d, parasitized host larvae were removed and placed on artificial diet in plastic cups (192 ml) with tight-fitting paper lids (Sweetheart Plastics, Wilmington, MA, USA). Every second day, larval frass was removed, diet replenished if needed, and parasite cocoons with insects to be used in bioassays transferred individually to capped plastic cups (30 ml) provisioned with sugar-water-soaked cotton wicks. Cocoons of insects to be used for mass rearing were placed in plastic Petri dishes (14 cm diam.) and food provisioned as described above. Rearing took place under a 16L:8D photoregime at 22–25°C and 50–70% RH.

Acquisition of Volatiles

Unmated, 1- to 2-d-old females (5–10) were placed into vertical cylindrical Pyrex glass chambers (10 ID × 6 cm), and were provisioned with a sugar-water-soaked cotton wick. Control chambers contained the same food source but no parasitoids. A water aspirator drew humidified, charcoal-filtered air at a rate of 1.5–2 l/min for 2 d through the chambers and a glass column (14 × 1.3 cm OD) filled with 150 mg of Porapak Q (50–80 mesh, Waters Associates Inc., Milford, MA, USA). Volatiles were eluted from the Porapak Q volatile traps with redistilled pentane (2 ml). The extract was concentrated under a stream of nitrogen such that 10 µl of extract contained one female hour equivalent (FHE) of volatile acquisition (= amount of volatiles released by one female during 1 hr).

Acquisition of Pheromone Extracts

Females (1- to 3-d-old) were macerated in vials containing hexane (ca. 10 µl per female) placed on dry ice. The extract was kept at room temperature for ~15 min. The supernatant was withdrawn, filtered through a small amount of glass wool in a pipette, and quantified to determine the volume representing one female body extract (FBE).

Video-recording of Trail-following Behavior by Males

To test the hypothesis that males follow a pheromone trail, their behavioral response was video recorded (Sony Digital Video Camera Recorder, DCR-VX 1000). Into each of 10 Pyrex glass dishes (9 × 2 cm high), 1 FBE was pipetted in trail-like pattern (Fig. 1). Additional 10 Pyrex glass dishes served as a control stimulus, with solvent

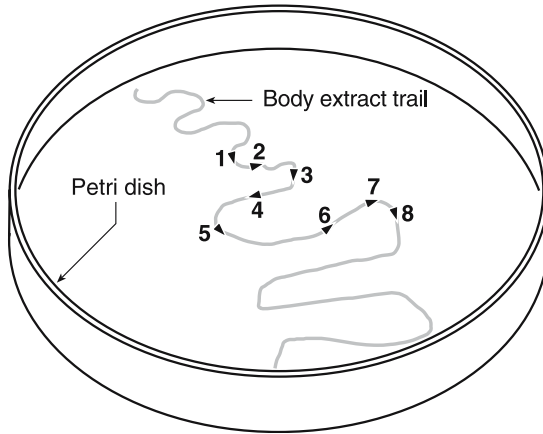
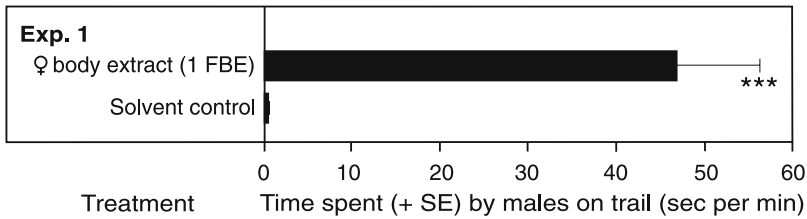


Fig. 1 (Top) Time spent by male *G. flavicoxis* ($n = 10$) on line drawings of a trail treated with one female body extract equivalent or a solvent control. Single-factor analysis of variance, $P < 0.05$. (Bottom) Representative example of “trail-following behavior” by a male (depicted as arrow head), with the position recorded every 2 sec

applied in the same way as the treatment. After the solvent had evaporated (~10 sec), a virgin 1- to 3-d-old male was released and video-recorded for 5 min. Recordings were analyzed for the time a male had spent on the trail and for other characteristic behavioral responses, such as wing fanning.

Y-tube Olfactometer Bioassays

All experiments were conducted during hours 2 to 6 of the insects' photophase (16L:8D). Anemotactic responses of males to odor sources were tested in vertical Pyrex glass Y-shaped olfactometers (stem: 20×2.5 cm ID; side arms at 120° : 18 cm long) positioned vertically 15 cm below a light source, consisting of one tube of fluorescent “daylight” (F40DX, H118; Osram Sylvania Ltd., Ontario, Canada) and one tube of “wide-spectrum grow light” (F40GRO/WS, H658; Osram Sylvania Ltd., Ontario, CA).

Treatment or control (solvent) stimuli were pipetted on white strips of paper (15×1 cm) placed in side arms of the Y-tube (experiments 1–28) or on filter paper discs (4.3 cm diam., Whatman No. 1, Whatman International Ltd., Maidstone, England, UK) placed near the orifice of side arms (experiments 29 and 30).

In experiment 31, two live 2- to 3-d-old females served as a test stimulus. They were transferred 10–15 min before experimental replicates into mesh-covered glass

tubes (6 × 2 cm ID) and provisioned with a sugar-water-soaked cotton wick. Treatment and control tubes (lacking females) were placed at the orifice of side arms of the Y-tube olfactometers.

In all experiments, a water aspirator drew air at ~1 l/min through the Y-tube to test anemotactic responses of parasitoids released individually into the stem of the Y-tube. An insect was classed a responder when it traversed the entire paper strip up to the orifice of the side arm within 10 min (experiments 1–28) or contacted the filter paper discs (experiments 29 and 30), or glass tube housing two females (experiment 31). All others were classified as nonresponders. For each replicate, a new insect, paper strip, filter paper disc, and clean (Sparkleen-washed and oven-dried) Y-tube or glass tube were used, with test stimuli randomly assigned to side arms.

To compare the attractiveness of test stimuli most rigorously, two to four experiments were often run in parallel over 2–4 d, alternating between replicates for each experiment. To gauge the relative attractiveness of two or more test stimuli, parallel experiments proved to be more effective than head-to-head comparisons of stimuli in the same Y-tube olfactometer.

Experiment 1 tested the “trail-following” response by males. Experiments 2 and 3 determined whether the females’ body extract in combination with the females’ effluvia, or synthetic effluvium component ethyl dodecanoate, were similarly effective in attracting males. Experiments 4 and 5 determined whether males or females respond to the pheromone. Experiments 6–8 explored the relative attractiveness of body extract, ethyl dodecanoate, or both.

Experiments 9–12 tested whether silica fraction 4 (containing candidate close-range pheromone components) and female body extract (containing candidate close-range pheromone components plus traces of ethyl dodecanoate and possibly other components) were equally attractive, at a low dose (1 FHE plus 1 FBE) or medium dose (5 FHE plus 5 FBE). Taking into account that silica fraction 4 at the medium dose was effective in attracting males, experiments 13 and 14 retested whether ethyl dodecanoate enhances the attractiveness of silica fraction 4. Although ethyl dodecanoate did not seem critical for male attraction, it was retained in subsequent experiments (15–27, 30) to ensure the best possible response of males to all test stimuli, and to allow the best comparison of results in all experiments.

Experiments 15 and 16 tested silica fraction 4 at the medium dose vs. the combination of all HPLC fractions that contained candidate close-range pheromone components (= effective blend). Considering the strong attractiveness of the effective blend, follow-up experiments 17, 19, 21, and 23 explored whether one or more of the candidate close-range pheromone components 1 (HPLC fractions 25–28), 2 and 4 (HPLC fractions 21–24), or 3 (HPLC fractions 16–20) could be deleted from the effective blend without affecting the males’ attraction or wing-fanning response. Experiments 25–27 tested the males’ attraction and wing-fanning responses to ethyl dodecanoate (experiment 27) alone, or in combination with either the effective blend (experiment 25) or most EAD-active pheromone component 3 (experiment 26).

Placement of test stimuli near (~1 cm) the junction of Y-tubes in experiments 1–28 was appropriate to test close-range anemotactic and wing-fanning responses of males, but not suitable to determine whether ethyl dodecanoate, or other female-produced components, might enhance the active space (mate-recruiting distance) of the entire pheromone blend. Thus, final experiments 29–31 tested the response of males to stimuli [silica fraction 4 on filter paper disc (experiment 29); silica fraction 4 plus ethyl dodecanoate on filter paper disc (experiment 30); two caged live females (experiment

31)] that were placed at the orifice of side arms >10 cm apart from the junction of the Y-tube.

Analyses of Pheromone Extracts

Aliquots of 1 FHE or 1 FBE were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975; Gries et al., 2002), employing a Hewlett Packard (HP) 5890A gas chromatograph equipped with a GC column (30 m × 0.25 or 0.32 mm ID) coated with DB-5, DB-17, DB-210, DB-23, or FFAP (J & W Scientific, Folsom, CA, USA). For GC-EAD recordings, a male's head was severed and placed into the opening of a glass capillary electrode filled with saline solution (Staddon and Everton, 1980). One antenna with its tip removed by spring microscissors (Fine Science Tools Inc., North Vancouver, British Columbia, CA) was placed into the opening of a second (indifferent) electrode.

EAD-active compounds were analyzed by (1) full-scan electron-impact and chemical ionization (CI, acetonitrile) mass spectrometry (MS) with a Varian Saturn 2000 Ion Trap GC-MS fitted with the DB-5 column referred to above; (2) retention index calculations (Van den Dool and Kratz, 1963); and (3) microanalytical treatments (hydrogenation, oxidation, reduction, acetylation, deacetylation) followed by renewed GC-EAD and GC-MS of the extract.

Aliquots of 100 FBEs with EAD-active components were fractionated through silica gel (0.5 g) in a glass column (14 × 0.5 cm ID). After the silica was prerinsed with pentane, the extract was applied, allowed to impregnate the silica gel, and then eluted with six consecutive rinses (1 ml each) of pentane/ether, with increasing proportion of ether, as follows: (1) 100:0; (2) 100:0; (3) 90:10; (4) 75:25; (5) 50:50; and (6) 0:100. This procedure generated fractions containing analytes of increasing polarity.

To determine silica fractions with candidate pheromone components, fractions were concentrated to the corresponding number of female equivalents processed in the initial extract and analyzed by GC-EAD, co-injecting as an internal standard ethyl dodecanoate (1 ng), which eluted 4–8 min earlier on the different GC columns than any of the four components. Fractions with more than one EAD-active compound (= candidate pheromone component) were fractionated further into 40 fractions (one fraction/25 sec) by HPLC, followed by renewed GC-EAD analyses of all HPLC fractions. HPLC fractionation employed a Waters LC 626 HPLC equipped with a Waters 486 variable-wavelength UV visible detector set to 210 nm, HP Chemstation software (Rev. A.07.01), and a reverse-phase Nova-Pak C18 column (60 Å, 4 µm; 3.9 × 300 mm) eluted with 1 ml/min of 100% acetonitrile.

Results

In experiment 1, 1 FBE induced wing-fanning and “trail-following behavior” by males (Fig. 1). Males also spent more time on trails of body extract of females than on solvent control trails (Fig. 1).

Effluvium (1 FHE) and body extract (1 FBE) of females in combination attracted more males than did the solvent control (Fig. 2, experiment 2). Similarly, ethyl dodecanoate plus female body extract attracted males (Fig. 2; experiments 3, 5, and 8), but not females (Fig. 2, experiment 4). Unlike female body extract, ethyl

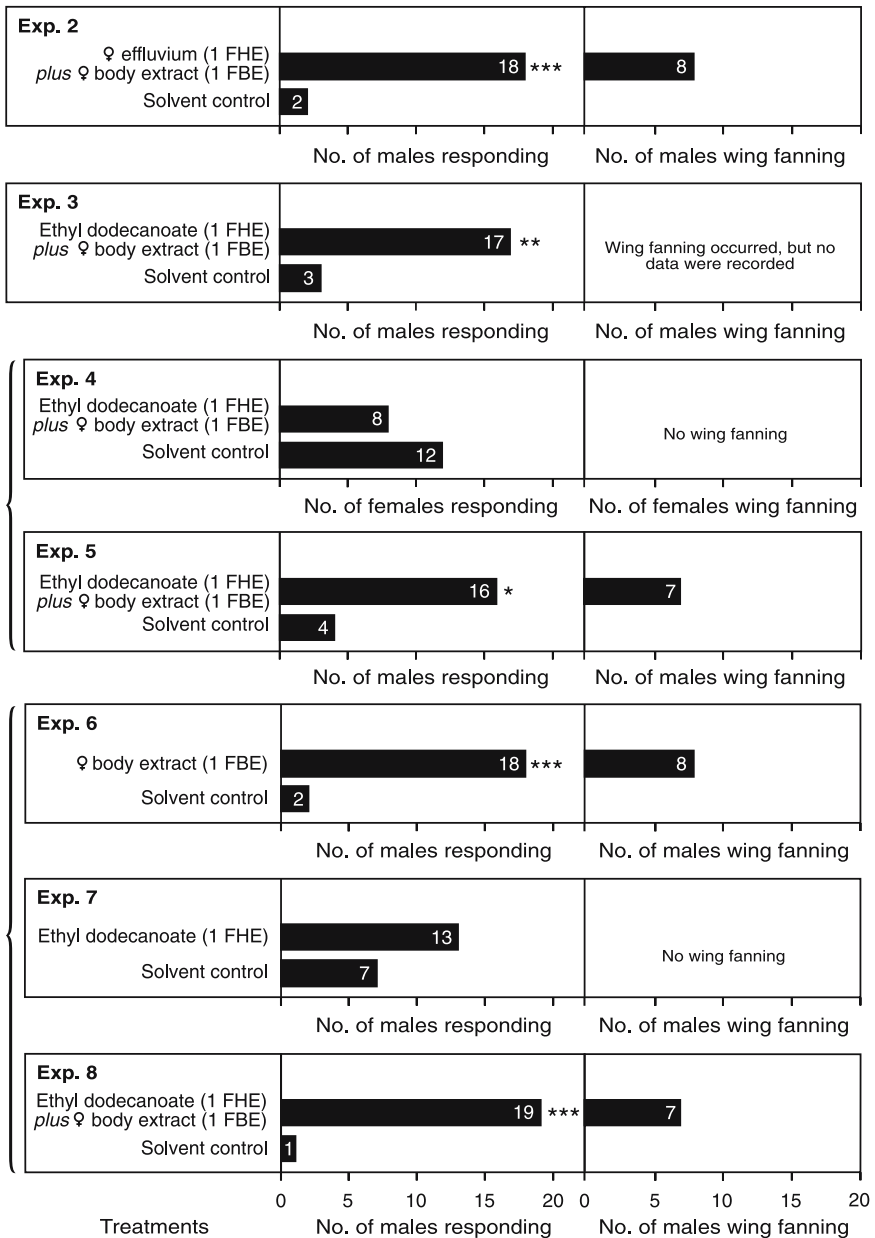
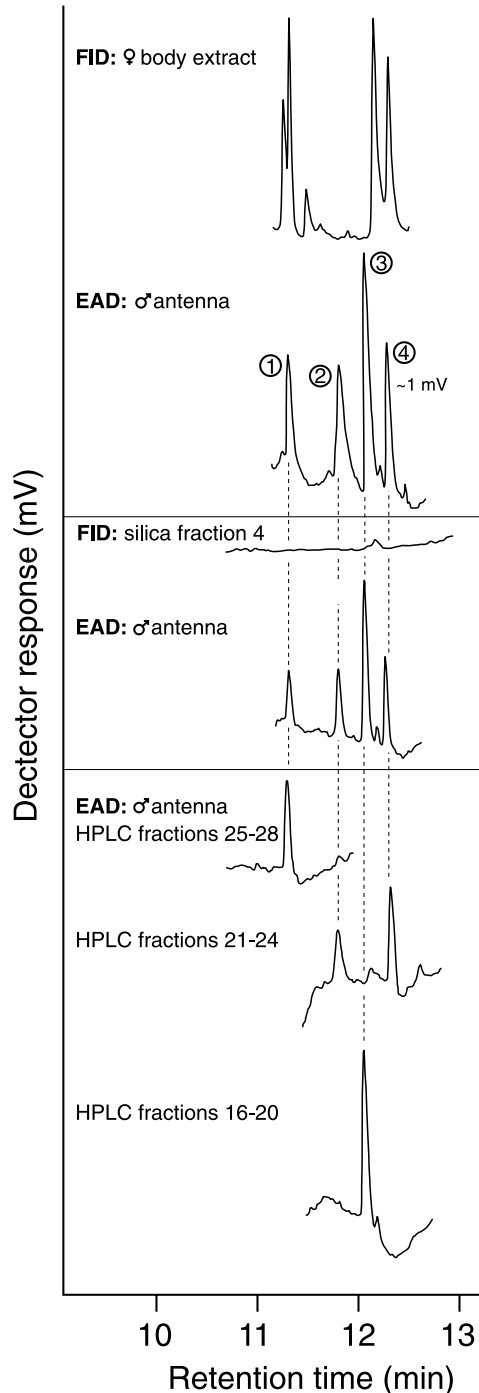


Fig. 2 Number of male or female *G. flavicoxis* that were attracted, or wing fanned, in response to test stimuli tested in Y-tube olfactometer experiments 2–8. 1 FHE = one female hour equivalent = pheromone component(s) released by one female during 1 hr; 1 FBE = one female body extract equivalent = pheromone component(s) contained in extract of one macerated female body. In each experiment, bars with asterisks indicate a significant response to a particular treatment; χ^2 test (experiments 2 and 3), heterogeneity χ^2 test with Yates' correction for continuity, treatment vs. control (Zar, 1996); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Note: (1) Experiments grouped by brackets were run in parallel; (2) only after completion of experiment 3 did we realize that we should have recorded wing fanning as a response criterion

Fig. 3 Flame ionization detector (FID) and electroantennographic detector (EAD: male *G. flavicoxis* antenna) responses to aliquots of female body extract (top), silica fraction 4 (middle), and HPLC fractions 16–20, 21–24, and 25–28 (bottom). Chromatography: Hewlett Packard 5890A equipped with a DB-23-coated column (30 m × 0.25 mm ID); linear flow velocity of carrier gas: 35 cm/sec; injector and FID detector temperature: 220°C; temperature program: 1 min at 100°C, 10°C/min to 220°C



dodecanoate by itself failed to attract males or to provoke wing fanning (Fig. 2; experiments 6, 7).

GC-EAD analyses of female body extracts revealed four components that elicited antennal responses from males (Fig. 3; Table 1). Although these components appeared to be abundant in the corresponding flame ionization detector (FID) trace, their mass spectra suggested that they were not pheromone components, but superimposed on them. GC-EAD analyses of all six silica fractions of female body extract revealed that fraction 4 contained the four EAD-active components, and that they indeed occurred below FID detection threshold (Fig. 3).

In Y-tube olfactometers, female body extract (at 1 FBE) combined with ethyl dodecanoate was attractive, whereas silica fraction 4 (at 1 FBE) with ethyl dodecanoate was not (Fig. 4, experiments 9, 10), suggesting that some active material had been lost during fractionation. However, silica fraction 4 at 5 FBE, together with ethyl dodecanoate, attracted males (Fig. 4, experiment 11), indicating that all essential components of the close-range pheromone were present in silica fraction 4. Female body extract at 5 FBE plus ethyl dodecanoate was not attractive (Fig. 4, experiment 12), suggesting that this dose might have exceeded a biologically relevant threshold. In experiments 13 and 14, silica fraction 4 with or without ethyl dodecanoate appeared equally attractive to males.

In GC-EAD analyses of HPLC fractions of silica fraction 4, component 3 was present in fractions 16–20 (elution time: 4–5 min), components 2 and 4 (not separable)

Table 1 Retention indices (relative to alkane standards) of pheromone components 1–4 in body extracts of female *Glyptapanteles flavicoxis* (Fig. 3), and ability of microanalytical treatments of silica or HPLC fractions of body extracts to alter the molecular structure of components 1–4, as determined by the presence or absence of respective antennal responses in GC-EAD recordings of such fractions

GC column	Retention indices of			
	Component 1	Component 2	Component 3	Component 4
DB-5	2068	2089	2083	2108
DB-17	2314	2358	2381	2393
DB-210	2406	2429	2429	2481
DB-23	2583	2658	2700	2731
FFAP	2529	2608	2657	2657
Microanalytical treatments of body extract ^{a,b}	Antennal response in GC-EAD recordings to ^c			
	Component 1	Component 2	Component 3	Component 4
Hydrogenation	Absent	Absent	Absent	Absent
Acetylation	Present	Present	Present	Present
Oxidation (PCC)	Present	Present	Present, but smaller	Present
Reduction (NaBH ₄)	Present	Present	Present	Present
Reduction (LiAlH ₄)	Absent	Absent	Absent	Absent
Deacetylation	Present	Present	Absent	Present

^a Details of these standard treatments are described elsewhere (Huwlyler, 1972; Corey and Suggs, 1975; Stanley, 1979; Bjostad et al., 1996; Millar and Haynes, 1998).

^b Each treatment was repeated at least two times with different extracts.

^c Each microtreated extract was tested with at least three male *G. flavicoxis* antennae in GC-EAD recordings.

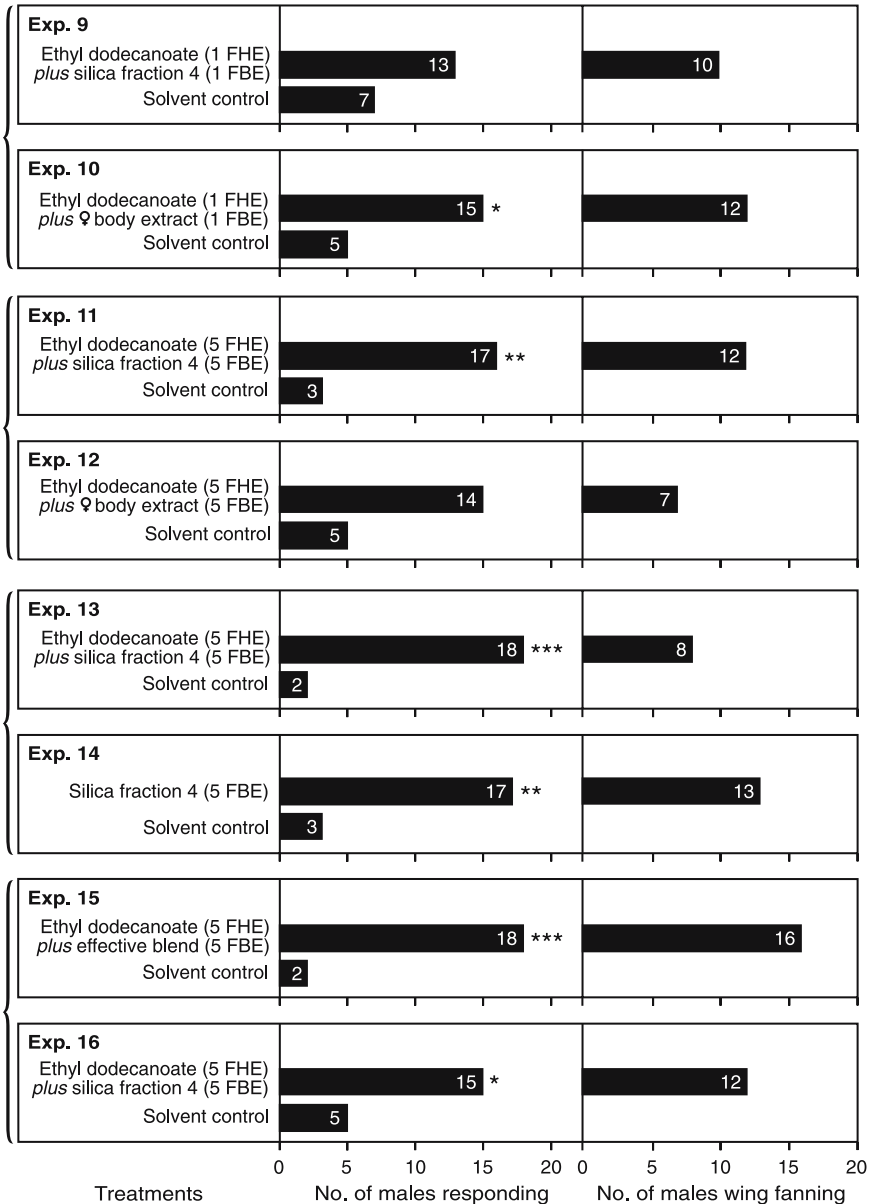


Fig. 4 Number of male *G. flavicoxis* that were attracted, or wing fanned, in response to test stimuli in Y-tube olfactometer experiments 9–16. Abbreviations as in caption of Fig. 2; effective blend = combined HPLC fractions 16–20, 21–24, and 25–28 (see Fig. 3). In each experiment, bars with asterisks (*) indicate a significant response to a particular treatment; heterogeneity χ^2 test with Yates' correction for continuity, treatment vs. control; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Note: (1) Experiments grouped by brackets were run in parallel; (2) one male in experiment 12 did not respond to test stimuli

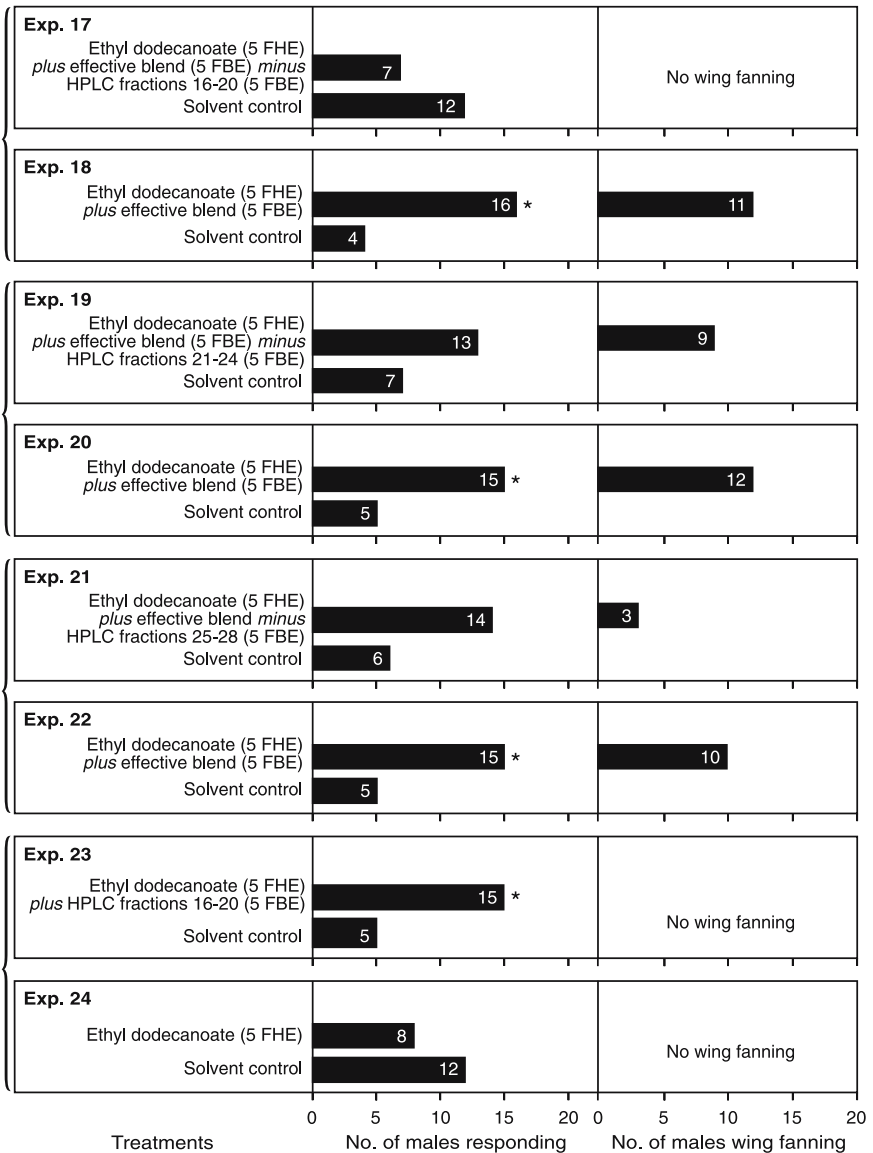


Fig. 5 Number of male *G. flavicoxis* that were attracted, or wing fanned, in response to test stimuli in Y-tube olfactometer experiments 17–24. Abbreviations as in caption of Figs. 2 and 4. In each experiment, bars with asterisks (*) indicate a significant response to a particular treatment; heterogeneity χ^2 test with Yates' correction for continuity, treatment vs. control; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Note: (1) Experiments grouped by brackets were run in parallel; (2) one male in experiment 17 did not respond to test stimuli

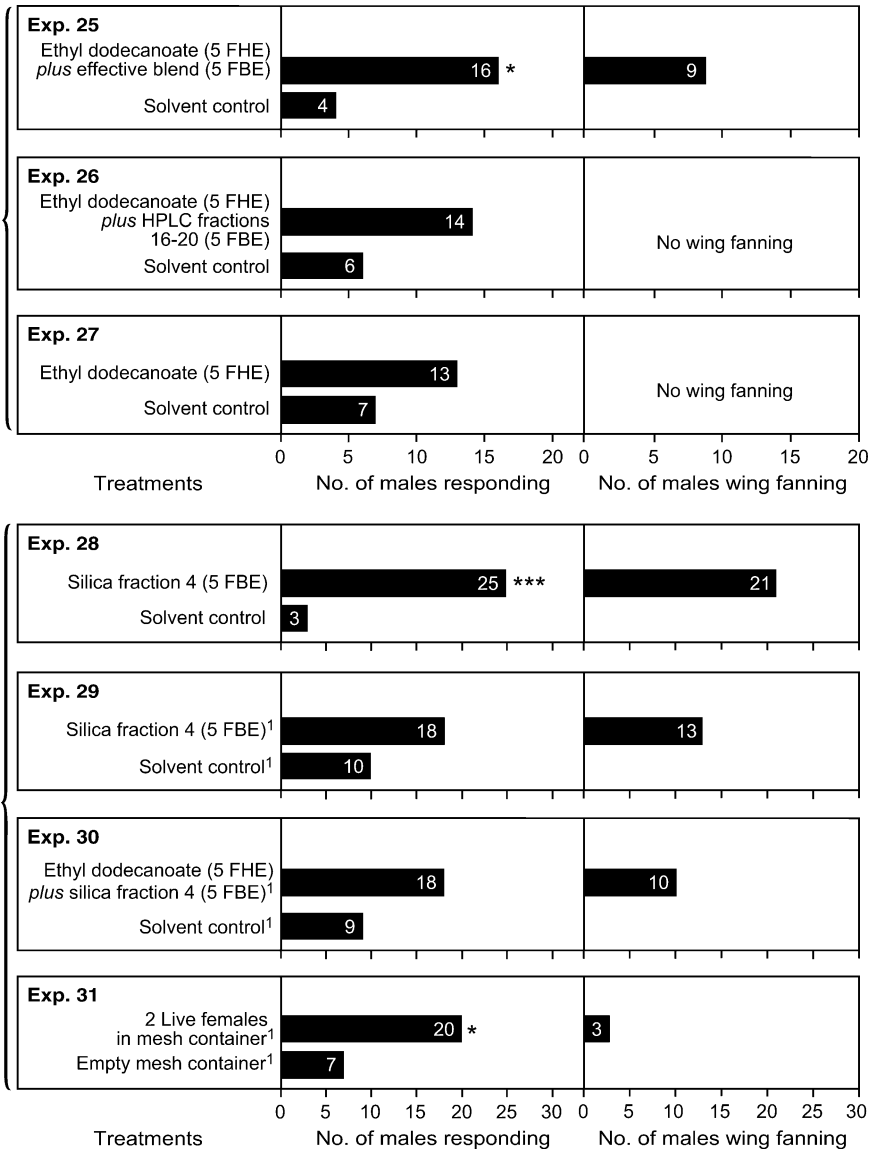


Fig. 6 Number of male *G. flavicoxis* that were attracted, or wing fanned, in response to test stimuli in Y-tube olfactometer experiments 25–31. Abbreviations as in caption of Figs. 2 and 4. In each experiment, bars with asterisks (*) indicate a significant response to a particular treatment; heterogeneity χ^2 test with Yates' correction for continuity, treatment vs. control; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Note: (1) Experiments grouped by brackets were run in parallel; (2) one male in each of experiments 30 and 31 did not respond to test stimuli. ¹Test stimuli placed at the orifice of the Y-tube's side arms

were present in fractions 21–24 (elution time: 5–6 min), and component 1 was present in fractions 25–28 (elution time: 6–7 min) (Fig. 3). In Y-tube olfactometers, all fractions with one or more EAD-active components recombined at 5 FBE, together with ethyl dodecanoate, attracted males (Fig. 4, experiment 15; Fig. 6, experiment 25). This effective blend was no longer attractive to males, when fractions 16–20 (containing component 3), 21–24 (containing components 2 and 4), or fractions 25–28 (containing component 1) were lacking (Fig. 5; experiments 17–22). Ethyl dodecanoate by itself, or in combination with HPLC fractions 16–20, failed to consistently attract males or to elicit wing-fanning responses (Fig. 2, experiment 7; Fig. 5, experiments 23, 24; Fig. 6, experiments 26, 27).

In experiment 28 (Fig. 6), silica fraction 4 applied on a paper strip (15 × 1 cm) in a Y-tube's side arm prompted strong anemotactic and wing-fanning responses by males (see also experiment 14), but failed to do so, with or without ethyl dodecanoate, when pipetted on a filter paper disc (4.3 cm diam.) at a side arm's orifice in parallel experiments 29 and 30. In contrast, two live females caged at a side arm's orifice were significantly attractive to males (experiment 31).

Discussion

Our data support the conclusion that female *G. flavicoxis* use a four-component pheromone blend that provokes strong close-range anemotactic attraction and wing-fanning responses by conspecific males (Fig. 4, experiment 14; Fig. 6, experiment 28). Response of males but not females to the pheromone (Fig. 2; experiment 4, 5) indicates that it is a sex rather than aggregation pheromone. Failure of these four components to attract males over a distance of 10 cm (Fig. 6, experiment 29), coupled with attraction of males to live females over the same distance (Fig. 6, experiment 31), suggests that females use one or more additional pheromone components for long-range attraction of males. Similarly complex sexual communication has been reported for the parasitic wasp *Aphidius nigripes* (Hymenoptera: Aphididae) (McNeil and Brodeur, 1995; Marchand and McNeil, 2000). Body extracts of females provoked wing fanning but not upwind flight by males, suggesting that female *A. nigripes* use both short- and long-range pheromone components.

Ethyl dodecanoate in the effluvia of female *G. flavicoxis* was a potential long-range pheromone component, but it did not affect the males' behavioral response in our experiments (Fig. 4, experiments 13, 14; Fig. 6, experiments 29, 30), and thus cannot be considered a pheromone component.

Video footage (graphical illustration not shown) revealed that females deposit, and males respond to, pheromone on substrate. It is, however, not likely that females deposit a continuous trail, as bioassayed in experiment 1. Males of the braconid *As. reticulatus* respond sporadically to substrate that females have frequented before, suggesting that females deposit traces rather than trails of pheromone (Kamano et al., 1989). Similarly, in *G. flavicoxis*, substrate-borne pheromone may signal the presence of, rather than provide long-range directional cues toward, females (Fig. 6; experiments 29, 30).

Intriguingly, the close-range pheromone blend of *G. flavicoxis* is bifunctional, also eliciting wing-fanning responses by males. The males' strong wing-fanning response, however, was dependent upon their close distance to the pheromone source (e.g., Fig. 4, experiment 14; Fig. 6, experiment 28). Even caged live females (and their

potential pheromone depositions on substrate) that remained inaccessible to males hardly elicited wing-fanning responses (Fig. 6, experiment 31). A strong wing-fanning response was also dependent upon the composition of the pheromone blend. It required the presence of component 3 and component(s) 1, or 2 and 4 (Fig. 5).

Wing fanning has been interpreted as a behavior that facilitates the males' orientation toward females. As demonstrated with fine chalk dust in the ichneumonid *Campoletis sonorensis*, wing fanning pulls air from front to rear, allowing directional orientation of males toward females (Vinson, 1972). This interpretation, however, does not explain completely why male *G. flavicoxis* were so discerning in their wing-fanning response to test stimuli (Fig. 5). Males wing fanned mostly in the presence of the complete pheromone blend, suggesting that they were motivated more by the quality of the female-produced signal than prospects of improved anemotactic orientation toward females. If true, the males' wing fanning could produce sound, possibly so specific that the female could use it to recognize conspecific males and discern between prospective mates (Sivinski and Webb, 1989).

Identification of the close-range sex pheromone components was attempted but failed despite the large sample size (4500 FE) that was analyzed. Nonetheless, numerous microanalytical treatments of, and electrophysiological recording with, pheromone extract (Table 1) suggested that all close-range sex pheromone components are unsaturated molecules of medium polarity, most likely esters. That these compounds remained below detection threshold of the mass spectrometer (~10 pg) even when 4500 FE were analyzed in a single injection, attests to the potency of the pheromone and the insects' sensitivity to it. Alternatively, the components are heat labile (as suggested by an anonymous reviewer), and defy identification by techniques involving gas chromatography.

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Hexyl Decanoate, the First Trail Pheromone Compound Identified in a Stingless Bee, *Trigona recursa*

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Abstract Foragers of many species of stingless bees guide their nestmates to food sources by means of scent trails deposited on solid substrates between the food and the nest. The corresponding trail pheromones are generally believed to be produced in the mandibular glands, although definitive experimental proof has never been provided. We tested the trail following behavior of recruits of *Trigona recursa* in field experiments with artificial scent trails branching off from natural scent trails of this stingless bee. First-time recruits (newcomers) did not follow these trails when they were laid with pure solvent or mandibular gland extract. However, they did follow trails made with labial gland extract. Chemical analyses of labial gland secretions revealed that hexyl decanoate was the dominant component ($72.4 \pm 1.9\%$ of all volatiles). Newcomers were significantly attracted to artificial trails made with synthetic hexyl decanoate, demonstrating its key function in eliciting scent-following behavior. According to our experiments with *T. recursa*, the trail pheromone is produced in the labial glands and not in the mandibular glands. Hexyl decanoate is the first component of a trail pheromone identified and proved to be behaviorally active in stingless bees.

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Introduction

Foragers of many species of stingless bees guide their nestmates to food sources using a scent trail deposited on solid substrates between the food and the nest (Lindauer and Kerr, 1958, 1960; Kerr and da Costa Cruz, 1961; Kerr et al., 1963; Kerr, 1969; Blum et al., 1970; Johnson, 1987; Jarau et al., 2003; Schmidt et al., 2003; Nieh et al., 2003, 2004; Sánchez et al., 2004). Many textbooks report that the pheromones involved in trail marking originate from the bees' mandibular glands (e.g., Wilson, 1971; Michener, 1974; Free, 1987; Roubik, 1989; Alcock, 2001; Wyatt, 2003). Definitive experimental proof of this assumption, based on conclusive trail-following bioassays of gland extracts or of synthetic compounds identified from mandibular glands, has never been provided (for a more detailed discussion see Jarau et al., 2004). In fact, Lindauer and Kerr (1958, 1960) reported in their pioneering investigations on stingless bee recruitment communication that recruits of *Scaptotrigona postica* never followed artificial scent trails made of mandibular gland secretions when these trails were laid in a direction other than that of the bees' natural scent trail.

Recently, Jarau et al. (2004) demonstrated that in *Trigona recursa*, a species that effectively recruits nestmates to food sources by means of scent trails (Jarau et al., 2003), mandibular gland extract had a repellent effect on both newcomers that arrived at a test feeder and on bees already sitting on the feeder to take up sugar solution. More importantly, however, feeders baited with labial gland extract clearly attracted the newcomers (Jarau et al., 2004). Labial gland secretions are released from the base of the glossa (Snodgrass, 1956; da Cruz Landim, 1967), and a forager rubs her extended glossa over exposed substrates like twigs, leaves, or blades of grass to deposit a scent mark on the feeder or along the way back to the nest (Jarau et al., 2004). From this observation and from the attractive effect of labial gland extract at the feeder, Jarau et al. (2004) concluded that the trail pheromone of *T. recursa* is produced in the labial glands and not the mandibular glands. This conclusion, however, was based on the behavior of newcomers at the food source and not on observations of trail-following behavior.

In the present study we used mandibular or labial gland extract to lay artificial scent trails branching off from the bees' natural scent trails to test whether newcomers follow such trails. In addition, we analyzed the components of mandibular and labial gland secretions and tested whether hexyl decanoate, which was found to be the dominant compound in the labial glands, releases trail-following behavior in recruited bees.

Methods and Materials

Study Site and Bee Nests

The experiments were carried out in March and April 2004 at the Ribeirão Preto Campus of the University of São Paulo with two nests (A and B) of *T. recursa* (Smith 1863) that naturally occurred there. Colonies of *T. recursa* usually consist of

about 3000 to 5000 adult individuals (Jarau et al., 2003). The two nests were separated from each other by approximately 900 m, and no additional nests were found near the test colonies. Bees marked with color during the experiments with one nest never showed up at the feeder during experiments with the other nest, nor were marked bees ever observed at the entrance of the wrong nest. Thus, it is highly unlikely that recruits from the nests would have been mixed in our experiments.

Test Substances for Bioassays

We tested whether one or more of the following substances release trail-following behavior in *T. recursa* recruits: (i) mandibular gland extract; (ii) labial gland extract; (iii) synthetic hexyl decanoate, the main compound identified from labial gland secretions (see “Results”); and (iv) pure solvent (pentane).

Gland extracts were prepared from *T. recursa* that had been collected during foraging at sugar solution feeders. The bees were sacrificed by freezing at -8°C and their glands dissected in saline solution under a microscope by carefully separating them from any tissue other than the respective glandular epithelia and reservoirs. Gland extracts from foragers of one nest were only used for bioassays with bees of the same nest. For all extracts, the amount of pentane was adjusted to 100 μl per pair of glands (i.e., nest A: 20 mandibular glands in 1000 μl pentane and 36 labial glands in 1800 μl pentane; nest B: 10 mandibular glands in 500 μl pentane and 18 labial glands in 900 μl pentane). Thus, 100 μl of extract corresponded to the gland content of one individual bee. The glands were left in the solvent for 24 hr at room temperature and the extracts subsequently stored in a freezer (-8°C) unless they were in use for the bioassays. For the tests with hexyl decanoate we diluted the synthetic ester in pentane (1 $\mu\text{g}/100 \mu\text{l}$).

Artificial Scent Trail Bioassays: Experimental Procedure

For each experiment we trained five marked foragers by stepwise moving a feeder with unscented 15% (w/w) aqueous sugar solution to a distance of 30 m from the nest. *T. recursa* recruits very well to food sources at this distance (Jarau et al., 2003). The location of the feeder relative to the nest was varied by experiment to exclude the possibility of a site preference by the bees. Recruitment never occurred during the training phase. Thus, recruited bees had not already been in the field searching for food when the artificial scent trails were installed. At its final location the training feeder was replaced with a clean feeder (henceforth the *recruitment feeder*) containing an unscented 40% (w/w) sugar solution, at which free recruitment (Jarau et al., 2003) was allowed. Unscented food was used to exclude an attraction of recruits to the feeders due to food volatiles, which they might have potentially encountered from the foragers inside the nest. As soon as the first forager began to lay a scent trail and to recruit nestmates from inside the nest, we installed an experimental scent trail between the nest and the recruitment feeder. The experimental trail branched off from the natural trail 20 m away from the nest, and led to a *test feeder* that was identical to the recruitment feeder in appearance and food supply (Fig. 1). The experimental trail consisted of 10 short branches of a Pitanga tree, *Eugenia uniflora* L., put into the ground at 1-m intervals and with their leaves baited with pentane (control experiments) or with one of the three test substances. To the leaves at each branch we applied 10 μl of the solvent control or

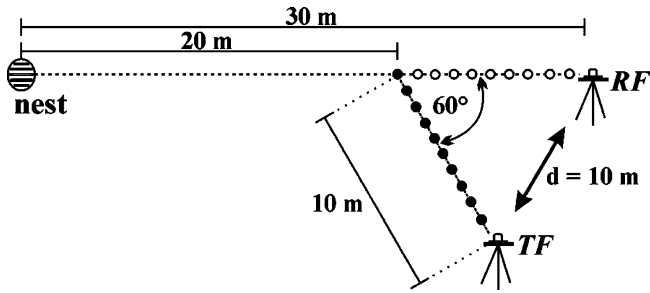


Fig. 1 Experimental setup for the behavioral assay of recruited *T. recursa* bees to artificial scent trails. Shaded circles represent baited and open circles represent unbaited short branches from a Pitanga tree, *E. uniflora*; RF, recruitment feeder; TF, test feeder; $d = 10$ m, distance between the two feeders

the test substance (corresponding to 0.1 bee equivalent mandibular or labial gland extracts or 0.1 μg hexyl decanoate). We used 0.1 bee equivalents because this concentration has been shown to be behaviorally active (Jarau et al., 2004). In addition, three scent marks (a total of 30 μl) were applied to the test feeder, because it was reported for several species of *Trigona* that the concentration of scent marks deposited by foragers is highest at the feeding table itself (Kerr et al., 1963; Johnson, 1987; Nieh et al., 2003, 2004). Untreated branches of *E. uniflora* were placed along the last 10 m towards the recruitment feeder (Fig. 1) in order to provide equal structural conditions for the 10-m test trail and for the corresponding route between the nest and the recruitment feeder. Five experiments (three with nest A, two with nest B) were carried out with each test substance and the control. On a particular day, a nest was used for only one experiment and the order of the substances tested on different days was randomized. Each trial with a substance lasted for 40 min, and the marks were renewed once after 20 min.

All bees arriving at the recruitment feeder for the first time were marked with colors that durably adhered to their thoraces and allowed to shuttle between the feeder and the nest without any interference. The bees that followed the experimental trail and arrived at the test feeder were captured, color marked, and only released when the experiment had ended. Thus, they were not allowed to return to the nest to report the location of the test feeder to nestmates during the experiment. Only unmarked recruits, which had never visited a feeder during a preceding experiment (henceforth called *newcomers*), were considered for the analyses. Thus, in order to minimize the impact of pseudoreplication in our experimental design, each individual bee was tested only once throughout the entire series of experiments. The percentage of bees that followed the experimental trail to the test feeder was calculated from the total number of newcomers that were counted at both feeders during an experiment.

Chemistry

For quantitative analyses, the labial glands of seven foragers and the mandibular glands of another seven foragers were dissected and extracted individually in 200 μl pentane (24 hr at room temperature). Prior to gas chromatographic analysis (Thermo Finnigan Trace GC, Rodano, MI, Italy) each extract was concentrated

to 30 μl , and 1 μg *n*-eicosane was added as an internal standard. The GC was equipped with a DB5 capillary column (30 m \times 0.32 mm, J&W Scientific, Folsom, CA, USA), and helium was used as carrier gas (constant linear flow rate 42 cm/sec). The GC was operated splitless at 50°C for 1 min, followed by a programmed increase to 310°C at a rate of 4°C/min and held at the final temperature for another 9 min.

For qualitative investigations, pooled extracts of the labial glands and the mandibular glands of 15 foragers were used. The samples were analyzed by using a Fisons Instruments gas chromatograph series 8000 linked to a Fisons MD 800 mass spectrometer (Fisons Instruments, Ismaning, Germany); 70 eV mass spectra were taken in EI mode. Separations were performed with a 30 m \times 0.25 mm fused silica column coated with DB5-MS, 0.25- μm thickness (J & W Scientific, Folsom, California, USA). The temperature was initially held at 60°C for 5 min, then increased by 10°C/min to 300°C and held at this temperature for 30 min. Helium served as the carrier gas. Identification of compounds was based on comparisons of mass spectra with the literature (McLafferty and Stauffer, 1989), as well as by comparing retention times and mass spectra using authentic reference substances. Esters that were not commercially available were synthesized from the corresponding acid chlorides and alcohols according to standard laboratory protocols. Mass and NMR spectra were in accordance with expected data (Francke et al., 2000). Racemic 2-octanol was purchased from Aldrich, whereas (*S*)-2-octanol was synthesized by Grignard reaction of pentyl magnesium bromide and commercially available (*S*)-methyloxirane (Aldrich). A mixture of diastereomeric derivatives of 2-octanol was prepared by reaction with (*S*)-2-acetoxypropanoic acid chloride (Aldrich). The corresponding derivative of (*S*)-2-octanol served as the reference. The derivatization was carried out as follows: To a natural extract containing one to two bee equivalents was added 100 μl carbon disulfide and a solution of 10 μl of diethylether and 1% of pyridine and 1 μl of (*S*)-2-acetoxypropanoic acid chloride. In a screw-capped 1-ml vial, the mixture was kept at 40°C for 12 hr. After cooling to room temperature, the mixture was diluted with 500 μl pentane and washed with two, 200- μl aliquots of water. The organic layer was filtered over 300 mg silica (Merck silica 60, 230-40 mesh). After additional elution with 2 ml diethylether, the resulting mixture was concentrated and submitted to GC/MS analysis. Separation conditions were the same as given above.

Statistical Analyses

Bioassay data were normally distributed with homogeneous variances. We therefore applied parametric statistical tests. One-way analysis of variance (ANOVA) was used to check for a significant effect of artificial trail treatment on the percentages of bees that landed on the test feeder during the experiments. Multiple comparisons between the single groups were carried out by using the Student–Newman–Keuls method to reveal significant differences among the mean percentages of bees following the control or various test substance trails.

Absolute amounts of the compounds detected in the gland extracts were determined by comparison of their peak areas with the peak area of the internal standard. The relative amount of a compound was calculated as the percentage of the total amount of all volatile gland constituents detected. For both variables we calculated the mean \pm SD ($N = 7$).

Results

Behavioral Bioassays

The total numbers of recruits reaching the two feeding tables in the single experiments proved to be similar for the different test substances (five experiments with each substance, mean \pm SD: mandibular gland extract: 42.8 ± 27.9 , range 23–91; labial gland extract: 48.4 ± 30.2 , range 18–94; hexyl decanoate: 35.8 ± 15.1 , range 19–54; pentane: 54.0 ± 21.1 , range 29–87). In all test series, the majority of the newcomers followed the natural scent trails made by the recruiting foragers. There was a highly significant difference, however, in the percentage of newcomers following the four different types of artificial scent trails [Fig. 2; one-way ANOVA: $F(3,16) = 45.599$, $P < 0.001$]. In the control experiments, only $1.4 \pm 1.7\%$ (mean \pm SD) of the bees arrived at the test feeder at the end of the pentane trail. This percentage is not significantly different (SNK method: $q = 0.407$, $P = 0.778$) from the $0.4 \pm 1.1\%$ of newcomers that reached the test feeder during the experiments with mandibular gland extract trails. In contrast, significantly more bees followed the labial gland extract trails ($35.4 \pm 9.7\%$; $q = 14.098$, $P < 0.001$) and the hexyl decanoate trails ($10.8 \pm 4.2\%$; $q = 3.867$, $P = 0.015$) than the control trails. Similarly, there were significant differences in the percentage of bees reaching the test feeders in the experiments using labial gland extracts and hexyl decanoate ($q = 10.231$, $P < 0.001$), labial gland and mandibular gland extracts ($q = 14.504$, $P < 0.001$), and hexyl decanoate and mandibular gland extracts ($q = 4.273$, $P = 0.021$).

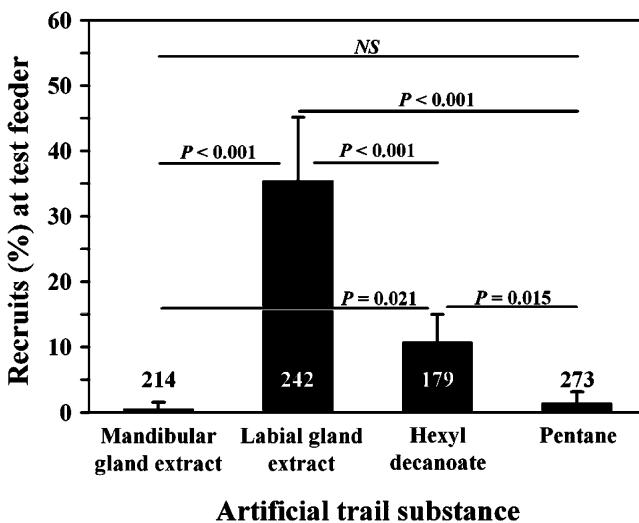


Fig. 2 Mean (\pm SD) response ($N = 5$ experiments per trail substance) to artificial scent trails measured as the percentage of newcomers of *T. recursa* that reached the test feeder (100% = newcomers at recruitment feeder + newcomers at test feeder). Numbers above or in the bars are the total numbers of the individuals tested; NS, not significant; P values for significant differences between groups (Student–Newman–Keuls method) are given

Table 1 Volatiles from labial gland secretions of *T. recursa* foragers^a

Number ^b	Compound name	Absolute amount (μg)	Relative amount (%)
1	Hexyl decanoate ^c	0.02 ± 0.01	0.50 ± 0.07
2	Hexyl decanoate	3.44 ± 1.02	72.38 ± 1.94
3	Octyl decanoate	0.22 ± 0.07	4.55 ± 0.27
4	Hexyl dodecanoate	0.09 ± 0.03	1.83 ± 0.06
5	Methyl octadecenoate ^c	0.03 ± 0.01	0.64 ± 0.23
6	Unidentified	0.09 ± 0.04	1.80 ± 0.42
7	Unidentified	0.26 ± 0.08	5.54 ± 1.30
8	Ethyl octadecenoate ^c	0.03 ± 0.02	0.58 ± 0.23
9	Isopropyl octadecenoate ^c	0.05 ± 0.03	1.06 ± 0.33

^a Components that make up at least 0.5% of the total gland content were quantified (mean ± SD, *N* = 7).

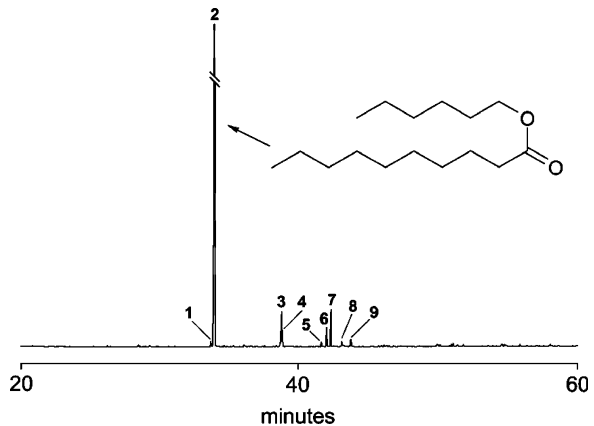
^b Numbering follows increasing retention times and is identical to peak numbering in Fig. 3. Trace components eluting before hexyl decanoate were identified to be decanoic acid, ethyl decanoate, isopropyl decanoate, propyl decanoate, methyl dodecanoate, isobutyl decanoate, hexyl octanoate, butyl decanoate, ethyl dodecanoate, isopropyl dodecanoate, and propyl dodecanoate. After ethyl octadecenoate, traces of decyl decanoate, octyl dodecanoate, hexyl tetradecanoate, and hexyl hexadecanoate also eluted.

^c Double bond position not identified.

Chemistry

The mean total amount of volatile constituents found in the labial glands was $4.76 \pm 1.39 \mu\text{g}$ (*N* = 7). Among the compounds that occurred in relative amounts of at least 0.5%, seven carboxylic acid alkyl esters were identified, whereas two substances remained unknown (Table 1). Hexyl decanoate was by far the dominant compound (Table 1, Fig. 3), comprising almost three quarters of all volatiles. The mass spectrometric fragmentation pattern of wax-type esters has been described from stingless bees (Francke et al., 2000). In addition to the seven quantified esters, trace amounts of decanoic acid and 14 additional esters were identified (see legend to Table 1). Traces of hexyl decanoate were also found in the extracts of mandibular

Fig. 3 Gas chromatogram of an extract of the labial glands from a *T. recursa* forager. The dominant compound (peak 2) is hexyl decanoate. See Table 1 for the remaining compound names



glands—possibly as a contaminant transferred from the labial glands during preparation. Volatiles of the mandibular glands were dominated by 2-octanol ($99.67 \pm 0.19\%$ of gland contents, $N = 7$). The diastereomeric (*S*)-acetyl lactic acid derivatives of 2-octanol were separated by GC, revealing that the natural product was 96% (*S*).

Discussion

Our experiments show that naïve recruits of a stingless bee can be misled by artificial scent trails during the natural process of recruitment to a food source. In contradiction to the prevailing opinion in the literature, however, the bees did not follow trails scented with substances from the mandibular glands. Instead, they followed trails made with labial gland extract, confirming our earlier hypothesis (Jarau et al., 2004) that the trail pheromone of *T. recursa* is produced in the labial glands. The effectiveness of the artificial scent trails made with natural extracts is even more remarkable because the bees had to leave their straight flight path towards the recruitment feeder and turn at an angle of 60° to follow the experimental trail. The nonnatural situation of a bent trail may explain why the majority of the recruits still arrived at the recruitment feeder instead of being equally distributed at the two feeders. It may also be possible, however, that additional information, such as guidance of recruits by the forager, is involved in the recruitment system of *T. recursa*. In *S. postica* (Lindauer and Kerr, 1958, 1960) and *T. corvina* (Aguilar et al., 2005), guiding flights most likely play an important role in addition to scent trails. On the other hand, information could have been lost or jammed in the extracts due to the work-up and application procedure.

The effectiveness of trails made with synthetic hexyl decanoate in releasing trail-following behavior clearly demonstrates that this substance is a key compound of the trail pheromone in *T. recursa*. Therefore, hexyl decanoate is the first trail pheromone component identified and proved to be behaviorally active in stingless bees. Its reduced attractiveness as compared to the labial gland extract indicates, however, that the complete pheromone contains additional substances that may specify and enhance the activity of the signal that is dominated by hexyl decanoate. In *T. recursa*, octyl decanoate and hexyl dodecanoate, along with two unidentified substances, are the next most abundant volatiles in the labial glands. These compounds along with various minor components might add species and/or nest-specific information, i.e., they “fine tune” the signal. Such details could be important in an environment shared with other species of stingless bees competing for the same resources and communicating by means of scent trails as well. Hexyl decanoate was also detected in the cephalic secretions of *Trigona hyalinata* and *Trigona hypogea* (Francke et al., 2000), which are partially sympatric with *T. recursa*. In order to keep the information about the food location “private,” the trail pheromone should contain a species-specific blend of different compounds or of the same compounds in different relative proportions. The gland secretions might also contain interspecifically active repellents. The activities of additional compounds in the trail pheromone of *T. recursa* and its species specificity will be investigated in future studies.

Many diverse chemical structures have been identified to function as trail pheromones in social insects, mainly in ants and termites (Moore, 1974; Hölldobler

and Wilson, 1990; Morgan, 1990; Keeling et al., 2004), but hexyl decanoate was not known as a trail pheromone in any species before. The compound (MW 256) is a typical low molecular weight wax-type ester, representing a structure that is particularly widespread among secondary metabolites produced by bees. It shows medium to low polarity and boils at about 300°C at atmospheric pressure. The compound has been described as a volatile component of the cephalic secretions of two other *Trigona* species, *T. hyalinata* and *T. hypogea* (Francke et al., 2000), but no biological significance has been reported. Future investigations with the aim to identify trail pheromones of other species of stingless bees should be performed in order to better understand the role of esters as marking substances in this group of insects.

The presence of 2-octanol in cephalic secretions of stingless bees has been reported for several *Trigona* spp. and *Frieseomelitta* spp. (Francke et al., 2000; Cruz López et al., 2002), but neither the enantiomeric composition of the compound nor its biological significance is known in these species. We did not test synthetic 2-octanol in our bioassays with *T. recursa*, but the failure of mandibular gland extracts in eliciting trail-following behavior demonstrates that 2-octanol, its almost exclusive component, does not act as a trail pheromone.

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Field Evidence of an Airborne Enemy-Avoidance Kairomone in Wolf Spiders

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Abstract *Hogna helluo*, *Pardosa milvina*, and *Trochosa ruricola* are co-occurring species of wolf spiders within agricultural fields in the eastern USA. The largest species, *H. helluo*, is a common predator of the two smaller species, *P. milvina* and *T. ruricola*. *H. helluo* frequently resides within soil fissures where *P. milvina* and *T. ruricola* may be attacked when they enter or walk near these fissures. We tested the ability of *P. milvina* and *T. ruricola* to avoid *H. helluo*-containing burrows by detecting airborne enemy-avoidance kairomones associated with *H. helluo*. To simulate soil fissures and control for visual and vibratory means of predator detection, we baited funneled pitfall traps with one of the following ($N = 20$ traps/treatment): (1) blank (empty trap); (2) one house cricket (*Acheta domesticus*); (3) one adult female *H. helluo*; and (4) one adult male *H. helluo*. Over two separate 3-d periods, we measured pitfall capture rates of *P. milvina* and *T. ruricola* as well as other incidentally captured ground-dwelling arthropods. During the day, male *P. milvina* showed significant avoidance of pitfall traps baited with *H. helluo* of either sex but showed no avoidance of empty traps or those containing crickets. At night, male *T. ruricola* showed a qualitatively similar pattern of avoiding *H. helluo*-baited traps, but the differences were not statistically significant. We found no evidence that other ground-dwelling arthropods either avoided or were attracted to *H. helluo*-baited traps. This study suggests that an airborne enemy-avoidance kairomone may mediate behavior among male *P. milvina* in the field.

Keywords Semiochemical · Lycosidae · Airborne · Chemical cue · Antipredator · Predation risk

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Introduction

Semiochemicals among insects have been studied extensively, but comparable studies among spiders have received less attention (Francke and Schulz, 1999; Dicke and Grostal, 2001; Schulz, 2004). Of the studies documenting behavioral responses to chemical cues among spiders, the vast majority (reviewed in Schulz, 2004) have examined pheromones rather than kairomones or other types of semiochemicals. Spider kairomones documented so far are of two types: foraging kairomones used to locate prey and enemy-avoidance kairomones used by prey species to avoid predators (Ruther et al., 2002). Foraging kairomones are known among ant-hunting spiders (Zodariidae) (Allan et al., 1996), jumping spiders (Salticidae) (Clark et al., 2000; Jackson et al., 2002), and wolf spiders (Lycosidae) (Persons and Uetz, 1996; Punzo and Kukoyi, 1997; Persons and Rypstra, 2000; Hoeffler et al., 2002). Enemy-avoidance kairomones have been found in bowl-and-doily spiders (Linyphiidae) (Suter et al., 1989), but have been studied more extensively in the Lycosidae (Punzo, 1997; Persons et al., 2001).

Pardosa milvina (Araneae, Lycosidae, Hentz, 1844) is a small wolf spider that exhibits a range of antipredator behaviors, such as freezing, vertical climbing, and reduced activity, when exposed to silk and excreta from the larger syntopic wolf spider *Hogna helluo* (Araneae, Lycosidae, Walckenaer, 1837) (Persons and Rypstra, 2001; Persons et al., 2001, 2002; Barnes et al., 2002; Lehmann et al., 2004). In these studies, *P. milvina* was in direct contact with substrates containing deposits from *H. helluo*, and, therefore, the methods allowed for the possibility that *P. milvina* gained information about *H. helluo* via tactile cues. However, one of the experiments revealed that *P. milvina* avoids substrates that have been occupied by *H. helluo* even before coming into physical contact with them (Persons and Rypstra, 2001). This finding suggests that *P. milvina* may be able to detect an airborne cue released by *H. helluo*.

Pardosa milvina can be expected to gain significant benefits by being able to detect airborne enemy-avoidance kairomones of *H. helluo*. *H. helluo* is a common ground-dwelling spider in agricultural systems in the eastern USA (Marshall et al., 2002) and a facultative burrower that often hides in soil fissures during the day when *P. milvina* is most active (Walker et al., 1999; Marshall et al., 2002). Many members of the genus *Pardosa*, including *P. milvina*, are active diurnal foragers that range widely across the ground surface and use soil fissures similar to those used by *H. helluo* as temporary retreats (Walker et al., 1999; Samu et al., 2003). Thus, it would be advantageous for *P. milvina* to detect volatiles emitted either directly from *H. helluo* or the material it leaves behind so that it could avoid entering burrows or soil fissures occupied by *H. helluo* as it navigates the landscape.

Trochosa ruricola (Araneae, Lycosidae, De Geer, 1778) is also a co-occurring agrobiontic wolf spider species intermediate both in size and abundance relative to *P. milvina* and *H. helluo*. Anecdotal field observations suggest that *T. ruricola*, unlike *P. milvina*, prefers burrowing or hiding under objects during the day and actively forages at night. Field and laboratory observations of *T. ruricola* indicate that they are also commonly preyed upon by *H. helluo* (personal observation) and may also benefit substantially from early detection of *H. helluo* via an airborne cue.

In an open plot field study, we tested the ability of *P. milvina* and *T. ruricola* to detect airborne enemy-avoidance kairomones associated with *H. helluo*. By

enclosing *H. helluo* in traps that allowed volatiles to escape but prevented free-ranging *P. milvina* and *T. ruricola* from having any direct contact cues, we were able to use differences in the rates at which these smaller wolf spider species were captured in the traps as evidence of either or both species using airborne information to avoid the predator. We also measured trapping frequencies of other incidentally captured ground-dwelling arthropods to assess if these other species can detect airborne volatiles from *H. helluo* and to control for the possibility that these other captured arthropods might serve as an attractant or deterrent for *P. milvina* and *T. ruricola*.

Methods and Materials

Adult male and female *H. helluo* were collected in late May 2003 from mixed corn and alfalfa fields in White Deer Township, Union County, PA, USA. They were maintained in individual opaque plastic containers (10.5-cm diam \times 7.5 cm deep) with a 1-cm-deep layer of moistened peat moss covering the bottom. Prior to being used as stimuli within pitfall traps, *H. helluo* were maintained at 22°C with a 14:10 hr light/dark cycle. When in the laboratory, spiders were fed with two to four adult house crickets *Acheta domesticus* (L.) (Orthoptera: Gryllidae) each week.

In an 8-ha fallow cornfield at Susquehanna University Ecology Research Center, Selingsgrove, Snyder County, PA, USA, a grid of 96 live-fall pitfall traps was established (12 columns \times 8 rows). This site was selected because of the high population density of *P. milvina* and *T. ruricola*. The rows and columns of traps were 12.5 m from each other. An individual trap consisted of a vertically oriented 0.7-cm-thick polyvinyl chloride (PVC) pipe (10.1-cm diam \times 18 cm deep) that was inserted into the soil flush with the ground surface. A plastic cup (9.8-cm diam \times 17 cm deep) was then inserted within the PVC sleeve and pushed to the bottom of the hole. Each pitfall trap was then capped with a small smooth plastic funnel (10-cm diam at top, tapering to an opening 2-cm diam at the bottom). The lip of the funnel was flushed with the ground and with the edge of the cup making it possible for wolf spiders and other small crawling arthropods to get in but not get out of the trap. This design also prevented spiders from viewing the contents of the cup prior to being trapped. Seismic detection of *H. helluo* from outside the trap was also highly unlikely because there was a small gap between the inner cup and outer PVC sleeve that would have impeded transmission of *H. helluo* movement across the barrier. Seismic information was further limited by restricting *H. helluo* to small containers that minimized their movement during the testing period. The relatively small funnel opening effectively prevented large predatory species from physically entering the trap and potentially preying on the smaller spiders.

We checked the empty traps in the grid for 3-d prior to the experiment to determine baseline trapping rates of *P. milvina* and to test for possible trap site effects. Traps remained open for an 8-hr sampling period (0900–1700 hr). A χ^2 analysis was performed on the 80 traps with the highest capture rates by using individual trap as the treatment. We found no significant difference in the total number of captured *P. milvina* spiders across traps ($\chi^2 = 84.683$, $df = 79$; $C_v = 100.749$, $P = 0.25$; $N = 670$). We caught 503 males, 153 females, and 14 unsexed juveniles. All 80 traps were then paired (highest and lowest, second highest and

second lowest, third highest and third lowest, etc.) to further minimize potential trap bias effects. We sequentially assigned each of these 40 paired pitfall traps to each of the assigned treatments and ran a second χ^2 test by using each of the 40 pairs of traps as a separate treatment. We found no significant difference in capture rates of *P. milvina* among these pairs, indicating no confounding trap bias among unbaited traps by treatment pairing ($\chi^2 = 9.317$, $df = 39$; $C_v = 54.572$, $P > 0.999$; $N = 670$).

The experiment was begun with one of the following stimuli placed in the bottom of each pitfall trap: (1) no added stimulus (negative control, $N = 20$); (2) one adult *A. domesticus* (positive control, $N = 20$); (3) one adult female *H. helluo* ($N = 20$); and (4) one adult male *H. helluo* ($N = 20$). The traps were baited by placing the stimulus arthropod in a plastic vial (4.5-cm diam \times 8 cm deep) that contained a folded moist paper towel as a water source. We then covered the vial with two layers of fiberglass screening and secured it with a rubber band. Each vial was placed in its assigned treatment trap. The fiberglass screening allowed any volatiles present to emanate and simultaneously served to further obscure visual detection of the stimulus from directly above. Each negative control trap contained a vial, moist paper towel, rubber band, and fiberglass screening, but was otherwise empty. All traps were live traps and devoid of preservatives or other chemicals.

The experiment was carried out from June 17 to 23, 2003. There was no rain during this period. Traps remained open daily during an 8hr period (0900–1700 hr). All traps were checked and emptied of arthropods three times daily during the daylight hours of June 17–19. Traps were checked at 1100, 1400, and 1700 hr. For June 21–23, traps were checked three times at night during an 8-hr sampling period from 1900 to 0500 hr. Traps were checked at 2300, 0200, and 0500 hr, primarily to assess *T. ruricola* trap catch rates, but all arthropods found in the traps were recorded during this period. One-way analyses of variance (ANOVAs) were used to analyze the differences in the total number of *P. milvina* and *T. ruricola* captured among the four trap treatments during the day and night, respectively. We then used a Tukey *post hoc* comparison of means test to compare significant differences among treatments. Additional arthropods caught incidentally in the traps were recorded and identified at least to class and, in some cases, to species. When sample sizes permitted, these taxa were also subjected to statistical analysis across treatments. For all arthropods other than *P. milvina* and *T. ruricola*, sample sizes were insufficient for ANOVA. Instead, captures from individual traps were pooled by treatment and subjected to a χ^2 analysis when possible. When sample sizes allowed, we also used binomial tests to compare the total number of spiders caught across all treatments during the day vs. the night for each age and sex category of *P. milvina* and *T. ruricola*. Using a binomial test, we also compared the number of males vs. the number of females captured for both diurnal and nocturnal testing periods.

Results

There was a significant effect of treatment on the total number of *P. milvina* per trap during the day (ANOVA, $F = 9.549$, $P < 0.001$, $N = 519$, $\beta = 0.998$; Tables 1 and 2). Fewer males were captured in traps with *H. helluo* (ANOVA, $F = 11.084$, $P < 0.001$, $N = 374$, $\beta = 1.00$; Table 1), but there was no difference in female *P. milvina* captures among treatments (ANOVA, $F = 1.548$, $P = 0.209$, $N = 140$, $\beta = 0.383$; Table 1).

Table 1 Number of *Pardosa milvina* and *Trochosa ruricola* (mean ± SE) caught per treatment^a per trap, June 17–23, 2003, Snyder County, PA, USA^b

Sex	Blank	Cricket	Female <i>Hogna helluo</i>	Male <i>Hogna helluo</i>	F	P
Diurnal (N)						
Total <i>P. milvina</i> (519)	9.0 ± 0.76a	7.9 ± 0.73a	4.65 ± 0.72b	4.4 ± 0.78b	9.55	<0.001
Male <i>P. milvina</i> (374)	7.0 ± 0.71a	5.65 ± 0.61a	2.9 ± 0.51b	3.15 ± 0.53b	11.08	<0.001
Female <i>P. milvina</i> (140)	1.95 ± 0.34	2.0 ± 0.39	1.5 ± 0.31	1.1 ± 0.31	1.55	0.21
Nocturnal (N)						
Total <i>T. ruricola</i> (217)	0.51 ± 0.06	0.52 ± 0.08	0.38 ± 0.06	0.40 ± 0.06	1.19	0.31
Male <i>T. ruricola</i> (187)	0.48 ± 0.06	0.44 ± 0.07	0.28 ± 0.05	0.36 ± 0.06	2.24	0.08
Female <i>T. ruricola</i> (27)	0.03 ± 0.02	0.08 ± 0.03	0.08 ± 0.03	0.03 ± 0.02	1.49	0.22

^a Treatments were empty trap (negative control), *Acheta domesticus* house crickets (positive control), female *H. helluo*, and male *H. helluo*.

^b Letters after means indicate significant differences between treatments based on Tukey *post hoc* test. Numbers in parentheses indicate sample size.

Sample sizes for *P. milvina* at night (Table 3) were too small to analyze statistically for age and sex effects by using ANOVA; however, by using a χ^2 test, we did find that significantly fewer *P. milvina* were captured at night in traps baited with *A. domesticus* compared to all other groups (Table 3), but we found no significant avoidance of traps baited with *H. helluo*.

A total of 597 *P. milvina* were captured during diurnal and nocturnal sampling periods (Tables 2 and 3). Significantly more (519 vs. 78) spiders were captured during the day than during the night (binomial test; $P < 0.001$) supporting our general field observations that *P. milvina* is active diurnally. Over the entire diurnal sampling period, 519 *P. milvina* were caught (374 males, 140 females, and 5 juveniles too young to accurately determine sex; Table 2). In comparison, over the entire nocturnal sampling period, 78 *P. milvina* were caught (12 males, 26 females, and 40 juveniles; Table 3). Although *P. milvina* is generally diurnal, we found significant differences in capture rates among adult males, adult females, and juveniles during the day and night. We captured significantly fewer juveniles during the day than at night (binomial test; $P < 0.001$). Significantly fewer females were captured than males during the day (binomial test; $P < 0.001$), but at night, this trend was reversed with significantly fewer males captured than females (binomial test; $P = 0.05$).

There was no significant effect of treatment on the total number of *T. ruricola* per trap during the night (ANOVA, $F = 1.186$, $P = 0.314$, $N = 217$, $\beta = 0.310$; Table 1). Adult male capture rates did not vary significantly among treatments (ANOVA, $F = 2.238$, $P = 0.083$, $N = 187$, $\beta = 0.558$; Tables 1 and 3), but fewer males were found in the *H. helluo*-baited traps. Fewer female *T. ruricola* were caught during the trapping period, and we found no difference in female trapping rates among treatments (ANOVA, $F = 0.725$, $P = 0.548$, $N = 27$, $\beta = 0.175$; Tables 1 and 3).

Table 2 Diurnal captures of arthropod and gastropod taxa in pitfall traps by treatment, June 17–19, 2003, Snyder County, PA, USA^a

Taxon	Treatment					
	Blank	Cricket	Female <i>H. helluo</i>	Male <i>H. helluo</i>	χ^2 ^b	<i>P</i>
Order Araneae						
Family Lycosidae						
Male <i>P. milvina</i>	140	113	58	63	50.62	<i>P</i> < 0.001
Female <i>P. milvina</i>	39	44	33	24	6.34	0.05 < <i>P</i> < 0.1
Juvenile <i>P. milvina</i>	1	1	2	1	*	
<i>P. milvina</i> total	180	158	93	88	49.45	<i>P</i> < 0.001
<i>Pardosa saxatilis</i>	0	0	1	0	*	
<i>Allocosa funerea</i>	3	3	3	0	*	
<i>Schizocosa avida</i>	1	0	1	1	*	
<i>T. rucicola</i>	0	0	1	2	*	
<i>Pirata</i> spp.	1	1	0	0	*	
<i>Gladicosa</i> spp.	0	0	1	1	*	
Family Thomisidae						
<i>Xysticus gulosus</i>	7	9	10	10	0.67	0.95 < <i>P</i> < 0.975
Male <i>Xysticus ferox</i>	32	25	22	25	2.08	0.50 < <i>P</i> < 0.75
Female <i>X. ferox</i>	22	14	11	12	5.07	0.10 < <i>P</i> < 0.25
<i>X. ferox</i> total	54	39	33	37	6.20	0.10 < <i>P</i> < 0.25
Family Gnaphosidae						
<i>Zelotes</i> sp.	7	5	4	6	0.91	0.95 < <i>P</i> < 0.975
Family Clubionidae						
<i>Clubiona</i> sp.	0	2	0	2	*	
Family Salticidae						
<i>Phidippus audax</i> juvenile	0	0	1	0	*	
Family Dictynidae sp.	4	1	0	0	*	
Order Opiliones	3	2	1	3	*	
Order Coleoptera						
Family Carabidae						
<i>Scarites subterraneus</i>	2	2	3	5	*	
Family Curculionidae						
Family Chrysomelidae	11	3	6	5	5.56	0.1 < <i>P</i> < 0.25
Order Hymenoptera						
Family Pompiliidae	13	23	17	17	2.91	0.25 < <i>P</i> < 0.50
Order Collembola sp.	0	1	0	0	*	
Order Hemiptera						
Family Pentatomidae						
<i>Acrosternum</i> sp.	0	1	0	0	*	
Order Orthoptera						
Family Gryllidae						
<i>Gryllus pennsylvanicus</i>	2	1	0	0	*	
Class Gastropoda	0	2	2	0	*	

^aTreatments were empty trap (negative control), *A. domesticus* house crickets (positive control), female *H. helluo*, and male *H. helluo*.

^b χ^2 tests were completed only for taxa where the expected frequencies of the treatments were greater than 5.

A total of 220 *T. ruricola* were captured during diurnal and nocturnal sampling periods (Tables 2 and 3). The species was caught in higher numbers during the night than during the day (binomial test; $P < 0.001$; Tables 2 and 3). Only three *T. ruricola* were caught over the entire 3-d diurnal period (Table 2), quantitatively supporting our hypothesis that *T. ruricola* is largely nocturnal. Over the entire nocturnal sampling period, 217 *T. ruricola* were caught (187 adult males, 27 adult females, and 3 late-instar juvenile females; Table 3). During nocturnal sampling, we captured more males than females (binomial test; $P < 0.001$).

A number of other arthropods were captured in the pitfall traps (Tables 2 and 3). In most cases, there were too few individuals to analyze statistically by treatment. Of those species with sufficient sample size for analysis, we found no differences in trapping rate across treatments (Tables 2 and 3). However, in males of the crab spider *Xysticus gulosus* (Keyserling, 1880), there appeared to be a nonsignificant trend to avoid *H. helluo*-baited traps (Table 3).

Discussion

Our results suggest that both sexes of *H. helluo* release an airborne kairomone that enables male *P. milvina* to detect and avoid *H. helluo* during the day, but we found no evidence that *P. milvina* or *T. ruricola* avoid *H. helluo* at night. Our data indicate that the detection by *P. milvina* is not very fine-tuned. Although we found significantly fewer male *P. milvina* in traps baited with *H. helluo*, *P. milvina* did not discriminate between male and female predators. In addition, they were not attracted to or repulsed by whatever cues might be released by adult crickets during the day. Although *T. ruricola* showed no statistically significant trap avoidance, the number of males found in *H. helluo*-baited traps was 19–40% lower than the number in cricket-baited or control traps, respectively.

Our finding that male, but not female, *P. milvina* detect and avoid *H. helluo* may be a statistical artifact because, throughout the study, male capture frequency was much higher than females and resulted in greater statistical power ($\beta = 1.00$ for males vs. 0.383 for females). This higher capture frequency suggests that males of *P. milvina* tend to be more active than females overall, as may be true of most wolf spiders (Hallander, 1967; Cady, 1984; Persons, 1999; Persons and Uetz, 1999). However, in laboratory studies, no differences in male and female *P. milvina* activity were detected (Walker and Rypstra, 2003), suggesting that pitfall traps may be an indicator of *P. milvina* space use rather than activity level *per se*, or that laboratory findings are not representative of movement patterns of spiders under field conditions. Notably, other studies have shown that an airborne pheromone attracts *P. milvina* males to female conspecifics (Searcy et al., 1999). In addition, males use pheromones to identify the mating status of females, whereas females assess male quality by using primarily visual cues (Rypstra et al., 2003). Thus, it may be that males are more sensitive to chemical cues in general and, specifically, more sensitive to airborne cues than are females.

Female *H. helluo* show significantly higher attack frequencies on *P. milvina* than male *H. helluo* (Walker and Rypstra, 2002; Lehmann et al., 2004); therefore, females pose a much greater predation risk. It was therefore surprising that *P. milvina* was unable to discriminate between male and female *H. helluo*. If an airborne sex

Table 3 Nocturnal captures of arthropod and gastropod taxa in pitfall traps by treatment, June 21–23, 2003, Snyder County, PA, USA^a

Taxon	Treatment					
	Blank	Cricket	Female <i>H. helluo</i>	Male <i>H. helluo</i>	χ^2 ^b	<i>P</i>
Order Araneae						
Family Lycosidae						
Male <i>P. milvina</i>	4	1	0	7	*	
Female <i>P. milvina</i>	8	2	10	6	5.38	0.10 < <i>P</i> < 0.25
Juvenile <i>P. milvina</i>	11	5	13	11	3.6	0.25 < <i>P</i> < 0.50
<i>P. milvina</i> total	23	8	23	24	11.19	0.01 < <i>P</i> < 0.025
Male <i>T. ruricola</i>	57	53	34	43	6.86	0.05 < <i>P</i> < 0.10
Female <i>T. ruricola</i>	4	9	10	4	4.56	0.10 < <i>P</i> < 0.25
Juvenile <i>T. ruricola</i>	0	0	2	1	*	
<i>T. ruricola</i> total	61	62	46	48	4.17	0.10 < <i>P</i> < 0.25
<i>A. funerea</i>	0	1	0	0	*	
<i>Sch. avida</i>	0	0	0	2	*	
Male <i>H. helluo</i>	1	0	0	0	*	
Thomisidae						
<i>X. gulosus</i>	15	19	6	11	7.27	0.05 < <i>P</i> < 0.10
Juvenile <i>Xysticus</i> spp.	5	5	7	10	2.48	0.25 < <i>P</i> < 0.50
<i>Xysticus</i> spp. total	20	24	13	21	3.33	0.25 < <i>P</i> < 0.50
Family Linyphiidae						
Ergioninae	4	2	3	2	*	
Gnaphosidae						
<i>Zylothes</i> sp.	0	1	0	0	*	
Order Coleoptera						
Family Chrysomelidae						
Unidentified flea beetle spp.	29	35	28	37	1.82	0.50 < <i>P</i> < 0.75
Family Carabidae	1	1	0	1	*	
<i>S. subterraneus</i>	1	1	0	1	*	
Order Orthoptera						
Family Gryllidae						
<i>G. pennsylvanicus</i>	8	10	5	6	2.03	0.50 < <i>P</i> < 0.75
Family Acrididae	3	1	1	2	*	
Order Hymenoptera						
Family Formicidae	7	6	9	11	1.79	0.50 < <i>P</i> < 0.75
Family Apiidae						
<i>Apis mellifera</i>	0	2	1	1	*	
Collembola	1	0	2	2	*	
Order Dermaptera						
Family Forficulidae	0	2	0	1	*	
Class Diplopoda	0	0	1	2	*	
Class Chilopoda	0	0	1	0	*	
Class Isopoda spp.	9	2	2	3	*	
Class Gastropoda: slug	0	1	0	0	*	
Class Clitellata						
Family Lumbriculidae						
<i>Lumbricus</i> spp.	1	0	1	1	*	

^aTreatments were empty trap (negative control), *A. domesticus* house crickets (positive control), female *H. helluo*, and male *H. helluo*.

^b χ^2 tests were completed only for taxa where the expected frequencies of the treatments were greater than 5.

pheromone of *H. helluo* were used as an enemy-avoidance kairomone by *P. milvina*, sex discrimination would appear to be readily achievable. Because volatiles from adult male or adult female *H. helluo* elicited similar avoidance responses by *P. milvina*, this suggests that the kairomone is not a sex pheromone.

Although it has been shown that *P. milvina* can recognize substrate-borne cues from prey items (Hoeffler et al., 2002), we uncovered no evidence that *P. milvina* responded either positively or negatively to the crickets contained in our traps during the day. We did find a significant tendency for *P. milvina* to avoid cricket-baited traps at night. The adult crickets that we used were much larger than those consumed by *P. milvina*. If *P. milvina* detected the crickets, and was able to determine their size, then they may have avoided them based on this criterion alone. Previous studies have demonstrated that *P. milvina* can estimate the size of *H. helluo* based on substrate-borne chemical cues alone by responding to the quantity of silk and excreta produced (Persons and Rypstra, 2001). Alternatively, *P. milvina* may not have had sufficient experience with crickets of this size and species to respond strongly to their emissions one way or another. Previous studies have found that *H. helluo* are attracted to volatiles from *P. milvina* but apparently not *A. domesticus* (Persons and Rypstra, 2000).

Although male *T. ruricola* and male *X. gulosus* showed the same general pattern as *P. milvina* with respect to trap capture rates, differences did not reach the level of statistical significance. This could be attributable to low statistical power. As an alternative hypothesis, *T. ruricola* and *H. helluo* are both nocturnally active and are closer in size to one another. As such, *H. helluo* may not present as great of a predation risk to *T. ruricola* as it does to *P. milvina*. *Xysticus gulosus* is smaller than *T. ruricola* and well within the prey size range of *H. helluo*. However, unlike *T. ruricola*, we have never observed *X. gulosus* predation by *H. helluo* in the field. Although we found no evidence that *Xysticus* spp. respond to *H. helluo* volatiles, there is field evidence that some *Xysticus* species are capable of detecting volatile chemicals (Aldrich and Barros, 1995).

Pardosa milvina failed to show significant avoidance of *H. helluo* at night. This also could have been attributable to a lack of statistical power because far fewer *P. milvina* were captured at night. Alternatively, diurnal and nocturnal conditions may have influenced kairomone production and persistence by *H. helluo*. Previous studies have found that *P. milvina* antipredator responses are reduced when they encounter *H. helluo* silk and excreta that have been wetted, but that subjecting *H. helluo* silk and excreta to heat has no effect on *P. milvina* response (Wilder et al., 2005). Based on these findings, dew at night may have reduced the effectiveness of any volatile kairomone produced by *H. helluo* resulting in less effective avoidance by *P. milvina* males, whereas the higher temperatures and sunlight during the day likely had no negative effect on *P. milvina* responsiveness to this kairomone. However, our use of moist paper towels in this study likely minimized extreme differences in ambient trap humidity levels during the day vs. the night, but humidity levels outside of the trap probably varied considerably.

We cannot completely rule out the hypothesis that *P. milvina* could have been attracted to insects or spiders captured in the traps other than *H. helluo*. Another alternative hypothesis is that differential predation by trapped larger spiders or ground beetles could have skewed the capture frequencies across treatments. However, both of these alternative interpretations of biased trap capture rates are

unlikely. First, we found no evidence for differential trapping rates by treatment for species other than *P. milvina*. Second, because the traps were designed to capture small wolf spiders, few larger potential predators were able to physically enter the traps. Third, because we cleared the traps frequently, arthropod densities never were sufficiently high to encourage predation. We also visited traps with sufficient frequency to observe any predation when it did occur. Only two instances of predation were found. This occurred twice in the control traps, and these were omitted from the analysis because the spiders could not be positively identified with respect to age, sex, or *Pardosa* species. Only six species found in the traps were capable of preying upon *Pardosa*: one beetle species, the carabid *Scarites subterraneus* (Fabricius, 1775); two species of crab spider, *Xysticus ferox* (Hentz, 1847) and *X. gulosus* (Keyserling, 1880); and three wolf spiders, *H. helluo* (Walckenaer, 1837), *Schizocosa avida* (Walckenaer, 1837), and *T. ruricola*. *Trochosa ruricola* and *S. subterraneus* occurred in sufficiently low numbers during the day as to be unlikely to have had a significant predation effect (three *Trochosa* during the day and 12 *Scarites* during the day). *Schizocosa avida* also occurred only in very low numbers during the day and night, and only a single *H. helluo* was captured making both unlikely to bias the results. The crab spiders, although numerous, were never larger than *Pardosa*, and, more importantly, they were either equally distributed across all treatment groups or showed trends toward the same capture bias as *Pardosa*, and thus, if anything, would underestimate the magnitude of the capture bias effect because predation would skew it in the opposite direction as what the results indicate. Furthermore, during trap checks, we found no evidence of intraguild predation occurring between *P. milvina* and either of the *Xysticus* spp.

Research on airborne chemical communication in spiders is in its infancy. Currently, there are few studies of communication among spiders via airborne pheromones (Tietjen, 1979; Watson, 1986; Willey and Jackson, 1993; Searcy et al., 1999; Papke et al., 2001; Becker et al., 2005) and even fewer that test for airborne chemical cues among different species of spider (Persons and Rypstra, 2000; Jackson et al., 2002; Li and Lee, 2004; Kasumovic and Andrade, 2004). Our study provides evidence that wolf spiders can and do use an airborne enemy-avoidance kairomone within an ecologically relevant context. We suggest that future studies of interspecific interactions among spiders should consider volatile cues as a possible mediator of observed behavioral responses.

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Kairomonal Response of Predators to Three Pine Bast Scale Sex Pheromones

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Abstract The kairomonal activity of the sex pheromones of three pine bast scales, *Matsucoccus feytaudi*, *Matsucoccus josephi*, and *Matsucoccus matsumurae*, as well as a new analog of the *M. feytaudi* sex pheromone, were investigated in pine forests of France, Portugal, and Italy. The response of the maritime pine bast scale predators, *Elatophilus* spp. and *Hemerobius stigma*, was used to test the influence of trapping methods, kairomone composition, and dose. Both predators showed significant attraction to all compounds except to the sex pheromone of *M. josephi*. Significant increase in captures was observed as a function of dose, and within the studied dose range, up to 2200 µg, no threshold saturation limits were observed for any of the attractive compounds. Trap design and size did not significantly influence predator captures, except for high population levels of *Elatophilus crassicornis*, when plate traps were more efficient than delta traps. Geographic variations were found in the kairomonal responses patterns of both predators, with the *M. matsumurae* sex pheromone being more attractive to the oriental populations from Corsica and Italy, whereas the western populations in Aquitaine and Portugal were more attracted to the *M. feytaudi* sex pheromone.

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Keywords Pine bast scales · Matsucoccidae · Sex pheromone · Kairomone · *Elatophilus* spp. · *Hemerobius stigma* · Predators · Trap design · Dose response

Introduction

Pheromones represent one of the major components of ecologically based insect pest management (Howse et al., 1998). Effectively, the use of these semiochemicals for monitoring, mass trapping, mating disruption, or lure-and-kill has been investigated or developed during recent decades (e.g., Taschenberg et al., 1974; Brockerhoff and Suckling, 1999; Millar et al., 2002). However, although pheromones are generally viewed as environmentally sound, some practical problems regarding their safety could arise from their kairomonal activity. In effect, there is increasing evidence that these pheromones attract natural enemies (e.g., Bakke and Kvamme, 1981; Hendrichs et al., 1994; Boo et al., 1998; Millar et al., 2001; Mendel et al., 2004). This is of importance, as catching large numbers could impact negatively the densities of these beneficial insects, and potentially result in increases in pest populations. However, this aspect has not been studied in great detail to date. The analysis of response patterns by natural enemies to the different compounds can further elucidate the role of these infochemicals in the behavioral mechanisms related to kairomonal attraction, prey detection, prey preference, and functional response.

In this work, we undertook an international study to determine if there is dose response to sex pheromones of hosts by natural enemies, by using the responses of *Elatophilus* spp. and *Hemerobius stigma*, to the pheromones of three pine bast scales, *Matsucoccus feytaudi* Ducasse, *Matsucoccus matsumurae* Kuwana, and *Matsucoccus josephi* Bodenheimer and Harpaz, and also to a new analogue of the *M. feytaudi* sex pheromone. We also examined the specific responses of these natural enemies to different types of traps in order to develop an optimal trapping method to monitor or attract natural enemies of *M. feytaudi* and to evaluate the adverse effect of trapping with *M. feytaudi* pheromone on its natural enemies.

Methods and Materials

The experiments were repeated in four European regions: Aquitaine and Corsica in France, Península de Setúbal in Portugal, and Liguria in Italy. In each region, a trapping line was set up at two different sites, with traps separated by about 25 m. At each site, we tested four compounds (synthetic sex pheromones of *M. feytaudi*, *M. josephi*, and *M. matsumurae*, as well as a new analogue of the *M. feytaudi* pheromone, Fig. 1) at four concentrations (400, 800, 1400, and 2200 µg), using two trap types (delta and plate) and two sticky surface areas (15 × 15 cm vs. 30 × 30 cm), for a total of 64 traps. In addition, there were four control traps (a delta and plate trap with either a 15 or 30 trapping surface). Traps were made of white polypropylene and covered with a sticky material. The active compounds were used in the form of racemic mixtures: 56:44 *EE*/*ZE* ratio for *M. josephi* sex pheromone and 65:35 *EE*/*ZE* ratio for the other three compounds.

Pheromones were synthesized at the UPMC, INRA-Versailles. The new analogue was synthesized by using a procedure similar to that used for the synthesis

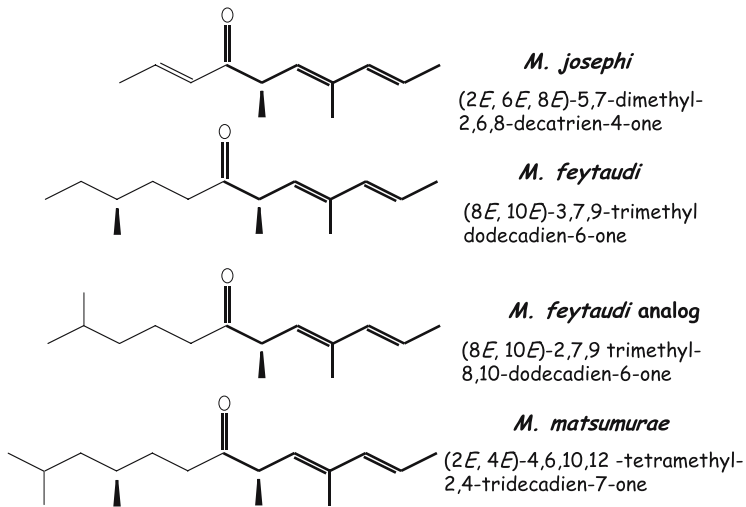


Fig. 1 Chemical structure of the four semiochemical compounds tested for attractiveness toward predator species of *M. feytaudi*

of *M. matsumurae* pheromone (Watanabe et al., 1997). Lures for *M. feytaudi*, *M. feytaudi* analogue, and *M. matsumurae* pheromones were loaded in caps of rubber septa (OD 8 mm, purchased from Sigma-Aldrich) in France (INRA-Versailles), and those for *M. josephi* were prepared in Israel (Volcani Center).

Trials were carried out between April and May, 2000. Sticky surfaces of traps were replaced twice a week, and the lures (rubber septa) replaced every 2 wk. In all sites, we recorded the number of brown lacewing, *H. stigma* Stephens. We also collected predaceous bug adults: *Elatophilus nigricornis* Zetterstedt in France and Italy and *Elatophilus crassicornis* Reuter in Portugal.

Data were analyzed by ANOVA, following logarithmic of $x + 1$ transformation and subject to *post hoc* Student–Newmann–Keuls multiple-range tests ($\alpha = 0.05$).

The *M. josephi*, *M. feytaudi*, and *M. matsumurae* pheromones have different relative volatiles (Dunkelblum et al., 1996). Therefore, to test lures with similar release rates, we compared the kairomonal response of the predators 400 and 800 μg of *M. feytaudi* sex pheromone with that of 1400 and 2200 μg of *M. matsumurae* sex pheromone. In this experiment, the data were analyzed using independent sample *t* tests.

Results

Kairomone Composition and Dose

There was significant region \times kairomone interaction for both *E. nigricornis* and *H. stigma* [$F(6,204) = 10.51$, $P < 0.001$ and $F(9,2720) = 7.13$ $P < 0.001$, respectively], so separate ANOVAs were conducted for each region. In the four regions, and for both groups of predators, the two main factors kairomone type and dose were significant (Tables 1 and 2). The mean catches increased with increasing dose of all pher-

Table 1 Analysis of variance to assess effect of trap type, trap size, kairomone type, and dose on *H. stigma* (Stephens) captures in four regions: Aquitaine, Corsica, P. Setúbal, and Liguria

Source	df	Region							
		Corsica		Liguria		Aquitaine		P. Setúbal	
		F	P	F	P	F	P	F	P
Type	1	0.03	0.857	5.03	0.028	0.86	0.356	1.08	0.303
Size	1	0.11	0.739	0.95	0.332	0.75	0.388	0.05	0.830
Kairomone	3	69.19	<0.001	42.81	<0.001	27.58	<0.001	20.17	<0.001
Dose	3	4.81	0.004	7.69	<0.001	12.43	<0.001	3.09	0.033
Type × size	1	0.15	0.702	0.81	0.371	0.04	0.849	0.01	0.914
Type × kairomone	3	0.13	0.944	1.72	0.172	0.30	0.822	0.06	0.983
Size × kairomone	3	0.10	0.962	0.53	0.665	0.24	0.868	0.16	0.924
Dose × kairomone	9	0.80	0.614	1.74	0.097	1.76	0.093	0.10	1.000
Type × dose	3	0.11	0.956	0.58	0.627	0.44	0.723	0.03	0.993
Size × dose	3	0.03	0.993	0.24	0.869	0.76	0.520	0.03	0.993
Error	68								

omones, except for that of *M. josephi*, which never differed from the control. The dose responses showed no clear saturation limit within the tested range (Figs. 2 and 3).

The highest *Elatophilus* species captures were in traps baited with the *M. feytaudi* pheromone, followed by the *M. feytaudi* analogue and *M. matsumurae* pheromone at all sites (Fig. 3). Similarly, in Aquitaine and Portugal populations, *H. stigma* were mostly attracted by *M. feytaudi* pheromone, followed by *M. feytaudi* analogue (Fig. 2). In contrast, especially at the higher doses, *H. stigma* from Corsica and Italy were mostly attracted by *M. matsumurae* pheromone (Fig. 2).

Table 2 Analysis of variance to assess effect of trap type, trap size, kairomone type, and dose on *Elatophilus* spp. (Reuter) captures in four regions: Aquitaine, Corsica, P. Setúbal and Liguria

Source	df	Region							
		Corsica		Liguria		Aquitaine		P. Setúbal	
		F	P	F	P	F	P	F	P
Type	1	0.00	0.999	0.02	0.887	1.00	0.322	33.22	<0.001
Size	1	0.00	0.959	0.69	0.411	0.01	0.917	0.54	0.466
Kairomone	3	90.27	<0.001	63.70	<0.001	283.20	<0.001	303.43	<0.001
Dose	3	4.34	0.007	7.20	<0.001	23.42	<0.001	22.84	<0.001
Type × size	1	0.08	0.782	0.03	0.865	0.00	0.963	0.47	0.497
Type × kairomone	3	0.18	0.910	1.18	0.322	0.36	0.783	0.40	0.756
Size × kairomone	3	0.08	0.972	0.35	0.792	0.45	0.721	0.11	0.953
Dose × kairomone	9	0.69	0.719	0.69	0.712	2.01	0.052	1.56	0.146
Type × dose	3	0.07	0.978	0.14	0.936	0.44	0.726	0.41	0.743
Size × dose	3	0.05	0.983	0.60	0.619	1.22	0.310	0.33	0.807
Error	68								

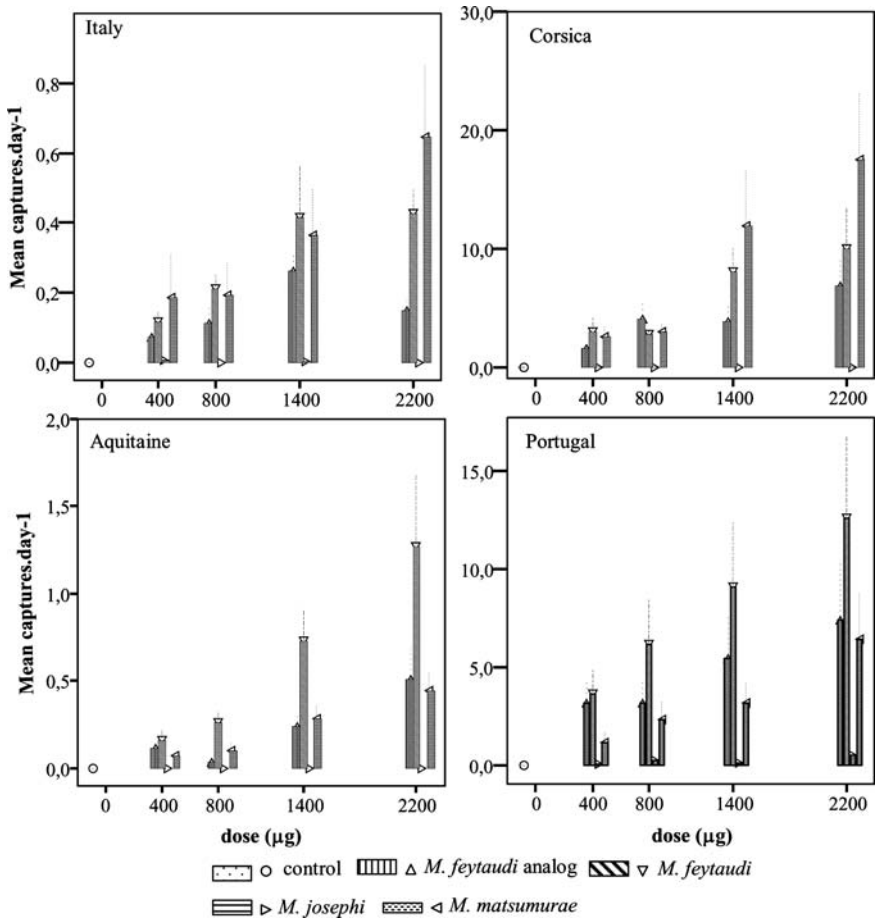


Fig. 2 Effect of kairomone and dose on *H. stigma* captures in Portugal, Corsica, Italy, and Aquitaine ($N = 8$), in relation to the compounds *Matsucoccus feytaudi* analog, *M. feytaudi*, *M. josephi*, and *M. matsumurae*

The comparison 400 and 800 µg of *M. feytaudi* sex pheromone with 1400 and 2200 µg of *M. matsumurae* sex pheromone, gave different results depending on the region. The western populations of the brown lacewing were equally attracted to both semiochemicals, whereas the eastern populations showed a significantly higher attraction to the *M. matsumurae* pheromone (Table 3). Furthermore, the eastern populations of *Elatophilus* species showed no preference for either kairomone, whereas the western populations were more attracted to the *M. feytaudi* pheromone.

Trap Size and Design

Captures of *H. stigma* were higher in Corsica and Portugal than in Aquitaine and Italy (Fig. 4). In Liguria, delta traps captured significantly more brown lacewings than plate traps (Fig. 4, Table 1), whereas no differences were observed in the other three regions. As for Portugal, where the *Elatophilus* spp. catches were two to three times higher than in the other three regions (Fig. 5), plate traps captured more bugs

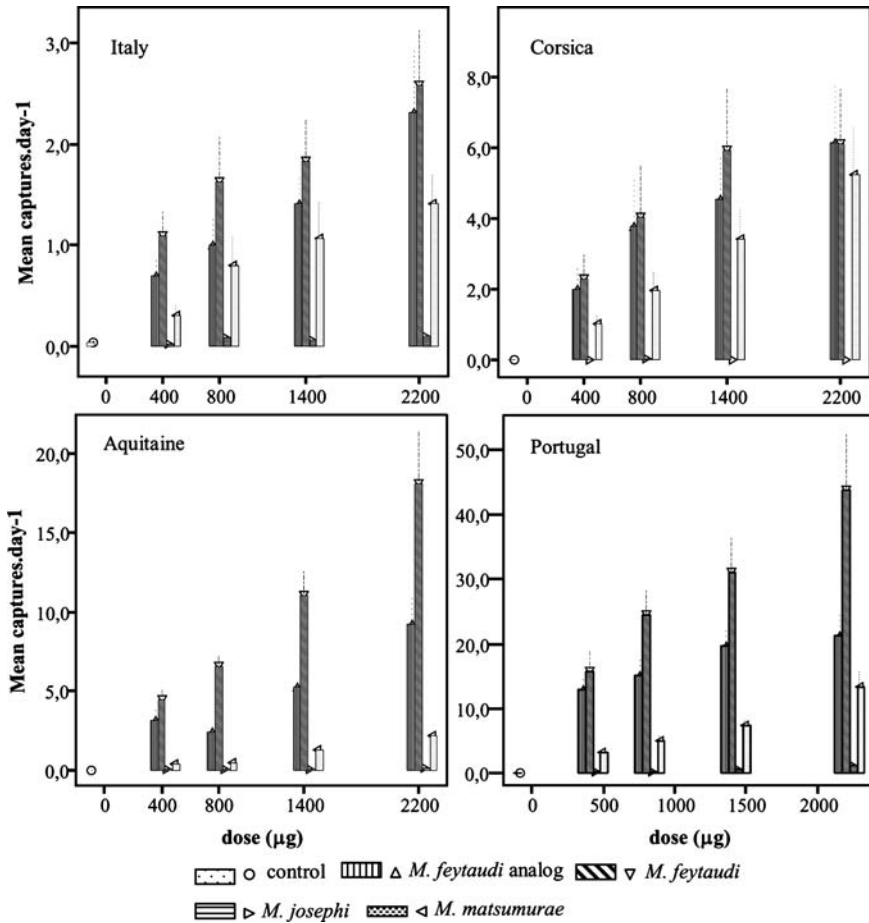


Fig. 3 Effect of kairomone and dose on *E. nigricornis* (Aquitaine, Italy, and Corsica) and *E. crassicornis* (Portugal) captures ($N = 8$), in relation to the compounds *Matsucoccus feytaudi* analog, *M. feytaudi*, *M. josephi*, and *M. matsumurae*

Table 3 Comparison of kairomonal activities of *M. feytaudi* and *M. matsumurae* pheromones

	Corsica	Italy	Aquitaine	Portugal
<i>H. stigma</i>				
Mm 1400	4.8 ± 0.55 ^a	2.4 ± 0.35	1.5 ± 0.26	3.8 ± 0.54
Mf 400	3.2 ± 0.28	1.6 ± 0.21	0.9 ± 0.31	3.7 ± 0.57
Mm 2200	5.2 ± 0.55 ^a	3.1 ± 0.28 ^a	1.9 ± 0.24	4.6 ± 0.46
Mf 800	3.7 ± 0.31	1.9 ± 0.36	1.5 ± 0.24	4.1 ± 0.46
<i>Elatophilus</i> spp.				
Mm 1400	4.1 ± 0.34	3.7 ± 0.38	3.1 ± 0.22	5.2 ± 0.14
Mf 400	3.4 ± 0.51	3.7 ± 0.24	4.3 ± 0.21 ^a	5.9 ± 0.22 ^a
Mm 2200	4.5 ± 0.34	4.3 ± 0.21	3.8 ± 0.12	5.7 ± 0.22
Mf 800	4.0 ± 0.46	4.0 ± 0.26	4.8 ± 0.16 ^a	6.4 ± 0.20 ^a

Values are log(mean captures per trap) ± SE. Mm, *M. matsumurae*; Mf, *M. feytaudi*.

^a $P < 0.05$; significantly different from the other treatments (independent sample *t* test).

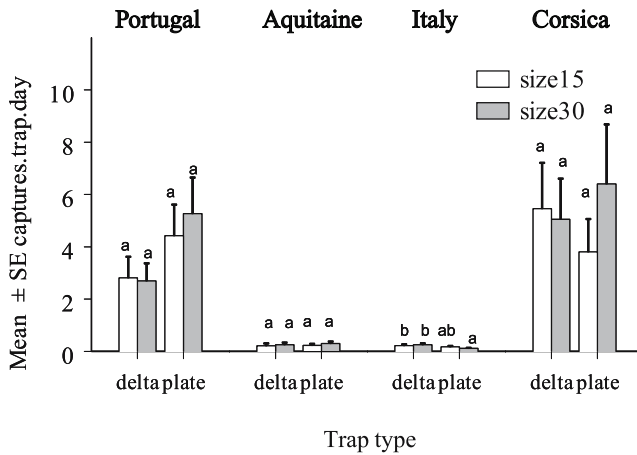


Fig. 4 Effect of region, trap type, and trap size on the mean catches of *H. stigma* ($N = 34$). Within each site, means with the same letter are not significantly different (SNK test, $\alpha = 0.05$)

than delta traps, thus explaining the observed trap type effect for this species (Table 2). Differences caused by trap size were not significant at any site.

Discussion

The results provide new evidence that predators respond to the sex pheromones of several scale species in several different geographic locations, suggesting that they play a role in host location. This kairomonal activity was previously demonstrated in Europe (Mendel et al., 2003, 2004) and Israel, where *E. hebraicus* responded to the same *Matsucoccus* pheromones (e.g., Dunkelblum et al., 1996; Mendel et al., 1997, 2003). Pine bast scales occupy an inconspicuous location under the bark of the trunk where they feed on the phloem, and in their native environment they generally

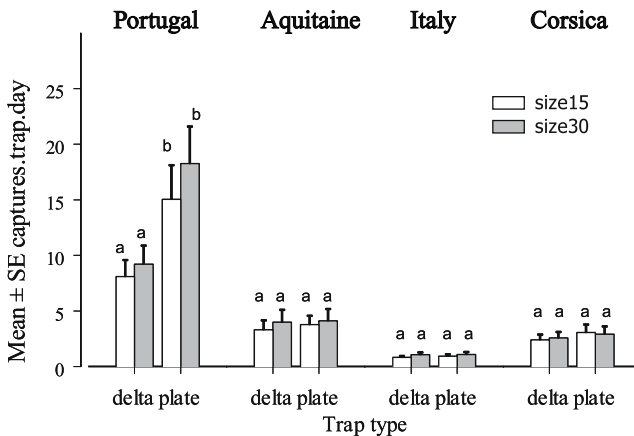


Fig. 5 Effect of region, trap type, and trap size on the mean catches of *E. nigricornis* (Aquitaine, Italy, and Corsica) and *E. crassicornis* (Portugal) ($N = 34$). Within each site, means with the same letter are not significantly different (SNK test, $\alpha = 0.05$)

occur in extremely low densities. Therefore, the use of the host sex pheromone would provide a reliable cue to locate prey, and the fact that they respond to the pheromones of several different scales would improve their ability to locate different host species. Predators require higher concentrations to locate the pheromone source than male pine scales, suggesting that scales may only be extensively exploited when densities are abundant enough to sustain natural enemy populations. However, the high kairomonal response of predators suggests that sex pheromone may also be used to draw natural enemies into the habitat and increase the local population of natural enemies. Further studies are needed to evaluate this possibility.

There are several nonmutually exclusive explanations for the observed intersite differences that are noted above: (i) the effect of prevailing climate on both the effectiveness/longevity of lures, as well as the behavior of the target species, (ii) the actual population densities of prey/predator populations, (iii) the relative importance of the scales as prey to these predators in each habitat, and (iv) the time that prey and predators have been in association at the different sites (Jonsson and Anderbrant, 1993; Sharov et al., 1996; Mendel et al., 1997; Krips et al., 1999). Although several examples of different geographic variation in intraspecific sex pheromones have been reported (McElfresh and Millar, 2001; Anderbrant et al., 2000; El Sayed et al., 2003), we believe that the marked differences in response of both *H. stigma* and *Elatophilus* spp. to different host pheromones is the first example of geographic variation in response to kairomones. These differences are possibly related to the fact that the western regions correspond to the original geographical range of *M. feytaudi*, whereas this species was only introduced into Italy and Corsica in the 1970s and 1990s, respectively (Covassi and Binazzi, 1992; Jactel et al., 1998; Burbán et al., 1999). Prior to the introduction of *M. feytaudi*, *M. pini* was the only pine bast scale present in these eastern regions (Rieux, 1976; Tranfaglia et al., 1985). The sex pheromone of *M. pini* is not known, but our results suggest that it may resemble the sex pheromone of *M. matsumurae*. This is also consistent with the observation that *Sympherobius fuscescens*, another potential predator of *M. pini* in the eastern areas, also responded to the sex pheromone of *M. matsumurae* but not to that of *M. feytaudi* (Mendel et al., 2004). The observation that the predators of *M. pini* responded to the *M. matsumurae* pheromone emphasizes the need to clarify the taxonomic status of these two species, as suggested by Kosztarab and Kozár (1988).

Dunkelblum et al. (2000) reported that the species specificity of the pheromone was dependent on the structure of the side chain, whereas the kairomonal effect was dependent on the ketodiene moiety. However, as all compounds tested in this study had a common ketodiene moiety yet differed markedly in kairomonal activity, this indicates that changes in the side chain also affect the response of natural enemies.

There are significantly different dose-dependent responses by scale predators. When sex pheromones are used as a pest management strategy, then the potential negative effects on the populations of natural enemies must take this into consideration when determining the most effective dose to use. Furthermore, no saturation level was observed by predators over the dose range tested, which contrasts with the observed response of *M. feytaudi* males to increasing dose of sex pheromone (Branco et al., 2004). The present results indicate that generally the size and type of trap did not influence efficiency, except for large plate traps, which

caught more *E. crassicornis* when there were high densities of this predator. A preference for vertical objects, as observed elsewhere among other groups of insects that use trees for shelter or feeding (e.g., Willis et al., 1994; Gross et al., 2001), may explain this result.

In summary, the present study provides information on the kairomonal responses of these entomophagous insects that could be useful in the pest management strategies against the pine bast scale. Additional research is required to determine the relative importance of biotic and abiotic factors affecting the responses of natural enemies to scale pheromones, and how these may differ at different geographic locations.

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Chemically Mediated Species Recognition in Closely Related *Podarcis* Wall Lizards

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Abstract In many animals, chemical signals play an important role in species recognition and may contribute to reproductive isolation and speciation. The Iberian lizards of the genus *Podarcis*, with up to nine currently recognized lineages that are often sympatric, are highly chemosensory and provide an excellent model for the study of chemically mediated species recognition in closely related taxa. In this study, we tested the ability of male and female lizards of two sister species with widely overlapping distribution ranges (*Podarcis bocagei* and *P. hispanica* type 1) to discriminate between conspecific and heterospecific mates by using only substrate-borne chemical cues. We scored the number of tongue flicks directed at the paper substrate by each individual in a terrarium previously occupied by a conspecific or a heterospecific lizard of the opposite sex. Results show that males of *P. bocagei* and *P. hispanica* type 1 are capable of discriminating chemically between conspecifics and heterospecifics of the opposite sex, but females are not. These results suggest that differences in female, but not male, chemical cues may underlie species recognition and contribute to reproductive isolation in these species. The apparent inability of females to discriminate conspecific from heterospecific males, which is not because of reduced baseline exploration rates, is discussed in the context of sexual selection theory and species discrimination.

Keywords Ethological isolation mechanisms · Cryptic species · Chemoreception · Species recognition · *Podarcis* · Lizards · Reptiles

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Introduction

The evolution of prezygotic reproductive isolation remains a central issue in the study of speciation and one of the least well-understood questions in evolutionary biology (Eady, 2001). Results from several taxa suggest that behavior plays a major role in preventing interspecific mating (so-called “ethological isolation mechanisms”): differences in mating signals between species or populations are often much more pronounced than morphological differences and provide an important barrier to gene exchange (e.g., Butlin and Ritchie, 1994; Ptacek, 2000; Shine et al., 2002). Furthermore, behavioral characters are increasingly recognized as useful tools for phylogenetic reconstruction (de Queiroz and Wimberger, 1993; Gittleman and Decker, 1994). Thus, behavioral assays of species recognition may provide important clues to the elucidation of the taxonomic status in complex groups.

In many animals, chemical signals play an important role in species recognition and speciation: the detection of species-specific chemical cues allows individuals to recognize each other as potential mates and promotes assortative mating (reviewed in Wyatt, 2003). This phenomenon has been widely studied, although with a strong taxonomic bias toward invertebrates, mainly moths (e.g., Phelan and Baker, 1987), and also flies (e.g., Mas and Jallon, 2005), beetles (Symonds and Elgar, 2004), spiders (e.g., Roberts and Uetz, 2004), and polychaetes (Sutton et al., 2005). Squamate reptiles have well-developed chemosensory systems and are among the most chemosensory of vertebrates: for many lizards and snakes, chemical stimuli are important sources of information about several aspects of the environment crucial to fitness (Halpern, 1992; Schwenk, 1995). Chemicals released at the time of reproduction may provide the basis for species recognition and avoidance of interspecific mating among closely related sympatric species (Cooper and Vitt, 1987; Mason, 1992; Labra et al., 2001) or between different populations of the same species (LeMaster and Mason, 2003). Among lizards, the discrimination of conspecifics from heterospecifics based on chemical cues alone has been reported in a few scincid (Cooper and Vitt, 1987), iguanian (Labra et al., 2001), and lacertid species (Gómez et al., 1993; Cooper and Pérez-Mellado, 2002; Barbosa et al., 2005).

The lizards of the genus *Podarcis* are the predominant lacertid group in southern Europe, and their taxonomy has remained unstable and controversial until recently. In the Iberian Peninsula, the combination of nuclear and mitochondrial molecular markers has allowed the identification of several cryptic forms, probably with specific status, confirmed also by morphological studies (Harris and Sá-Sousa, 2002; Sá-Sousa, 2001; Sá-Sousa et al., 2002; Pinho et al., 2003, 2006; Kaliontzopoulou et al., 2005).

Although the chemosensory abilities of *Podarcis* lizards have been previously studied (e.g., López and Martín, 2001; Font and Desfilis, 2002; Desfilis et al., 2003), only a few studies have dealt with the issue of species recognition. Gómez et al. (1993) provided the first evidence of chemically mediated species recognition in a lacertid: male *Podarcis hispanica* (*sensu lato*) were capable of detecting and discriminating conspecific from heterospecific (*Psammotromus algirus*) chemicals. However, the two species used in this study were not even congeneric. More recently, Cooper and Pérez-Mellado (2002), working also with *P. hispanica* (*sensu lato*), reported that males of this species have the ability to discriminate among conspecifics and sympatric female congeners (*Podarcis carbonelli*) based on chemical cues alone. These findings point to a possible role of chemoreception as an ethological isolating mechanism in this complex genus.

In a previous study (Barbosa et al., 2005), we tested the ability of males of two closely related species, *P. bocagei* and *P. carbonelli*, to discriminate between substrates labeled by conspecific and heterospecific females. Our results demonstrated the existence of reciprocal chemical discrimination between these two species. In the present study, we focused on *P. hispanica* type 1 and *P. bocagei*, a monophyletic and closely related species pair (in fact, sister taxa) within this genus, (Pinho et al., 2006), and tested the ability of individuals of either sex to discriminate between conspecifics and heterospecifics of the opposite sex. Because they are sister species, have largely overlapping distributions that includes contact even at a microscale (personal observation), no introgression events have been detected so far, and no extrinsic barriers to hybridization have been described, this species pair presents an interesting case study for research on the role of chemical cues in species discrimination and reproductive isolation.

Methods and Materials

Study Species

Podarcis hispanica type 1 and *P. bocagei* are small-sized lizards, which have a widely overlapping distribution in the NW Iberian Peninsula, living in sympatry over large areas. *P. hispanica* type 1 is a rock-dwelling lizard found in Galicia (Spain), northern Portugal, and the western Castilian plateau and Central System (Spain). *P. bocagei* is a ground-dwelling lizard occurring in western Asturias, Cantabria, Galicia (Spain), and northwestern Portugal (Galán, 1986; Sá-Sousa, 2000). Although very closely related, the two species can be easily identified by their morphological characteristics (for details, see Sá-Sousa, 2001; Sá-Sousa et al., 2002).

Animal Capture and Maintenance

Lizards were collected at the beginning of the reproductive season (February 2005) in the coastal area of Moledo do Minho (northern Portugal), where both species occur syntopically. They were transported to and housed at the laboratory in Valencia, Spain. Lizards were transported in individual plastic terraria (20 × 12 × 16 cm) with damp paper to avoid dehydration and then housed in glass terraria (40 × 20 × 25 cm for females and 35 × 20 × 20 cm for males), with a permanent supply of water and a rock for basking and shelter. Incandescent (40-W) bulbs placed above each terrarium provided light and heat. Lights were scheduled to provide a 12-hr light/12-hr dark photoperiod cycle. Terraria were kept in a temperature-controlled room (temperature cycle of 24–6 hr: 19°C; 6–11 hr: 23°C; 11–20 hr: 28°C; 20–22 hr: 23°C; 22–24 hr: 22°C) at ambient humidity (2–45%). Lizards were fed daily with mealworm (*Tenebrio molitor*) larvae dusted with vitamins.

Experimental Design

The experimental trials were conducted during March and April 2005. Trials consisted of gently picking up a lizard and transferring it to a test terrarium (40 × 20 × 25 cm) for a 10-min observation period. The test terrarium was kept in the same room where lizards were housed and had a 40-W incandescent bulb suspended

ca. 20 cm above the floor of the terrarium as a source of heat and light. The floor of the test terrarium was covered with a paper substrate. The stimulus conditions were prepared by placing an odor donor inside the test terrarium in the evening preceding an experimental trial and allowing it to remain there until 15 min before the trial. Shed skin, feces, and other obvious visual stimuli left by the donor were removed prior to the trial. For control trials, the test terrarium was fitted with a clean paper substrate. At the end of each trial, the paper substrate was discarded, and the terrarium was washed thoroughly with water and alcohol to eliminate residual chemical traces. Trials were conducted between 11:00 and 13:30 hr GMT, when the lizards were fully active. Room temperature at the time of testing was maintained between 26 and 29°C to minimize variability arising from thermal dependence of tongue flick rates (Cooper and Vitt, 1986a).

Each trial was video-recorded, and the lizards' behavior was later analyzed with the aid of a portable computer equipped with JWatcher event-recording software (Blumstein et al., 2000). As we were interested in the lizards' chemosensory responses, we focused our behavioral observations on tongue flicks. Tongue flicking functions to acquire chemicals for analysis by the vomeronasal organ and provides an observable index of the chemosensory investigation of a stimulus (Cooper and Burghardt, 1990; Halpern, 1992). We scored the number of tongue flicks directed at the substrate, which is indicative of chemosensory investigation of traces left by other individuals, and the number of tongue flicks directed at the air (air licks). Although results were similar for both variables, based on our previous work (Font and Desfilis, 2002; Barbosa et al., 2005), we restricted further analyses to the number of tongue flicks directed at the substrate in each experimental condition. Moreover, it has been suggested that air licks may be more influenced by stress than specific stimuli, at least in other lizards (Greenberg, 1985).

In the first set of experiments, we tested the ability of *P. hispanica* type 1 and *P. bocagei* males to chemically discriminate conspecific from heterospecific females. Thirteen females (five *P. hispanica* type 1 and eight *P. bocagei*) acted as donors of chemical stimuli for the trials (no female was used more than five times as a donor). Males were tested three times: once in a clean test terrarium (control), once in a test terrarium bearing chemical stimuli from a conspecific female, and once in a test terrarium bearing chemical stimuli from a heterospecific female. Each individual was tested only once per day with an intertrial interval of 2 d. The order of stimulus presentation was partially counterbalanced to avoid sequence effects. Eleven *P. hispanica* type 1 and 11 *P. bocagei* males were tested.

In the second set of experiments, we tested the ability of females of both species to discriminate chemical stimuli of conspecific and heterospecific males. The procedure was identical to that described for the first experimental set, except for the sex of donors and experimental lizards. Thirteen males (six *P. hispanica* type 1 and seven *P. bocagei*) acted as donors of chemical stimuli for the trials (no male was used more than five times as a donor). Twelve *P. hispanica* type 1 and 11 *P. bocagei* females were tested.

Statistical Analyses

We fitted a robust partly nested analysis of variance (ANOVA) model to rank-transformed data using SPSS 11.5. We used a group \times trials repeated-measures design with responses to each of the three stimulus conditions as the repeated

measure and with individual males/females (random factor) nested within species (Quinn and Keough, 2002). The sphericity assumption was tested with Mauchly’s test. In those cases in which significant departures from sphericity were detected, we tested the significance of treatment effects and of the treatment × species interaction using adjusted univariate *F* ratios and multivariate ANOVA statistics (Pillai Trace). Following detection of significant treatment effects, we conducted pairwise comparisons between the different stimulus conditions. Power analyses were performed using *R*.

To compare the rate of tongue flicks of males and females in control conditions (which indicates the level of baseline chemosensory investigation by each sex), we used a Mann-Whitney nonparametric test for two independent samples (Siegel and Castellan, 1988).

Significance level for rejection of the null hypothesis was set at 0.05, and all tests were two-tailed. Data are presented as mean ± SEM.

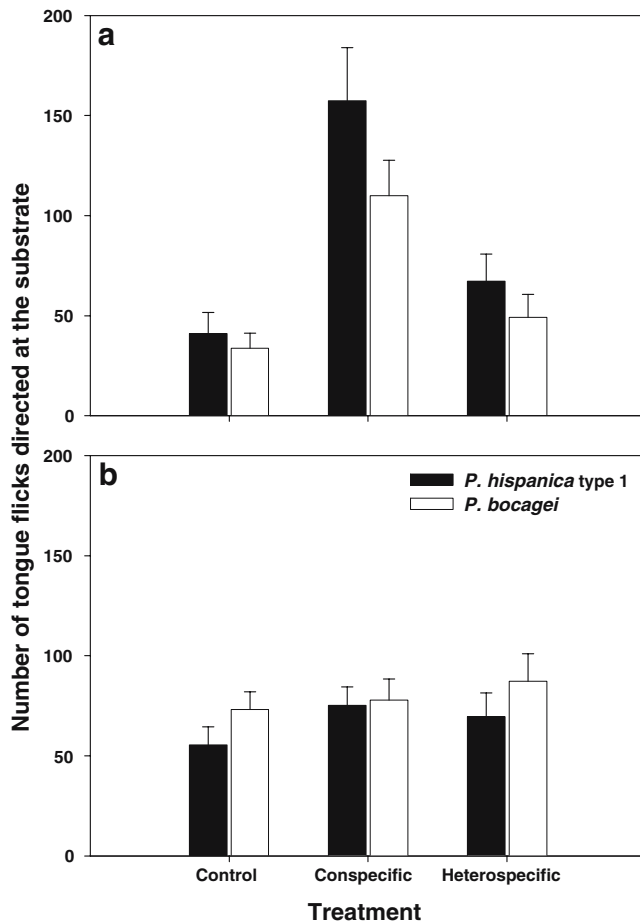


Fig. 1 Number (mean ± SEM) of tongue flicks of *P. hispanica* type 1 and *P. bocagei* males (a) and females (b) directed at the clean substrate (control) or at the substrate labeled by a conspecific or a heterospecific of the opposite sex

Results

Five *P. bocagei* (two males and three females) exhibited persistent escape attempts during the trials and were discarded from the analyses.

The males' responses to the treatments are illustrated in Fig. 1a. Table 1 shows *F* ratios adjusted by means of two commonly used types of correction (Quinn and Keough, 2002), although sphericity could be assumed (Mauchly's test: $W = 0.939$; $df = 2$; $P = 0.587$). All tests indicate a highly significant treatment effect. Lizards of both species directed more tongue flicks at substrates bearing chemical stimuli from conspecific females than to clean (control) substrates ($F = 65.68$, $df = 1$, $P < 0.001$) or substrates labeled by heterospecific females ($F = 36.706$, $df = 1$, $P < 0.001$). Moreover, lizards directed more tongue flicks to substrates labeled by heterospecifics than to clean substrates ($F = 5.079$, $df = 1$, $P = 0.037$). In no case was the species and treatment \times species interaction term significant.

The females' responses to the treatments are depicted in Fig. 1b. Table 1 shows adjusted *F* ratios (as in the case of males). As we could not assume sphericity (Mauchly's test: $W = 0.507$; $df = 2$; $P = 0.003$), we tested for treatment and treatment \times species interaction effects on the number of tongue flicks directed at the substrate using adjusted univariate and multivariate statistics. There were no significant species, treatment, or treatment \times species interaction effects. This test had sufficient power (0.883) to detect a difference of the same magnitude as that found in the males' experimental set.

There was a statistically significant difference in the number of tongue flicks performed in control conditions between sexes in *P. bocagei* (females perform more tongue flicks than males: Mann-Whitney's test, $U = 8.5$, $P = 0.008$); in the case of *P. hispanica* type 1, no differences were detected between sexes (Mann-Whitney's test, $U = 44.5$, $P = 0.185$).

Table 1 ANOVA table for the number of tongue flicks directed at the substrate (after rank transformation) by *P. hispanica* type 1 and *P. bocagei*

Sex	Males			Females		
	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>
Source of variations						
Between subjects						
Species	0.819	1	0.378	0.957	1	0.341
Within subjects						
Treatment						
Sphericity assumed	33.024	2	<0.001	–	–	–
Greenhouse-Geiser	33.024	1.885	<0.001	1.846	1.339	0.187
Huyn-Feldt	33.024	2	<0.001	1.846	1.488	0.184
Pillai Trace	37.685	2	<0.001	5.63	2	0.013 ^a
Treatment \times Species						
Sphericity assumed	0.144	2	0.866	–	–	–
Greenhouse-Geiser	0.144	1.885	0.855	0.683	1.339	0.458
Huyn-Feldt	0.144	2	0.866	0.683	1.488	0.472
Pillai Trace	0.109	2	0.897	1.827	2	0.191

^a Note that the multivariate adjustment (Pillai Trace) indicates a significant treatment effect. However, because there is a disagreement between the univariate and multivariate adjusted results, we conclude, following Quinn and Keough (2002), that there is a lack of treatment effect.

Discussion

The results show that *P. hispanica* type 1 and *P. bocagei* males respond differentially to chemical cues of conspecific and heterospecific females, exhibiting more chemosensory behaviors in response to conspecifics. These results clearly indicate the males' ability to discriminate between conspecific and congeneric females of a sympatric, closely related species using only substrate-borne chemical cues. Thus, differences in female chemical cues may underlie specific recognition in these species. The lack of significant species or treatment \times species interaction effects further indicates that males of the two species show consistent responses to the paper substrates. Moreover, the difference between the number of tongue flicks directed at substrates labeled by heterospecific females and controls indicates that the males also respond to heterospecific cues, which may suggest their detection as a biologically relevant stimulus. These findings are in agreement with previous results of reciprocal chemical discrimination in *P. carbonelli* and *P. bocagei* (Barbosa et al., 2005) and underscore the importance of chemical cues in species discrimination by *Podarcis* males, even in closely related lineages. These results also suggest that the chemicals deposited by females on the paper substrate vary in composition. These differences may be related to the genetic differences between the two species, as has been reported in mammals (Bininda-Emonds et al., 2001; Heth et al., 2001), although this hypothesis is still to be tested in lacertids.

Cooper and Pérez-Mellado (2002) showed that *P. hispanica* (*sensu lato*) males are capable of discriminating between chemicals of conspecific females and those of sympatric *P. carbonelli* presented on cotton swabs. However, these authors did not test the ability of *P. carbonelli* males to discriminate between chemical cues of their own females and those of the other species. In closely related sympatric species, an ability to distinguish between conspecifics and heterospecifics may be important for reducing energy costs of finding potential mates (Labra et al., 2001; Cooper and Pérez-Mellado, 2002) or sexual rivals (Cooper and Garstka, 1987) and for preventing interspecific courtship or fighting. Although vision and chemoreception have been recognized as important sensory modalities in courtship and mating in lacertids (Verbeek, 1972; López and Martín, 2001), the fact that *Podarcis* females belonging to different species are generally more similar in morphology and coloration than males (Sá-Sousa, 2001; Sá-Sousa et al., 2002) suggests that there may be a selective pressure for chemical recognition mechanisms to take precedence over visual recognition when a male is searching for a potential mate.

Chemical species recognition has been reported in other squamates, particularly in complex groups such as tropidurids of the genus *Liolaemus* and scincids of the genus *Eumeces*. *Liolaemus jamesi* and *L. bellii* are capable of discriminating conspecifics from sympatric heterospecifics (*L. alticolor* and *L. nigroviridis*, respectively) based on chemical cues, manifested by higher chemical exploratory behavior in enclosures previously occupied by conspecifics (Labra et al., 2001). In the species of the *Fasciatus* group of the genus *Eumeces*, lizards exhibit higher tongue flick rates to conspecifics than to members of closely related species (Cooper and Vitt, 1987). In addition, the sympatric sibling species of sea snakes *Laticauda colubrina* and *La. frontalis*, so similar in morphology that they were considered conspecific until recently, are reproductively isolated because of species-specific chemical cues (female skin lipids acting as pheromones) that elicit courtship by males of their own species (Shine et al., 2002).

Contrary to males, females of *P. hispanica* type 1 and *P. bocagei* did not respond differentially to conspecific and heterospecific male chemical cues. The contrasting discrimination by males and apparent lack thereof by females require an explanation. It could be argued that, compared to males, females have reduced chemosensory exploration rates when placed in a novel environment. However, this hypothesis is not supported by our results because a comparison of the basal rate of tongue flicks (number of tongue flicks performed in control conditions) of males and females revealed that females explore as much or even more than males.

Tongue flicks provide a convenient assay of the ability to respond differentially to biologically relevant stimuli and have been extensively used in the study of chemoreception in squamates (Cooper and Burghardt, 1990). Whereas significant results are usually taken as a *bona fide* evidence for discrimination, the interpretation of nonsignificant results in experiments where tongue flick rates are the dependent variable is not straightforward. In a thoughtful review of methodological issues relating to the study of squamate chemoreception, Cooper (1998) argued that, although differential tongue flick rates may indicate discrimination, their absence does not necessarily indicate a lack of discriminatory ability. On the other hand, several authors have suggested that the discrimination of individual characteristics of conspecifics, such as sex (Mason, 1993), body size and shape (Shine et al., 2003), or recent reproductive history (Shine et al., 2000), may require only a few tongue flicks. According to this interpretation, the differential tongue flicking rates would not indicate semiochemicals' discrimination but rather an interest in pursuing a scent trail and locating its source, as suggested by Cooper and Vitt (1986b). Thus, females may detect differences through vomerolfaction, but not respond by differential tongue flicking.

An alternative explanation is that species discrimination in females is based on sensory modalities other than vomerolfaction. Lacertids have well-developed olfactory systems (Gabe and Saint-Girons, 1976), and it is conceivable that olfaction alone may be sufficient to make adaptively significant discriminations (Halpern, 1992). This type of discrimination would not be detectable through varying tongue flicking rates, although other behaviors, such as buccal pulsing, could serve as observable indices indicating olfactory discrimination (Dial and Schwenk, 1996; Desfilis et al., 2003). The role of visual stimuli may also be important and cannot be discarded, especially considering that *Podarcis* males are, in general, more colorful and display much more interspecific variation in coloration patterns than females (Sá-Sousa, 2001; Sá-Sousa et al., 2002). Because males and females undergo different selective pressures to optimize different aspects of reproduction, it is conceivable and even likely that they use different criteria to assess potential mates and, thus, use different sensory modalities in mate choice/recognition (for an example in spiders, see Rypstra et al., 2003).

The apparent inability of females to discriminate substrate-borne chemical cues of males of their own and of a closely related species could be due to a lack of species specificity of semiochemicals released by males. According to this hypothesis, male-produced semiochemicals would have not diverged sufficiently to allow females to discriminate conspecific from congeneric males by using substrate-borne chemicals alone. This hypothesis seems unlikely for two reasons: (1) males are capable of species chemical discrimination of congeneric females (which suggests that female chemicals do have diverged), and (2) several studies with lizards have revealed the

presence of variability in the chemical composition of male-produced semiochemicals, even among closely related species (Alberts, 1991; Labra et al., 2001).

Differences in discriminatory abilities of males and females have been reported in other studies. For example, Verrell (2003) found that males of the salamander *Desmognathus ocoee* discriminate conspecific from heterospecific and heterotypic females based on chemical cues, but females cannot make a similar discrimination. Similarly, studies of sex recognition in lizards and snakes have reported differences in the discriminating abilities of males and females. Females of *Eumeces laticeps* (Cooper and Vitt, 1984) and *Eublepharis macularius* (Steele and Cooper, 1997) do not discriminate sexes by differential tongue flicking, while males of these species do. In garter snakes, *Thamnophis sirtalis*, males discriminate and follow female trails during the breeding season, whereas females, in general, do not trail either sex (Mason, 1992; LeMaster et al., 2001). These findings may reflect the lack of a selective pressure for females to locate mates by scent tracking (Cooper and Vitt, 1986b). Likewise, it is conceivable that in the species studied here, there has not been a selection pressure for the evolution of female chemical discrimination.

Although a naive interpretation of sexual selection theory would lead to the expectation of female mate choice and, thus, discrimination, the apparent lack of chemically based species recognition shown by female *Podarcis* would seem to agree with reports that female lizards are generally indiscriminate and lack mate choice based on male quantitative traits (Olsson and Madsen, 1998; Tokarz, 1995; LeBas and Marshall, 2001). However, the evidence regarding female mate choice in lizards is controversial (see, for example, Hamilton and Sullivan, 2005), and studies of the mating systems of autarchoglossans, and lacertids in particular, are scarce.

Although chemically mediated species discrimination may not function as a complete reproductive barrier, our study demonstrates the contribution of chemical stimuli in species discrimination and reproductive isolation in a complex and diverse genus. The relative roles of chemoreception and of other sensory modalities in species discrimination by males and, especially, females are still not clear. Further studies are clearly necessary to clarify the role of different sensory systems in specific recognition between *Podarcis* species leading to the maintenance of reproductive isolation between lineages.

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Pheromone-Induced Priming of a Defensive Response in Western Flower Thrips

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Abstract The Western flower thrips *Frankliniella occidentalis* produces conspicuous anal droplets that function as a direct defense against various predators. These droplets also function in pheromonal communication in that they contain a mixture of decyl acetate and dodecyl acetate, which acts as an alarm. Exposure of thrips to synthetic pheromone is known to promote takeoff or refuge seeking, but the effect of the natural pheromone has not yet been studied. Here, we not only studied the response to natural pheromone, but also tested the new hypothesis that the alarm pheromone primes a defensive response in thrips. This test was carried out by measuring the reaction time to a simulated predator attack after exposure to synthetic or natural alarm pheromone (against a control with no pheromone at all). The reaction was quantified in terms of the time it takes a thrips larva to produce a droplet after attack. We found that thrips larvae produce droplets of alarm pheromone faster when cues associated with danger are present. There were no significant differences in reaction times of responses to synthetic pheromone, natural pheromone, or odors from a patch with a predator attacking a thrips larva. This implies that the synthetic pheromone mimics the natural pheromone, and that other cues emanating from the predator play a minor role. We conclude that the alarm pheromone increases the vigilance of the thrips, and this may promote its survival.

Keywords Alarm pheromone · Thrips · *Frankliniella occidentalis* · Predator · *Orius laevigatus* · Antipredator response · Defense priming

Introduction

Much intraspecific communication in insects involves chemical messengers, called pheromones, which elicit attraction of the other gender, aggregation, or alarm.

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Alarm pheromones are animal-produced chemicals that intentionally or inadvertently communicate danger. Their release usually results in conspecifics showing antipredator behavior, such as fleeing, hiding, remaining motionless (e.g., in cryptic species), aggregating, changing group structure, or even mobbing predators (Lima and Dill, 1990). This behavior is generally assumed to promote survival.

The Western flower thrips *Frankliniella occidentalis* produces conspicuous droplets that are excreted anally. Upon encounter with a predator, thrips quickly move their abdomen from side to side (swing), trying to hit the predator, and they produce droplets when the threat of predation persists. Predators contaminated with such a droplet retreat to groom, and in this way, the droplets serve as a defense (Bakker and Sabelis, 1987, 1989). The droplets contain a solution of decyl acetate and dodecyl acetate (Teerling et al., 1993a,b) in a ratio that varies with the age of the larva (Macdonald et al., 2003). When a synthetic formulation of these compounds is present, thrips will increase their movements, reduce oviposition, decrease landing rates, increase takeoff rates, or hide (Teerling et al., 1993a,b; Macdonald et al., 2002), suggesting that the chemicals function as an alarm pheromone.

Although the behavior of thrips toward the synthetic alarm pheromone is well documented, there are no reports on the response of thrips to natural pheromone. It is also not known whether the alarm pheromone primes defensive responses. For instance, upon exposure to alarm pheromone, a thrips larva may become alerted and may produce an anal droplet faster upon actual attack. This hypothesis on priming of the defensive response was tested by assessing the effect of synthetic pheromone as well as natural alarm pheromone on the time elapsed from a simulated attack of a thrips larva until it produced an anal droplet. This effect is taken as a measure for the vigilance of thrips larvae because it demonstrates the degree to which they are prepared to defend themselves.

Methods and Materials

Thrips

Thrips were collected from cucumber plants in a commercial greenhouse in Pijnacker, The Netherlands, in May 1994. They were reared in the laboratory under constant climatic conditions (25°C and 60% humidity at 16-hr light/8-hr dark) on a cucumber leaf provided with cattail pollen (*Typha latifolia*). The leaf was cut to fit in a Petri dish with a layer of wet cotton wool on the bottom. Cohorts of thrips were obtained by introducing thrips pupae, allowing the emerging adults to oviposit and the larvae to develop until the pupal stage, after which this process was repeated.

Predatory Bugs

Orius laevigatus (Fieber), obtained from Koppert BV, was reared by using eggs of the flour moth *Ephestia kuehniella* (Zeller) as prey and bean pods as an oviposition substrate. Bean pods with *Orius* eggs were regularly collected from cages with adults in the reproductive phase, replaced by fresh ones, and used to start new age cohorts (van den Meijracker, 1994; Venzon et al., 1999).

Synthetic Alarm Pheromone

According to Macdonald et al. (2003), the alarm pheromone of Western flower thrips contains decyl acetate and dodecyl acetate in a molar ratio ranging from 0.4:1 (first instar) to 1.5:1 (second instar). Because we used second instars in our behavioral experiments, we used 0.05 μl of the 1.5:1 mixture dissolved in 500 μl pentane following the procedure described by Teerling et al. (1993a). Decyl acetate (98.1% pure), dodecyl acetate (95%+ pure), and pentane (98% pure) were obtained from Sigma-Aldrich, Los Angeles, CA, USA.

Pheromone Production after Simulated Attack

Five treatments were applied to test the response of thrips larvae to attacks simulated by the experimenter with the aid of a wooden toothpick. Four second instars were transferred to a leaf disk (24-mm diam) cut from the cotyledons of a cucumber plant. Per replicate and per treatment, one focal individual larva was challenged repeatedly by contacting it with a toothpick until it responded by producing an anal droplet. In the first three treatments, the toothpick was dipped in synthetic alarm pheromone (dissolved in pentane) or solvents (water or pentane) as controls. Each focal thrips larva was challenged with a toothpick that had received one treatment only. Per treatment, 15 larvae on 15 different leaf disks were challenged. These tests were performed “blind,” i.e., without the observer knowing which liquid was applied to the toothpick. Therefore, variation in the way the thrips were challenged was independent of the treatment of the toothpick. In a fourth treatment, the toothpick was put in the anal droplet released by a thrips larva upon challenge with a toothpick. Subsequently, this toothpick was used to challenge another thrips larva, of which the response was recorded. For obvious reasons, this treatment could not be performed blind. In the fifth treatment, the focal thrips larva was challenged with a water-dipped toothpick, while one of the other three thrips was being attacked simultaneously by a predatory bug (*O. laevigatus*) and usually produced an anal droplet. Results of this treatment were compared with the controls with a toothpick dipped in water prior to challenge (see above).

All observations were performed with a binocular microscope with magnification 6.3. The time elapsed between challenge with a toothpick and the production of a droplet by the focal thrips larva was measured and subjected to a one-way analysis of variance (ANOVA). Multiple comparisons among treatments were made using a Tukey’s honestly significantly different *post hoc* test.

Results and Discussion

The time elapsed between challenging thrips larvae with a toothpick and droplet production varied among treatments (Fig. 1; ANOVA: $F_{4,70} = 13.7$, $P < 0.001$). Compared to the controls (pentane, water), the response time was significantly shorter in treatments involving natural pheromone (pheromone droplet collected from another thrips larva), synthetic pheromone (decyl acetate and dodecyl acetate dissolved in pentane), and signals released nearby during the attack of another thrips larva by a predator (Fig. 1). However, Tukey tests reveal no difference among

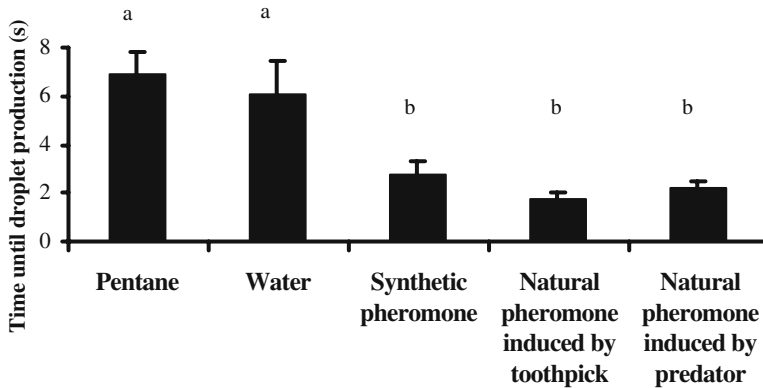


Fig. 1 Response time of thrips larvae to a simulated attack, expressed as the number of seconds between attack and the production of an anal droplet containing the putative alarm pheromone. See text for explanation of the treatments; each treatment involves 15 replicates. Vertical bars represent standard errors. Bars with different letters differ significantly

these three treatments. Hence, other cues emanating from the predator appear to play a minor role in inducing vigilance in thrips larvae. Furthermore, the synthetic pheromone elicits responses similar to those elicited by the natural pheromone, indicating that the concentrations are in the same range. These results provide additional evidence that decyl acetate and dodecyl acetate act as an alarm pheromone. Our results also demonstrate that the response of a simulated attack with a toothpick is context dependent; whereas attacks with a toothpick dipped in water elicit a slow response, attacks with a similarly treated toothpick elicit a fast response when nearby thrips larvae produce a droplet.

If the anal droplet contains alarm pheromone, the response of the thrips larvae to the pheromone is expected to result in increased survival. We think this is the case for two reasons. First, thrips larvae with a fast response were observed to escape more often from an attack by the predatory mite *Iphiseius degenerans* (personal observation, PJAdB). Second, females of other predatory mite species have been reported to retreat upon contamination with the anal droplet (Bakker and Sabelis, 1987, 1989). However, releasing a pheromone also makes the thrips larvae more conspicuous to predators because predatory bugs (*Orius tristicolor*) and predatory mites (*Neoseiulus cucumeris*) are attracted to a source of pheromone (Teerling et al. 1993a,b). Thus, we hypothesize that the release of pheromone-containing droplets upon predator attack depends on: (1) the probability to escape because of increased alertness or predator retreat; (2) the increased vigilance induced in conspecifics; and (3) the increased conspicuousness to predators other than the attacker. It remains to be investigated whether the balance of these costs and benefits explains the behavior of thrips larvae.

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RAPID COMMUNICATION

INTERSPECIFIC PHEROMONE CROSS-ATTRACTION
AMONG SOYBEAN BUGS (HETEROPTERA): DOES
Piezodorus hybneri (PENTATOMIDAE) UTILIZE THE
PHEROMONE OF *Riptortus clavatus* (ALYDIDAE)
AS A KAIROMONE?

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Abstract—The chemical and ecological function of cross-attraction of *Piezodorus hybneri* (Pentatomidae) to the *Riptortus clavatus* (Alydidae) pheromone (a mixture of three components) was studied. In a field attraction test using traps with synthetic pheromone components, *P. hybneri* was attracted to (*E*)-2-hexenyl (*E*)-2-hexenoate, a component of the *R. clavatus* pheromone. Other components had neither an additive nor a synergistic effect on the attraction of *P. hybneri*. Neither (*E*)-2-hexenyl (*E*)-2-hexenoate nor other components of the *R. clavatus* pheromone were detected in volatiles or whole-body extracts of *P. hybneri* adults by gas chromatographic analysis. In addition, (*E*)-2-hexenyl (*E*)-2-hexenoate could not be found in volatiles of soybean plants. Therefore, it appears that *P. hybneri* responds to a component of the *R. clavatus* pheromone that is not emitted by *P. hybneri* itself. We discuss this interspecific pheromone cross-attraction of the soybean bug and hypothesize that *P. hybneri* utilizes the pheromone of its competitor as a kairomone for host location.

Key words—*Piezodorus hybneri*, *Riptortus clavatus*, Pentatomidae, Alydidae, aggregation pheromone, kairomone, cross-attraction, soybean, (*E*)-2-hexenyl (*E*)-2-hexenoate, host finding.

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INTRODUCTION

A complex of bugs (Heteroptera) often threatens soybean production in Japan. The most important species are the bean bug *Riptortus clavatus* (Thunberg) (Alydidae) and the stink bug *Piezodorus hybneri* (Gmelin) (Pentatomidae) (Wada et al., 2006). Pheromone-based monitoring is a significant component of the integrated pest management of these bugs in soybean.

The adult male of *R. clavatus* releases an aggregation pheromone to which conspecific adults and nymphs are attracted (Leal et al., 1995). The pheromone is a mixture of three components: (*E*)-2-hexenyl (*Z*)-3-hexenoate, (*E*)-2-hexenyl (*E*)-2-hexenoate, and tetradecyl isobutyrate in a ratio of 1:5:1 (Leal et al., 1995). These compounds have been synthesized, and the synthetic pheromone is now commercially available (Fuji Flavor Co., Ltd.).

Endo et al. (2003) revealed that the synthetic pheromone of *R. clavatus* attracts not only conspecifics but also *P. hybneri*, which is one of the major competitors of *R. clavatus* in terms of consuming soybean resources. Among the three pheromone components, (*E*)-2-hexenyl (*E*)-2-hexenoate seemed to be responsible for attracting *P. hybneri*. The male-produced sex pheromone of *P. hybneri* has also been identified as a mixture of β -sesquiphellandrene, (*R*)-15-hexadecanolide, and methyl (*Z*)-8-hexadecenoate (Leal et al., 1998). No components in the *P. hybneri* pheromone system are identical with those of the *R. clavatus* pheromone system.

In addition, *Ooencyrtus nezarae*, one of the dominant egg parasitoids of *R. clavatus*, is attracted to the synthetic pheromone of *R. clavatus* (Leal et al., 1995). However, another pheromone component, (*E*)-2-hexenyl (*Z*)-3-hexenoate, was responsible for the attraction in this case (Mizutani et al., 1997). It was suggested that the egg parasitoid utilizes this component of the host pheromone as a kairomone to find host eggs (Leal et al., 1995; Mizutani et al., 1997).

The objectives of the present study were (1) to determine whether or not (*E*)-2-hexenyl (*E*)-2-hexenoate is the only component responsible for attracting *P. hybneri* and (2) to confirm the absence of the pheromone components of *R. clavatus* in body extracts and volatiles emitted from *P. hybneri*. On the basis of our findings, we discuss the ecological significance of this interspecific cross-attraction among soybean bugs.

METHODS AND MATERIALS

Chemical. The pheromone components of *R. clavatus* and *P. hybneri* were synthesized as described previously (Leal et al., 1995, 1998).

Field Experiment. A field experiment using traps baited with the pheromone components of *R. clavatus* was conducted in soybean fields of the National Agricultural Research Center for Kyushu Okinawa Region (KONARC), Kumamoto, Japan. Synthetic (*E*)-2-hexenyl (*E*)-2-hexenoate (EE), (*E*)-2-hexenyl (*Z*)-3-hexenoate (EZ), and tetradecyl isobutyrate (14iBu) were incorporated into plastic pellets (4- to 5-mm diam, containing 5 mg chemical) made of a copolymer of polyethylene and ethyl acetate and used as bait for the traps. Treatments were EE (50 mg), EZ (10 mg) plus 14iBu (10 mg), *R. clavatus* pheromone blend (EZ/EE/14iBu = 1:5:1, total 70 mg), and an empty (blank) trap. A plastic bottle (4 × 3.3 cm ID) with plastic pellets containing the chemicals was placed 5 cm above the water surface of a water-pan trap (16 × 35 cm ID). One set of traps was spaced 20 m apart and placed randomly around the soybean field. The numbers of adults and nymphs of *P. hybneri* and *R. clavatus* captured in traps were counted and removed every 3 d (15 October to 26 November 2004, a total of 14 replicates). Pheromone lures were renewed every 2 wk.

Insects. Adults of *P. hybneri* and *R. clavatus* were caught in soybean fields of the KONARC, and their progenies were kept in the laboratory (25 ± 1°C, 16-hr light/8-hr dark photoregime). Both species were reared on a diet of soybean seeds, red clover (*Trifolium pratense*) seeds, and water. After molting to the adult stage, the bugs used for experiments were transferred and kept individually in plastic containers to prevent sexual contact.

Extracts. Whole-body extracts of *P. hybneri* and *R. clavatus* were prepared by immersing five mature adults (15 d old) in hexane for 3 min. Airborne volatiles from *P. hybneri* (10 bugs) were collected on a Tenax TA (GL Sciences Inc., Japan) column as follows. Cohorts of 10 virgin adults (15 d old) and five soybeans were placed in individual 250-ml glass vessels. Activated charcoal-filtered, humidified air (100 ml/min) was pulled through the system by vacuum, over the insect, and out through the Tenax TA (60/80 mesh, 100 mg) column. Bugs were aerated for 24 hr, and then the column was eluted with hexane (1 ml × 3). Volatiles from soybean plants (leaves and pods) were collected in the same way as for the bugs.

Analysis of Extracts. Hexane extracts of bugs and soybean plants were concentrated and, subsequently, analyzed by gas chromatography–mass spectrometry (GC-MS). GC was performed on a Hewlett-Packard 5890 Series II Plus instrument equipped with an Equity-5 column [30 m × 0.25 mm × 0.25 µm; split mode (41:1); Supelco Inc., Bellefonte, PA, USA] with helium carrier gas. The column temperature was held at 50°C for 1 min, increased to 180°C (5°C/min) and held for 1 min, and increased to 230°C (10°C/min) and held for 5 min. MS spectra were recorded on JMS-600W (JEOL, Japan) in the electron impact (EI) mode (70 eV). Compounds were identified by comparison of retention times and mass spectra with those of authentic standards.

TABLE 1. NUMBERS^a OF *Piezodorus hybneri* AND *Riptortus clavatus* CAUGHT IN THE TRAPS BAITED WITH *R. clavatus* PHEROMONE COMPONENTS

Treatment ^b	<i>Piezodorus hybneri</i>				<i>Riptortus clavatus</i>			
	Male	Female	Nymph	Total ^c	Male	Female	Nymph	Total ^c
EZ + EE + 14iBu	0.86	0.57	0.50	1.93 ± 0.63a	0.50	0.36	0.57	1.43 ± 0.45a
EZ + 14iBu	0.14	0	0.14	0.29 ± 0.13b	0.21	0.21	0.50	0.93 ± 0.22a
EE	0.57	0.57	0.64	1.79 ± 0.53a	0.07	0	0.14	0.21 ± 0.11b
Blank	0	0	0.14	0.14 ± 0.14b	0	0	0	0.00 ± 0.00b

^aEach numeral shows the mean of the bugs caught per 3 d (14 replicates from 15 October to 26 November).

^bEZ: (*E*)-2-hexenyl (*Z*)-3-hexenoate; EE: (*E*)-2-hexenyl (*E*)-2-hexenoate; 14iBu: tetradecyl isobutyrate.

^cMeans (±SE) followed by the same letter are not significantly different at 5% level by ANOVA and Tukey-Kramer test. The data were analyzed after $\log(x + 0.5)$ transformation.

RESULTS

Field Experiment. Trap catch data indicated that the *R. clavatus* pheromone blend (EZ/EE/14iBu = 1:5:1, total 70 mg) and EE (50 mg; equal to the amount in the pheromone blend) attracted adults and nymphs of *P. hybneri* (Table 1). The mean catch of *P. hybneri* did not differ significantly between EE and the *R. clavatus* pheromone blend. A few bugs were caught in the trap baited with the mixture of 14iBu (10 mg) and EZ (10 mg), but these values were not significantly different from that of the control.

The *R. clavatus* pheromone blend (70 mg), as well as the mixture of 14iBu (10 mg) and EZ (10 mg), attracted adults and nymphs of *R. clavatus*. EE

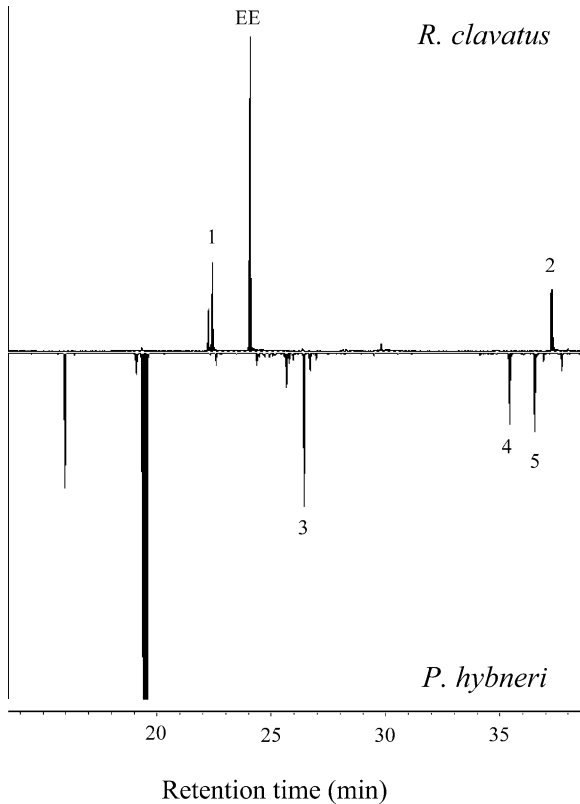


FIG. 1. Gas chromatograms of extracts from *Riptortus clavatus* (top) and *Piezodorus hybneri* (bottom) males. EE: (*E*)-2-hexenyl (*E*)-2-hexenoate; 1: (*E*)-2-hexenyl (*Z*)-3-hexenoate; 2: tetradecyl isobutyrate; 3: β -sesquiphellandrene; 4: (*R*)-15-hexadecanolide; 5: methyl (*Z*)-8-hexadecenoate.

(50 mg) alone did not attract *R. clavatus*, consistent with previous reports (Mizutani et al., 1997; Endo et al., 2003)

Chemical Analysis. The presence of the three pheromone components, EE, EZ (**1**), and 14iBu (**2**), in the extract from *R. clavatus* males, as reported by Leal et al. (1995), was confirmed (Figure 1, top). In *P. hybneri* males, EE was not detected in either the body extracts (Figure 1, bottom) or headspace volatiles (not shown). The three pheromone components of *P. hybneri* (Leal et al., 1998) were detected: β -sesquiphellandrene (**3**) (R_t 26.26 min), (*R*)-15-hexadecanolide (**4**) (R_t 35.26 min), and methyl (*Z*)-8-hexadecenoate (**5**) (R_t 35.26 min; Figure 1, bottom). In addition, neither EE nor the other two components of the *R. clavatus* pheromone were found in any of the extracts from *P. hybneri* females or from soybean plants (not shown).

DISCUSSION

Interspecific pheromone attraction has been reported for some true bugs. Such cross-attraction has been observed between species of the same genus (Aldrich et al., 1991) and even among species in different families (Borges et al., 1998; Zhang and Aldrich, 2003). These cross-attraction phenomena have been attributed to common pheromone components shared by the species involved (pheromone sharing). For example, synthetic pheromone of the milkweed bug *Oncopeltus fasciatus* (Lygaeinae) attracts males of the plant bug *Phytocoris difficilis* (Miridae). This phenomenon has been explained in part by the coexistence of (*E*)-2-octenyl acetate in both pheromone systems (Zhang and Aldrich, 2003). In the Brazilian soybean stink bug complex, *Piezodorus guildinii* is attracted to a synthetic racemic mixture of methyl 2,6,10-trimethyltridecanoate, which is a pheromone component of *Euschistus heros* (Borges et al., 1998). Further study (Borges et al., 1999) revealed that *P. guildinii* has the same chemical compound in its pheromone.

Interspecific pheromone attraction among *R. clavatus* and *P. hybneri* seems to be a phenomenon that differs from the pheromone sharing described above. Our field data clearly indicated that *P. hybneri* was attracted to (*E*)-2-hexenyl (*E*)-2-hexenoate, a component of the *R. clavatus* pheromone. Other components had neither additive nor synergistic effects on the attraction to *P. hybneri*. However, (*E*)-2-hexenyl (*E*)-2-hexenoate was not detected in volatiles or extracts from *P. hybneri* adults, despite the detection of the three pheromone components from *P. hybneri* males. In addition, the chemical structure of (*E*)-2-hexenyl (*E*)-2-hexenoate is quite different from the structures of the *P. hybneri* pheromone components. Thus, it is unlikely that *R. clavatus* and *P. hybneri* share a common compound in their pheromone blends, as exemplified by

pheromone sharing. One likely possibility is that *P. hybneri* is attracted by a pheromone component of *R. clavatus*, which is a competitor in soybean fields.

A possible explanation for the interspecific attraction found between *R. clavatus* and *P. hybneri* is that *P. hybneri* utilizes the *R. clavatus* aggregation pheromone as a kairomone to search for food plants. Both species have similar host ranges, feeding mainly on leguminous plants including soybean (Kono, 1991). Males of *R. clavatus* release a pheromone only when food is available (Leal et al., 1995). Therefore, if *P. hybneri* responds to a component of the *R. clavatus* pheromone, an individual can increase its probability of finding food. Thus, the response to the component of the *R. clavatus* pheromone is adaptive for *P. hybneri*.

Further studies will be needed to verify the hypothesis that *P. hybneri* utilizes the pheromone component of its competitor as a kairomone. However, our finding of interspecific cross-attraction between soybean bugs provides new insights into the diverse communication systems of heteropteran bugs.

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On the Definition and Measurement of Human Scent: Comments on Curran et al.

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A recent article by Curran et al. [*J. Chem. Ecol.* vol. 31(7); 1607–1619, 2005] describes the collection and chemical analysis of “human scent.” Contrary to the authors' claims, a great deal is known about the chemical constituents of human scent and its measurement. Here we clarify what is known about human scent and highlight several shortcomings concerning the authors' analysis related to (1) the definition of human scent, (2) chemical analysis of human scent, and (3) conclusions about individual differences.

Human Scent

More than 15 years of research has presented both organoleptic and analytical evidence that a mixture of C₆–C₁₁ normal, branched, hydroxy- and unsaturated acids present in axillary sweat constitutes the characteristic axillary odor (Zeng et al., 1991, 1992, 1996a,b; Natsch et al., 2003). In addition to this mixture of major odor constituents are trace amounts of thioalcohols (Hasegawa et al., 2004; Natsch et al., 2004; Troccaz et al., 2004) with high odor impact (low olfactory threshold). The details of the chemical identification, exact structures, and synthesis (of noncommercially available compounds), as well as biogenesis of many of these compounds, have been described in the above cited manuscripts.

Importantly, these characteristic axillary odorants are volatile organic compounds. Thus, we read with dismay the authors' comments that there is “limited understanding of how the body produces the volatile organic compounds present in human odor.” Furthermore, their comment on the bottom of page 1608 to the top of 1609, “Although the components of human sweat have been studied extensively, comparatively little work has been carried out

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to determine the volatile organic compounds (VOC's) present in human odor," is also incorrect. The compounds that give the underarm its characteristic odor are volatile organic compounds (VOC's).

We have previously demonstrated that the characteristic odor components are not the most abundant compounds found in underarm extracts (see Figure 2 in Zeng et al., 1991). Hence, their definition of "human scent" as the most abundant VOC's is inconsistent with past studies and unfounded. It also contradicts numerous studies of food, beverages, plants, and malodors that have used the nose as a detector to determine which compounds have the greatest odor impact. The "scent" is often found in the minor constituents of complex mixtures from natural products. Likewise, Zeng et al. (1991) used gas chromatography olfactometry (GC-O) to identify the characteristic "human scent" constituents, which turned out not to be the most abundant compounds.

Analytical Techniques to Measure Scent

To measure scent, Curran et al. used gauze pads to swipe armpits of subjects following exercise, and then used solid phase microextraction (SPME)–gas chromatography/mass spectrometry to sample the headspace above the gauze pads. It is possible that many of the compounds identified by this approach have no relevance to human odor for at least three reasons.

First, and the most serious, the characteristic axillary odorants are predominately a variety of polar, acidic compounds that will bind to the gauze material used for collection. Therefore, SPME headspace analysis over the gauze pads would not be adequate to sample and detect these types of compounds, which offers one possible explanation for their nondetection of 3-methyl-2-hexenoic acid (3M2H). We demonstrated more than 25 years ago (Labows et al., 1979) that the major compounds collected by sweeping and collecting the headspace above either cotton pads worn in the underarm or the underarm itself are not characteristic of axillary odor. In model experiments, we also noted that aliphatic acids were not readily transferable from cotton pads to headspace collection devices. In that study, most compounds observed in the headspace were exogenous constituents because of residual VOCs from cosmetic products. The important odor constituents are polar molecules that tend to bind to pad material. In Curran et al. (2005), dodecanoic and tetradecanoic acid were found only in the axillary volatiles of one subject (out of eight). In a previous study (Zeng et al., 1996a), these acids were found in each gender. The authors do not comment on the sensitivity of their instrumentation, but it is likely that the nondetection of these two compounds is an example of polar compounds remaining on absorbent surfaces.

Second, the sampling procedure of wiping the underarm after exercise is more likely to collect eccrine sweat than apocrine secretions, the well-documented source of axillary odor precursors (Shehadeh and Kligman, 1963; Zeng et al., 1992, 1996b). In an eccrine-enriched sample, odorants derived from the interaction of axillary bacteria and apocrine secretions are not given time to form. To this point, the absence of 3M2H in their analysis, one of the most abundant characteristic axillary odorants, should have been an immediate indication that something was amiss with their measurement technique, and that the measured compounds might not be characteristic of human scent.

Third, prior to sampling, subjects were instructed to discontinue use of deodorants, lotions, and perfumes for at least 48 hr. This "washout" period is likely too short to prevent collection of exogenous compounds, so that many of the compounds detected might actually be artifacts of cosmetic products. Labows et al. (1979) showed that the less volatile components of some

cosmetic products may still be found in axillary headspace analyses 10–14 days after subjects stop using them.

Individual Differences

In their abstract, Curran et al. state, “Qualitative differences noted between the males and females studied, along with differences in chemical ratio patterns among the common compounds, demonstrated the ability to differentiate between the individuals through the examination of VOC’s,” which is an apparent overgeneralization of their reported data. Any conclusion about differences between individuals needs to account for intrasubject variability, i.e., variability between samples from the same subject collected on different days or conditions. This is illustrated in Figure 2 of the paper, but no data were provided, nor were they factored into statements about individual differences. [The authors cite a previous study in their lab comparing two samples from one subject with one sample from another subject as evidence for repeatable individual differences.] Beyond statistical significance—which is not demonstrated—is the task of accurately discriminating individuals from each other, which is generally more difficult to accomplish, and again not demonstrated by the authors.

For these data, it seems the best we can do is look for qualitative or quantitative differences between *genders*. Four males and four females were used in the study. Table 2 in Curran et al. provides *qualitative* comparisons between the eight subjects, showing which of the 47 compounds were detected for each subject. No compounds show statistically significant qualitative differences between genders.

Table 3 in Curran et al. shows the relative ratios of 22 compounds (relative to decanal) for all eight subjects. Some of these ratios are reported as 0 where the compound was not detected, which can complicate quantitative comparisons: it is possible that the compound is present in trace amounts below detection limit but greater than 0. With this in mind, and using two standard two-sample comparison techniques (*t*-test and Wilcoxon nonparametric test based on ranks), we found only one compound to be marginally significant (nonanoic acid–methyl ester; *t*-test, $P = 0.09$; Wilcoxon, $P = 0.11$), but after adjusting for the large number of hypothesis tests, no compound is significant.

In summary, it is possible, perhaps even likely, that people have distinct genetically determined odor profiles—or *odorprints*. Sweat contains a complex mixture of compounds whose expression is influenced by a person’s genetic makeup. The profile of volatile metabolites is also likely to reflect, in part, a person’s genotype. Because it is difficult to determine whether a VOC profile is really measuring “scent” without behavioral confirmation (e.g., perception tests using GC-O), the authors may have been a bit overzealous in their definition of “scent.” From a forensics perspective, however, it does not really matter whether the VOC profile measures “scent” or something else, as long as it can be used to differentiate individuals. Similarly, it might not matter which secretions the profile of constituents is obtained from, although, again, the “scent” moniker is not justified for all chromatographable constituents.

Whatever the definition, the individuality of human scent as measured by VOC’s has yet to be demonstrated.

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On the Definition and Measurement of Human Scent: Response by Curran et al.

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Many of the concerns raised by Preti et al. in their letter to the editor of *JCE* entitled “On the definition and measurement of human scent” appear to be the result of misunderstandings regarding the objectives of the published work as well as the different terminology used in this multidisciplinary research project. Perhaps, we could have been clearer in describing the overall goals of the project. However, the purpose of the paper (Curran et al., 2005b) was not to identify the components of perspiration, nor to differentiate subjects, but rather to provide a limited qualitative and semiquantitative population study of the volatile components above collected axillary samples via headspace solid phase microextraction-gas chromatography/mass spectrometry (SPME-GC/MS), and to analyze the pattern differences among primary odor compounds.

Although we recognize that it is important not to be overzealous in using the terms odor or scent, to limit these terms to only those chemicals eliciting responses from an animal severely restricts their use. For example, Preti’s use of the term “characteristic axillary odor” should be restricted to discussions of human perception of malodor since insects and canines may not use the same chemicals for detection. When referring to human odor, many of the studies referenced by Preti et al. mistakenly interchange the terms human odor and “characteristic axillary odor.” This is illustrated in the Letter to the Editor itself when citing a paper by Zeng et al. (1991) that used GC olfactometry “to determine which of the chromatographic effluents had odors resembling axillary extract” (p. 1474), and was not intended to identify the characteristic “human scent” constituents as claimed. The papers cited by Preti et al. are focused on studies intended to determine the specific compounds

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that contribute to human axillary malodor and are, therefore, limited in application to the broader research field of human scent detection that includes canines and machines.

In law enforcement, as well as the forensic science community, the terms human scent and human odor are commonly used and often interchanged. The Locard exchange principle is fundamental to the field of forensic science, and proposes that a person can not enter or leave an area or come in contact with an object without an exchange of materials. In the case of human scent evidence, the suspect leaves scent in the location of the crime scene itself or on objects found therein. Because our research interests entail the use of human scent for forensic purposes, our paper (Curran et al., 2005b) states “The authors have defined *human scent* to be the most abundant volatile organic compounds (VOCs) identified in the headspace above *scent* samples; however, other substances that have a relatively low volatility or are present in low concentrations may make contributions to *human odor*.” In our paper, human odor was used as a general term to describe overall body odor, and not region-specific. Although axillary sweat was collected, and thus a scent sample from the axillary region, the purpose of our study was never to analyze “characteristic axillary odor.”

Human Scent

The 15 years of research conducted and cited by the authors of the Letter to the Editor pertain to compounds that are characteristic of axillary odor as described by humans. It is known that human secretions and emanations contain a vast number of compounds as stated in the previously referenced papers as well as by Bernier et al. (1999, 2000). The elucidation of how the body produces a handful of these (as described in the papers referenced by Preti et al.), which have been determined to be characteristic of axillary odor, does not translate into a complete understanding of how the human body produces all of the volatile organic compounds in human odor. Thus, our statements pertaining to “a limited understanding in how the body produces the volatile organic compounds in human odor” are founded.

Another point of contention by Preti et al. is the statement in our paper (Curran et al., 2005b) on pp. 1608–1609, “Although the components of human sweat have been studied extensively, comparatively little work has been carried out to determine the VOCs in human odor.” The compounds that give the body its detectable odor are anticipated to fall into the volatile to semivolatile range; yet, the majority of work conducted in the arena of determining the VOCs present in human odor has been carried out with emphasis on the components of sweat. Consequently, our manuscript goes on in the next sentence to say: “Knowing the contents of human sweat may not accurately represent the nature of what volatile compounds are present in the headspace above such samples, which would comprise the odor.” This is not a new concept, as it was stated as the reasoning for not conducting solvent extractions of sweat in *Analytical Chemistry* by Bernier et al. (1999). While investigating the volatile compounds emitted by humans that function as mosquito attractants, they reported that “these volatiles may not be present in the perspiration aqueous phase.”

Advances in the field of machine olfaction or mimicking the sense of smell further complicate the use of the terms odor and scent. Instruments that are capable of distinguishing odors via electronic detection (including arrays of sensors) and suitable signal processing (typically a statistical system based on multivariate analysis) units are commonly referred to as electronic noses. This instrumental “olfaction” of chemicals capable

of differentiating individuals may utilize different chemicals from those utilized by biological systems. It is well established that different organisms may utilize distinct targets with invertebrates capable of both excitatory and inhibitory responses to odors via multiple transduction pathways. In addition, the number of detectable odors may exceed 100,000 depending on the training of the organism to discriminate odors and on the physical chemistry, which acts as a limiting factor on an odor chemical's ability to act on olfactory systems based on volatility, solubility, and stability (Firestein, 2001).

Olfactory studies of food, beverages, plants, and studies of the compounds with the greatest human determined odor impact may have no bearing on the differentiation of humans by scent identification canines. In addition, the study of chemicals that have been used by human beings as odor judges may not be applicable to studies relating to the ability of canines to differentiate humans based on scent. Previous headspace studies that compare volatile profiles have demonstrated pattern differences among individuals (Sommerville and Gee, 1987; Sommerville et al., 1994). In addition, pattern matching recognition software has been applied to the volatile patterns obtained from the headspace analysis of collected axillary sweat among people. This research has determined that both qualitative and quantitative differences are important factors when evaluating identity through sweat analysis (Sommerville et al., 1994). We have extended this work to identify specific chemical components that may be important in identifying individuals.

Analytical Techniques

Sample Collection on Cotton Sorbents

Human scent evidence collected from a crime scene can be evaluated through the use of specially trained canines to determine an association between the evidence and a suspect (Sommerville and Gee, 1987; Stockham et al., 2004a; Curran et al., 2005a). Canines can successfully utilize human scent collected on a cotton material to differentiate among individuals (Kalmus, 1955; Settle et al., 1994; Schoon and De Bruin, 1994; Schoon, 1996, 1998; Harvey and Harvey, 2003; Stockham et al., 2004a,b). If what the authors of the Letter to the Editor are proposing by stating that nondetection of acids is “an example of polar compounds remaining on absorbent surfaces” is that the acids are binding to cotton materials rendering them unavailable in the headspace, then it is reasonable to infer that these compounds are also unavailable to canines and, thus, possibly not utilized for human scent discriminations.

It has been shown that males can be distinguished through human evaluation of odors collected on cotton from both the torso and axillary area based on the number of matching major histocompatibility complex (MHC) alleles (McClintock et al., 2005); the odors evaluated were described by the female odor judges as “mild scents” and seldom associated with human malodor. In 1979, when Labows and Preti published their report, suggesting that polar compounds cannot be determined through headspace analysis with a Tenax Trap from cotton materials, it is possible that their technique was not as sensitive as those available today. A recent study by a group from the Federal Bureau of Investigation in collaboration with Oak Ridge National Labs has shown that cotton gauze pads have the ability to trap and release isovaleric acid as well as hexanoic acid, as detectable through headspace GC/MS analysis (Eckenrode et al., 2006). Preti et al. also state that “the important odor constituents are polar molecules that tend to bind to the (cotton) materials,” referring to a previous study into characteristic axillary odors, which we (again) emphasize was not the subject of our study, and, thus, compounds deemed to be “important odor constituents” may have no consequence.

Non-Detection of 3-Methyl-2-Hexenoic Acid

A study conducted by Munk et al. (2000) to determine the compounds present in residual axillary odor on cotton (polar backbone) and polyester (nonpolar backbone) after wearing and then washing failed to detect acid compounds. The study utilized human odor judges, and was followed by subsequent liquid extraction of swatches deemed to contain an axillary odor quality and these swatches did not contain organic acids. The compound classes identified in the extract were esters, ketones, and aldehydes, thus suggesting that human perception of axillary malodor may not be limited to the acid class. Direct sampling of the volatile organic compounds released by the skin through SPME-GC/MS, without the use of a collection material, has been conducted by various groups in which 3-methyl-2-hexenoic acid (3M2H) has not been reported (Ostrovskaya et al., 2002; Zhang et al., 2005). Nondetection of 3M2H by the headspace SPME/GC-MS method employed in our research (Curran et al., 2005b) does not mean the compounds are not present, but rather, that they are minor components of the headspace and may be below the detection limit of the instrument. Likewise, nondetection by an animal does not necessarily mean that the compounds are not odor compounds or scent components, but rather, the delivered amount may be below—or in some cases, above—the range of detection for the animal. Our definition of human scent acknowledges the fact that some components, due to low volatility and or concentration, which are not detected, may contribute to human odor.

Sampling Procedure

Preti et al. cite a study by Labows and Preti (1979) and state that most compounds observed in the headspace of cotton pads worn in the axillary area were exogenous. However, the sampling method used by Labows and Preti lends itself to the collection of exogenous compounds, as the cotton pads were worn overnight, thus coming into contact with various types materials (bedding, sheets, clothing, etc.), which would contribute exogenous or “tertiary odor” components to the collection materials. With the sampling procedure followed in our experiment (Curran et al., 2005b), the axillary area is wiped for sample collection, thus limiting the opportunity for tertiary odors to influence the sample. We acknowledge that the influence of deodorants may not have been completely removed through discontinued use; however, other studies (Sommerville et al., 1994) on the volatile components of human sweat also used the same method of no deodorant for a 48-hr period. It was suggested by Preti et al. that, because our samples were eccrine-enriched, adequate time was not given for the interaction of axillary bacteria and apocrine secretions to allow for these types of odorants to be formed. The samples collected in our study were allowed to sit for 24 hr at room temperature prior to extraction, and no attempt was made to control microbial activity inside the vials. This was modeled from the fact that suspect samples are generally kept no longer than 48 hr before canine human scent lineup evaluations by most law enforcement agencies in Europe, including the Netherlands National Police Agency. The sampling method was also created for the purpose of collecting a concentrated sweat sample by using the materials and storage techniques employed for collecting human scent evidence for canine evaluation, which was discussed on p. 1611 (Curran et al., 2005b).

As described in our introduction (Curran et al., 2005b), canines have the ability to differentiate human odors (Kalmus, 1955; Sommerville and Gee, 1987; Settle et al., 1994; Schoon and De Bruin, 1994; Schoon, 1996, 1998; Hepper, 1998; Harvey and Harvey, 2003; Stockham et al., 2004a,b). By utilizing human scent collected from objects and/or people,

canines have also demonstrated the ability to generalize odors (Settle et al., 1994) collected from different regions of the body with an 85% accuracy. These studies suggest that there are common elements to human odor produced and/or released among the various body regions that contain differing types and/or quantities of secretion glands. The purpose of our study was not to analyze apocrine sections, but the secretions of the axillary region as a whole, which is composed of apocrine, eccrine, and sebaceous secretion. As has been presented elsewhere (Curran et al., 2006), in the United States more than 70% of items from which human scent is collected for forensic purposes have been handled by people and, thus, contain odor imparted from the secretions of eccrine and sebaceous glands. Since our primary interest lies in being able to apply the information gained from our study to forensic science and the canine community, it was expected that the samples would be eccrine-enriched and, thus, not an oversight or a flaw in experimental design.

Individual Differences

The focus of our study (Curran et al., 2005b) was neither to determine differentiation between subjects nor to determine stability of a profile for an individual over time. Rather, our study demonstrates that through the use of SPME as an extraction method, it is possible to analyze human scent and measure ratio pattern differences among subjects. The 23 compounds compared for ratio differences among the eight individuals were selected because of their probability as being “primary odor” components, as their presence was detected in weekly scent samples for an individual, thus reducing the chance of selecting an exogenous, “tertiary odor” components for comparison. The primary odor of an individual is the portion of the odor that is most likely to be stable over time, and is not influenced by external factors. To be considered a primary odor component, a compound must be present in all samplings for that individual, and it may differ among subjects (Curran et al., 2005a).

While Preti et al. were unsuccessful in differentiating individuals by applying a *t*-test and Wilcoxon nonparametric test to our data, if one utilizes nonparametric Spearman ranking correlation to our data, there is a statistically significant difference between the individuals in the study. Comparing the results of eight subjects in the possibility of 28 pairs and at a correlation threshold value of 0.9, there are no Type II errors (100% distinguished), whereas a threshold of 0.8 results in two Type II errors at correlation values of 0.81 and 0.83 (92.8% distinguished). Preti et al. correctly state that intrasubject variability is important when discussing the significance of intersubject differences, and this was qualitatively displayed in Figure 2 of our paper. However, quantitative comparisons were not presented, as this was not the focus of the presented work.

In summary, the concerns stated by Preti et al. in their Letter to the Editor appear to be the consequence of differences in terminology and misinterpretation regarding the objectives of the published work. We consider the study of human scent to be a multi-disciplinary field; if relevant research is restricted only to those studies on human perceived malodorous axillary substances, then there will be no occasion for comprehensive understanding of the compounds that emanate from the human body as a whole. Preti et al. draw conclusions from our study that are beyond the scope of the paper. While interpretive differences exist between Preti et al. and our group, there are agreements as well. This may represent a convergence between research groups addressing different aspects of this important subject. We agree with Preti et al. that it is likely that people have distinct genetically determined odor profiles made up of a complex mixture of volatile metabolites reflecting, in part, a person’s genotype. We also agree that, from a forensic science perspective, it does not matter whether the VOC profiles measure “scent” or something else, as

long as it can be used to differentiate humans. We disagree, however, that a VOC profile cannot be referred to as measuring “scent” without a human perception test such as GC-O and, rather, describe this as one measure of scent. Finally, while “the individuality of human scent as measured by VOCs has yet to be demonstrated,” this was not our intention. The reevaluation of the data through statistical analysis as presented here supports the hypothesis of the uniqueness of human scent. Larger-scale studies are ongoing to evaluate the differentiation of human scent samples by using precleaned collection materials, an optimized SPME-GC/MS method and multivariate statistical analysis.

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Geographic Variation in Attraction to Human Odor Compounds by *Aedes aegypti* Mosquitoes (Diptera: Culicidae): A Laboratory Study

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Abstract Previous investigations of *Aedes aegypti* response to human odor components have revealed a number of compounds that attract host-seeking females. However, such studies have utilized only a small number of long-term laboratory *Ae. aegypti* colonies. Using laboratory y-olfactometers, we studied the attraction of four different *Ae. aegypti* populations (North Queensland, Australia; Florida, USA; Singapore; and Minas Gerais, Brazil) to a key attractant compound from human skin, lactic acid. Combinations of lactic acid with ammonia and a fatty acid (caproic acid) were also investigated. The aims were to determine the extent of variation in lactic acid dose response among populations and to see whether all four populations responded equally to combinations of human odor components. Although all *Ae. aegypti* populations were attracted to lactic acid, there were differences in the threshold dose: Florida 0.03 $\mu\text{g}/\text{min}$, Singapore 0.17 $\mu\text{g}/\text{min}$, North Queensland 1.92 $\mu\text{g}/\text{min}$, and Brazil 10.27 $\mu\text{g}/\text{min}$. Attraction to lactic acid alone

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(maximum <40%) was significantly lower than for human odor (>87% for all populations). Significant increases in attraction were observed when lactic acid was combined with ammonia or caproic acid, although not for all populations. In addition, the highest doses of caproic acid tested decreased attraction when combined with lactic acid. The divergent responses to host kairomones seen here may be evidence of adaptation to locally available hosts in different parts of the geographic range of *Ae. aegypti*.

Keywords *Aedes aegypti* · Ammonia · Caproic acid · Host-seeking · Lactic acid · Y-olfactometer

Introduction

The yellow fever mosquito *Aedes aegypti* (L.) is a prominent vector of dengue viruses throughout tropical regions, resulting in human illnesses ranging from mild fever to fatal hemorrhagic disease (Gubler and Kuno, 1997). The role of *Ae. aegypti* as a dengue vector is related to its ability to breed in water-filled habitat associated with human habitation and its preference for human blood hosts (Gubler and Kuno, 1997). This host preference is facilitated by strong olfactory responses to human skin emanations (Skinner et al., 1965; Schreck et al., 1990; Geier and Boeckh, 1999).

The surveillance of *Ae. aegypti* populations has traditionally relied on labor-intensive methods, such as the sampling of immature stages from breeding sites, and on the use of ovitraps that sample the egg-laying portion of the population. However, some of the indices obtained lack epidemiological sensitivity (Focks, 2003). Sampling techniques for adult *Ae. aegypti*, such as human-biting catch and aspiration samples, can be unsafe and labor intensive for operators (Focks, 2003). Hence, there is a need to develop efficient sampling methods for adult *Ae. aegypti* that can be readily related to dengue epidemiology. The development of traps that specifically attract host-seeking *Ae. aegypti* through the use of kairomone lures would provide a more efficient and epidemiologically relevant alternative, and possibly augment current population reduction methods.

A number of compounds identified from human skin emanations attract host-seeking *Ae. aegypti*. Lactic acid was identified as a key kairomone for *Ae. aegypti* host finding (Acree et al., 1968; Geier and Boeckh, 1999), given that the addition and removal of this compound from human skin extracts respectively increases and decreases attraction in laboratory olfactometers (Geier et al., 1996; Steib et al., 2001). However, it is much less attractive alone than whole human odor extracts (Eiras and Jepson, 1991; Geier et al., 1996), indicating that other kairomones are important in *Ae. aegypti* host finding. The combination of lactic acid with carbon dioxide creates a powerful attractive synergism (Acree et al., 1968; Smith et al., 1970; Eiras and Jepson, 1991) as do other compounds from human skin, namely ammonia (Geier et al., 1999a), fatty acids of various chain length (Bosch et al., 2000), dimethyl disulfide, and acetone (Bernier et al., 2003). The response to lactic acid alone or when combined with other kairomones is dose dependent (Geier et al., 1996, 1999a; Bosch et al., 2000). However, the importance of such compounds in nature is not as certain, so extrapolation from laboratory studies must be performed with caution, especially as all research to date has been carried out with a single *Ae. aegypti* strain that has been reared in the laboratory for several decades.

To develop attractive kairomone blends for global use in *Ae. aegypti* trapping, the response of different populations to key kairomones should be assessed to evaluate

geographic variability in responses and to determine whether specific blends are needed for different populations. Furthermore, such a study may detect differences in the sensory physiology of *Ae. aegypti* throughout its geographic range. Given that genetic differentiation may exist between *Ae. aegypti* populations only a few kilometers apart (Tabachnick and Powell, 1978; Paupy et al., 2004), and that other mosquito species show heterogeneity in odor-mediated host preferences throughout their geographic range (e.g., Williams et al., 2003), it seems likely that *Ae. aegypti* populations could become adapted to particular kairomones exuded by locally available resources (i.e., humans). Any geographic variability in human odor profiles could facilitate such adaptation, which would be manifested in divergent responses to certain kairomones.

The aims of this study were to describe the response of host-seeking *Ae. aegypti* from four populations of distinct geographic origin to host kairomones, using laboratory y-olfactometers. The response of each population to increasing doses of lactic acid alone as well as in combination with ammonia or a fatty acid (caproic acid) was to be established. A combination of all three compounds forms a proprietary blend (Geier and Eiras, 2003; BioGents GmbH, Regensburg, Germany) and is the focus of a subsequent study.

Methods and Materials

Mosquito Rearing Methodology

Four populations of *Ae. aegypti* mosquitoes, which varied in their laboratory colonization history, were used in these studies at the University of Regensburg (September–November 2004; Table 1). Although it would have been ideal for all populations to be tested as field F_1 generations, this was not practical. Larvae were fed with Tetramin fish food. Adults were maintained in plastic containers (50 × 40 × 25 cm) at 26–28°C, 60–70% relative humidity, light/dark 12:12 hr, and with continuous access to a 10% glucose solution.

Olfactometers

A series of four identical, Plexiglas[®], y-olfactometers (Geier and Boeckh, 1999) on white tables was used (Fig. 1). Eighty l/min of charcoal-filtered, heated ($27.6 \pm 0.1^\circ\text{C}$), and humidified ($63.7 \pm 0.6\%$ relative humidity) air was passed through two parallel stimulus chambers, in which test compounds mixed with the air. These two parallel airstreams (0.2 m/sec) then passed through the common rectangular branch of the olfactometer and into the stem (0.4 m/sec) that was attached to the release chamber holding the mosquitoes. Rotating screens in the release chamber and in both arms of the upwind end enabled the release and recapture of mosquitoes.

Table 1 Summary data for *Ae. aegypti* populations used in this study

Population	Source	Length of time in laboratory
North Queensland, Australia	Cairns	Nil (F_1 generation)
Florida USA	Orlando	Since ca. 1960 (regularly supplemented with field material until ca. 1992)
Minas Gerais, Brazil	Belo Horizonte	Nil (F_1 generation)
Singapore	Singapore	ca. 20 generations

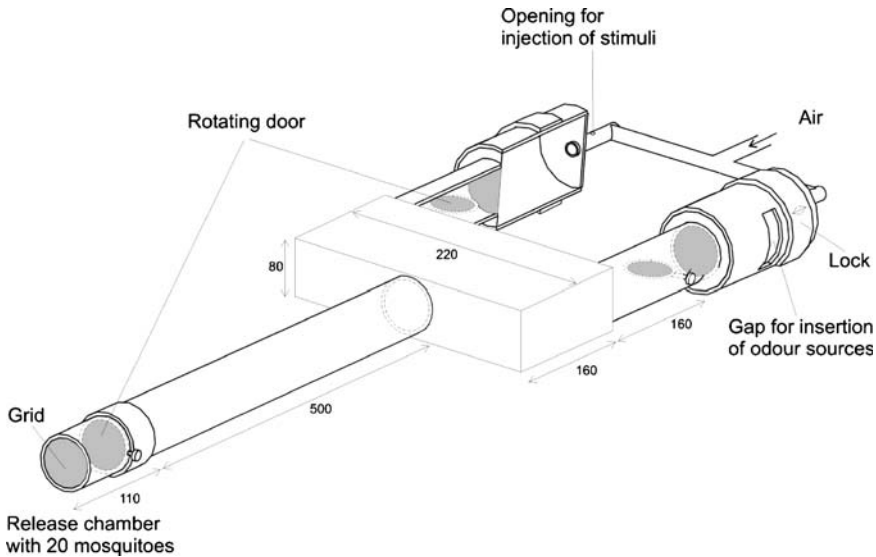


Fig. 1 Schematic drawing of the y-olfactometer design used to test geographically disparate populations of *Ae. aegypti* (Geier and Boeckh, 1999). Dimensions are given in millimeters

Volatile Stimulus Delivery

For each test, volatile stimuli were introduced into one of the two parallel stimulus chambers, with the other remaining as a blank control. Air was passed through a 200-ml Erlenmeyer flask containing 20 ml of the compound under study, and carried the headspace volatiles to ports immediately proximal to the treatment arms of the olfactometers by Teflon tubing (Geier et al., 1999a; Bosch et al., 2000). The delivery rate of volatile compounds to the olfactometers was determined by the airflow rate through the flask. When combinations of compounds were tested, each was delivered through separate flasks and tubing that met at a single release point in the olfactometer.

Bioassay and Statistical Analyses

Previously published methods (Geier et al., 1999a; Bosch et al., 2000) were used. Groups of 17–25 avid female *Ae. aegypti* (10–20 d postemergence, not blood fed, nulliparous) were lured from their holding cages by human odor, thereby selecting host-seeking mosquitoes for tests. This was achieved by fitting the y-olfactometer release cage onto a specially designed fitting on the holding cage, and creating airflow with a small fan that carried human odor from a hand through the release cage into the holding cage. Attracted mosquitoes then entered the release cage, which was then removed.

Before tests, the release cage containing mosquitoes was attached to the olfactometer. Mosquitoes were allowed 20 min to acclimatize, with clean air flushing through. When odor stimuli were introduced, mosquitoes were released from their chamber and allowed 30 sec to respond, after which the rotating screens were closed, trapping those in the treatment and control arms, and in the release cage. Odor stimuli were tested in balanced, randomized block designs. Each experiment involved the use of all four *Ae. aegypti* strains,

allocated to y-olfactometers in a randomized block design. Those captured in the treatment arm of the olfactometer were deemed to have been attracted to the stimulus. The small numbers captured in the control arm were also recorded. Mosquitoes were lured back into their release cage in response to human odor (hand of CRW) by reversing the air flow. Groups of mosquitoes were retested up to five times in this manner, with a 30-min “rest” period between tests, during which clean air continued to flow through the olfactometer. This method verified that mosquitoes would readily respond to natural human odors in between each trial. Previous studies with this apparatus have demonstrated that there are no carryover effects of contamination or behavioral conditioning in between replicate trials (Geier 1995; Geier and Boeckh, 1999).

Furthermore, previous studies have shown that a single laboratory strain of *Ae. aegypti* will respond in a consistent manner to a variety of kairomones (M. Geier and C. Aigner, unpublished data, University of Regensburg, 2001). This was demonstrated by testing cohorts from the same colony repeatedly over 5 mo in the same y-olfactometers used here.

Percentage data (number of mosquitoes in the test arm as a percentage of those released) were arcsine-transformed to normality before ANOVA with least significant difference (LSD) *post hoc* tests performed using SPSS statistical software (Release 11.0.1, 2003, SPSS, Inc., Chicago, IL, USA).

Experiment 1: Lactic Acid

Lactic acid (analytical reagent grade, Merck, Germany) dose was manipulated by adjusting flow rate through the Erlenmeyer flask. Five flow rates ranging from 0.2 to 300 ml/min were used. The size of the lactic acid dose (in $\mu\text{g}/\text{min}$) was determined by using calibration values taken from Geier et al. (1999b).

Human odor was tested as a gold standard control to determine the optimal behavioral response of each population in the y-olfactometers. This was introduced into stimulus chambers by inserting an index finger (always that of CRW) through a snug-fitting hole. A 20 ml water control (air flow: 10 ml/min) was also used to ascertain the baseline level of upwind flight. A minimum of 10 replicate trials was performed for each population for each treatment. Human odor was used despite the risk of subtle variations in kairomone output. The balanced, randomized block design used here ensured that all four *Ae. aegypti* populations would be subject to the same variations in output from the human hand, thereby controlling for this factor. Human hands have been previously used as an odor source in similar experiments (e.g., Geier and Boeckh 1999; Geier et al. 1999b).

The lowest lactic acid dose required to cause attraction significantly greater than the water control is hereafter termed the “attraction response threshold” dose. Means were separated using LSD *post hoc* tests.

Experiment 2: Lactic Acid + Ammonia, and Experiment 3: Lactic Acid + Caproic Acid

The attraction of a fixed dose of lactic acid (determined from experiment 1) with four 10-fold increasing flow rates of ammonia (experiment 2) or caproic acid (experiment 3) ranging from 0.3 to 300 ml/min was investigated. Unlike for lactic acid, we could not determine the ammonia and caproic acid doses in micrograms per minute because of a lack of calibration data. Ammonia (analytical reagent grade, Merck, Germany) was a 25% solution of ammonium hydroxide further diluted 1:100 in water. Caproic acid (Fluka Chemika, Germany) was also of analytical reagent quality.

Results

Experiment 1

All four mosquito populations responded to both lactic acid and human odor (Fig. 2). In all cases, the responses to human odor were significantly higher than to any concentration of lactic acid tested. Mean percentage capture in the control arm was $\leq 6.4\%$ for the lactic acid treatments.

Each of the populations demonstrated different attraction response thresholds. These varied from 0.03 $\mu\text{g}/\text{min}$ for Florida USA ($F = 68.86$, $d.f. = 6$, $P < 0.001$) to 0.17 $\mu\text{g}/\text{min}$ for Singapore ($F = 35.964$, $d.f. = 6$, $P < 0.001$), 1.92 $\mu\text{g}/\text{min}$ for North Queensland Australia ($F = 57.04$, $d.f. = 6$, $P < 0.001$), and 10.27 $\mu\text{g}/\text{min}$ for MG Brazil *Ae. aegypti* ($F = 66.42$, $d.f. = 6$, $P < 0.001$; Fig. 2).

There is no one common flow rate that is more attractive than others for all populations. Therefore, a dose of 50 ml/min (10.27 $\mu\text{g}/\text{min}$) was chosen for experiments 2 and 3, because it was greater than or equal to the attraction response threshold dose for all groups.

Experiment 2

For the North Queensland Australia population, there was no statistically significant increase in attraction when ammonia was added to lactic acid ($F = 1.86$, $d.f. = 4$, $P = 0.131$; Fig. 3), in contrast to the marked increase in attraction seen with the Florida USA population ($F = 12.97$, $d.f. = 4$, $P < 0.001$). For the MG Brazil and Singapore populations, lactic acid and ammonia combined provided greater attraction than lactic acid alone ($F = 2.59$, $d.f. = 4$, $P = 0.048$ for both) although only at the lower ammonia concentrations (Fig. 3).

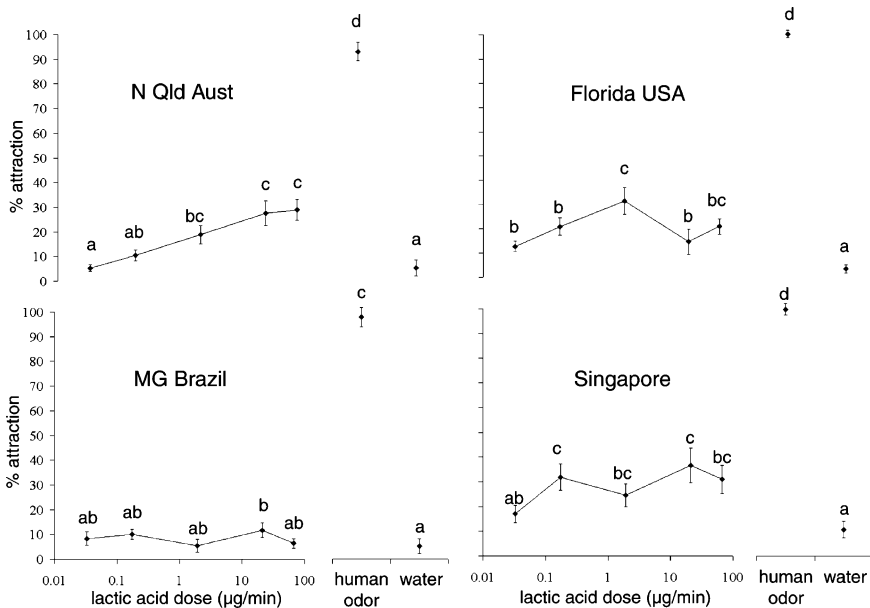


Fig. 2 Mean percentage attraction (\pm SE) of four populations of *Ae. aegypti* to various doses of lactic acid, as well as human odor and water. Different letters indicate significant differences within a population

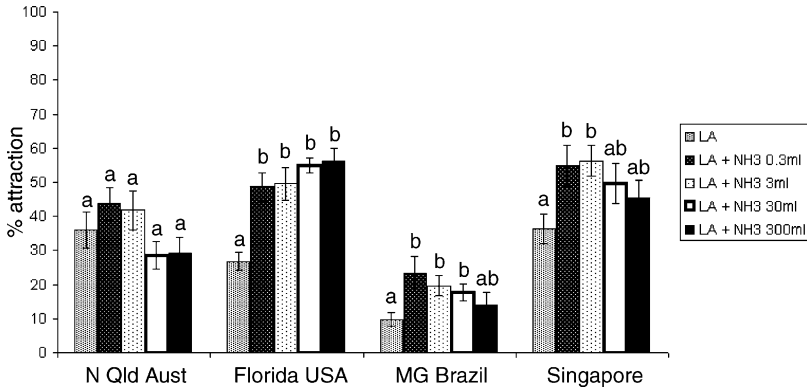


Fig. 3 Mean percentage attraction (\pm SE) of four *Ae. aegypti* populations to mixtures of lactic acid (LA) at 10.3 μ g/min and ammonia (NH₃) in y-olfactometers. Different letters indicate statistically significant differences within a population

When tested alone (0.3 ml/min), ammonia proved slightly attractive to some populations: North Queensland Australia $28.3 \pm 3.9\%$, Florida USA $19.7 \pm 6.2\%$, Singapore $23.1 \pm 3.9\%$, and MG Brazil $8.1 \pm 2.7\%$. These data enabled calculation of the expected additive attraction (E) for lactic acid and ammonia (Colby 1967). These values were then compared with the observed attraction percentage ($O \pm 95\%$ confidence interval) to determine the presence of synergistic attraction. Comparisons were not performed for North Queensland Australia *Ae. aegypti*, as ammonia did not improve attraction for this group (Fig. 3). Singapore ($E = 51.1\%$, $O = 54.8 \pm 11.7\%$), Florida USA ($E = 41.3\%$, $O = 48.7 \pm 8.1\%$), and MG Brazil ($E = 17.1\%$, $O = 23.4 \pm 9.6\%$) *Ae. aegypti* all showed slightly greater attraction (O) than the expected additive effect (E). However, overlapping 95% confidence intervals were suggestive of an additive rather than synergistic effect.

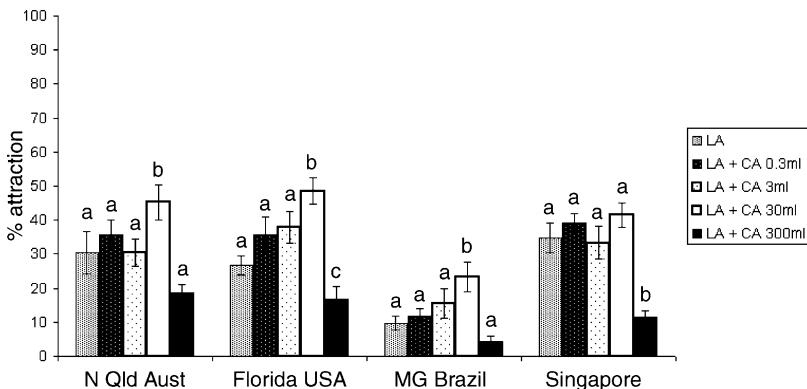


Fig. 4 Mean percentage attraction (\pm SE) of four populations of *Ae. aegypti* to lactic acid (LA) at 10.3 μ g/min and caproic acid (CA) in y-olfactometers. Different letters indicate significant differences within a population

Experiment 3

Lactic acid plus caproic acid (30 ml/min) significantly increased attraction over lactic acid alone or with caproic acid at 0.3 or 3 ml/min, in all populations except Singapore (Fig. 4). Increasing the concentration of caproic acid to 300 ml/min caused a significant decline in attraction, compared with lactic acid plus caproic acid (30 ml/min) in all populations, and was less attractive than lactic acid alone for Florida USA and Singapore populations. ($F = 11.19$, $df = 4$, $P < 0.001$).

Caproic acid alone (0.3 ml/min) showed little or no attraction for *Ae. aegypti*: North Queensland Australia $16.2 \pm 7.8\%$, Florida USA $3.2 \pm 1.9\%$, Singapore $7.8 \pm 5.9\%$, and MG Brazil $6.5 \pm 2.6\%$. Furthermore, caproic acid did not improve the attraction of lactic acid at this flow rate (0.3 ml/min, Fig. 4) and no synergism calculations were possible.

Discussion

Geographically disparate populations of *Ae. aegypti* responded to lactic acid but differed in their sensitivities. The attraction response threshold dose for Florida USA and Singapore strains was below the lowest rate of lactic acid emission from human hands ($0.38 \mu\text{g}/\text{min}$; Smith et al., 1970). Human individuals differ in their level of emission of L-lactic acid from their skin by at least a factor of 5 (Acree et al., 1968; Smith et al., 1970; Steib et al., 2001). However, we do not know how big the differences in the lactic acid emission rates are between different geographic subpopulations of human hosts. MG Brazil mosquitoes showed a much lower response to lactic acid at any dose when compared with other populations. Given the avid response to human odor by this population (Fig. 4), the low response to lactic acid cannot be attributed to some behavioral anomaly hampering y-olfactometer flight. Rather, it indicates that other kairomones may be more important in host finding for this population.

The results presented here concur with those of Geier et al. (1996), who demonstrated that increasing the dose of lactic acid past the attraction response threshold does not significantly increase the attraction of *Ae. aegypti* (Bayer strain). The level of attraction (ca. 25%) reported in Geier et al. (1996) was similar to the level reported for three of the four populations studied here. Also, a similar mean level of attraction (20.5%) was reported by Bernier et al. (2003) for *Ae. aegypti* from the same Florida USA laboratory population by using similar apparatus. This finding demonstrates consistency in response of this *Ae. aegypti* strain to lactic acid when tested in different laboratories. This consistency is further illustrated by similar responses to a human hand in both laboratories (this study; Kline 1998).

The greater attraction of the lactic acid–ammonia combination compared with lactic acid alone, first described for the Bayer strain of *Ae. aegypti* by Geier et al. (1999a), has been confirmed here. An exception is the North Queensland Australia population in which the lack of effect may reflect the good response of North Queensland Australia *Ae. aegypti* to lactic acid alone, or that ammonia is not an important attractant for this population. A significant combination effect was demonstrated for the MG Brazil population, although its attraction both to lactic acid and the lactic acid–ammonia combination was poor. This possibly reflects a poor response to lactic acid in general. The attraction to ammonia when presented alone, up to ca. 28% (North Queensland Australia), is much greater than the ca. 5% presented by Geier et al. (1999a). This may be attributed to behavioral variability between *Ae. aegypti* populations. The increased attraction of lactic acid with the addition of

caproic acid, first described by Bosch et al. (2000), has been confirmed here for at least three *Ae. aegypti* populations, although too much caproic acid may cause reduced attraction.

Because of the varied colonization history of the populations studied here, and the significant genetic differentiation that exists even between *Ae. aegypti* populations separated by just a few kilometers (Tabachnick and Powell, 1978; Paupy et al., 2004), conclusions about naturally occurring heterogeneity in response to attractants throughout the geographic range of *Ae. aegypti* must be made with caution. Nor can generalizations be made about *Ae. aegypti* response to kairomones from particular regions, as population genetic studies would be needed to determine how representative these laboratory populations are for each region.

Groups of *Ae. aegypti* may respond differently to the same volatile compounds, and statements concerning responses to kairomone combinations must be specific to the population in question. The divergent responses to host kairomones seen here may be evidence of adaptation to locally available hosts in different parts of the geographic range of *Ae. aegypti*. However, a study of human odor components in different regions (particularly lactic acid emission levels), paired with population genetic studies and behavioral responses of mosquito strains from different regions, is needed to address such a hypothesis. The results presented here indicate that the development of trapping lures for host-seeking *Ae. aegypti* may require some specialization for the region of use. The validity of laboratory olfactory studies in terms of field behavior has been demonstrated directly for at least one mosquito species (*Culex annulirostris* in Australia; Williams et al., 2003). Nonetheless, we report here the response of *Ae. aegypti* to human odorants over a very short range in an artificial environment. It follows that field trials are required to validate the attraction of *Ae. aegypti* to these odorants. Last, it is clear that human odor is significantly more attractive to *Ae. aegypti* than any of the odorants tested here, confirming that a complex suite of kairomones is required to maximize the attraction of this species.

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Olfaction and Identification of Unrelated Individuals: Examination of the Mysteries of Human Odor Recognition

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Abstract Although several studies have examined the effect of human odor on kin recognition and mate choice, few have focused on the impact of familiarity on recognition of nonrelatives by olfactory cues. As part of a program designed to engage students in scientific research, 53 high school students researched, planned, and implemented a project to analyze the effect of odor on human recognition of, and preference for, friends, sex, and self. A total of 37 students, including friends of their choosing, wore T-shirts for three consecutive nights. During that time, subjects were controlled for exposure to extraneous perfumes, household odors, and other humans. The students were then asked to smell a series of five shirts and evaluate them with respect to pleasantness. Students were also asked to identify the shirts belonging to themselves and their friend, and determine the sex of the person who wore each shirt. Although subjects were unable to distinguish sex by olfactory cues alone, a significant percentage of subjects were able to identify their own odor (51.6%), as well as distinguish the odor cue of their friend (38.7%). Additionally, subjects who could not identify their friend's cue were apt to choose the odor of a member of the opposite sex as their friend. This result was not believed to rely on odor preference as neither individual, friend, nor stranger odors were rated significantly different with respect to odor pleasantness. The ability to recognize friends via odor cues lends credence to the hypothesis that association and familiarity are important aspects of conspecific olfactory recognition in humans. Furthermore, this study augments evidence that olfaction may supplement visual and auditory cues used in human conspecific and kin recognition.

Keywords Kin recognition · Human social behavior · Association · Familiarity

This research was designed and implemented by 53 high school General Biology and Advanced Placement students who contributed as fellow authors to this project. Refer to Acknowledgments for a full list of names.

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Introduction

“Reason is sight. Instinct is touch. Intuition is smell.” Mason Cooley (2001)

Humans are creatures of sight and sound. Yet, of all the human senses, olfaction is considered the most ancient, possessing links to several areas of the brain, including those responsible for cognitive, visceral, emotional, and homeostatic behaviors (Purves et al., 2001). Moreover, although it is often assumed that humans have a poor sense of smell, we possess an amazingly acute sensitivity to some chemicals, such as vanillin and 2-bromophenol (sensitivity thresholds of $10^{-5.6}$ and $10^{-4.5}$ ppm, respectively; Devos et al., 1990; Jacob et al., 2002). In fact, humans can recognize at least 10,000 different odors (Prasad and Reed, 1999).

Humans may also use olfactory cues for conspecific recognition and communication (see Hays, 2003; Wysocki and Preti, 2000, 2004 for recent reviews). With its persistence and complexity, the olfactory cue is suitable for producing highly individualized and long-lasting signals. Even with voices and faces, olfaction may function throughout life to supplement these cues. Furthermore, olfactory cues learned in the first few hours of life, or even prenatally, might impact our earliest lessons of whom kin and non-kin are (refer to Porter, 1999).

Odor-mediated recognition of conspecifics is ubiquitous throughout the animal kingdom. However, the mechanisms for discrimination can be diverse. For example, *Polistes metricus* paper wasps use the scent of hydrocarbons from their nest to recognize kin (Espelie et al., 1990). Salmonid fishes, well known for their use of olfaction in kin recognition, potentially use odors mediated by major histocompatibility complex (MHC) alleles (immunoresponse genes) to determine kin (Olsen et al., 1998; Rajakaruna et al., 2001). However, another fish, the Trinidadian guppy (*Poecilia reticulata*), uses odor to identify and school with *familiar* conspecifics, whether they are genetically related or not (Griffiths and Magurran, 1999).

In mammals, there are several examples of conspecific recognition via olfaction. Golden hamsters (*Mesocricetus auratus*) appear to use self-referent phenotype matching of odors (the “armpit effect”) to determine kin (Mateo and Johnston, 2000a). Belding’s ground squirrels (*Spermophilus beldingi*) respond toward littermate odors even after hibernation (Mateo and Johnston, 2000b). These squirrels can also discriminate degrees of relatedness through odor alone (Mateo, 2002). In this species, precise recognition may be mediated by both kin association and self or kin phenotype matching (Mateo and Johnston, 2000b).

There is a growing body of evidence that humans can also recognize each other via olfactory cues. Several studies have shown evidence for odor-mediated self-recognition (Russell, 1976; Hold and Schleidt, 1977; Schleidt, 1980; Schleidt et al., 1981; Lord and Kasprzak, 1989; Platek et al., 2001). Mothers can recognize olfactory cues from their infants within a few hours of birth (Porter and Cernoch, 1983; Russell et al., 1983; Kaitz et al., 1987). Likewise, infants will preferentially orient their heads toward maternal odors (Cernoch and Porter, 1985; Porter et al., 1992). There is also evidence that siblings and other relatives can recognize each other using odor cues (Porter and Moore, 1981; Porter et al., 1986).

The mechanisms underlying human odor recognition have not been elucidated. A number of studies have suggested that odor is influenced by the MHC, a series of genes also involved in immune response (see Penn, 2002 for review). In addition, both phenotype matching and association have been suggested to play a role in human kin recognition (Weisfeld et al., 2003). However, few studies have examined odor-mediated recognition of nonrelatives. Studies of relatives show that we can recognize conspecifics, but are unable to

distinguish the mechanisms behind this recognition. Genetically related individuals might employ both association and phenotype matching to identify each other. Tests of non-relatives are imperative to understand the impact of familiarity on human odor recognition, as phenotype matching is impossible where no genetic relatedness exists. Wallace (1977) found that subjects could distinguish between the hand odor of two different individuals. Kaitz and Eidelman (1992) showed that nonparturient strangers with little infant experience could distinguish a newborn's undershirt from other infants' after holding them for only 45 min. Verron and Gaultier (1976) found that 4- to 5-yr-old children were able to identify peers to some extent, although it was unclear how they controlled for extraneous scents due to foods, perfumes, and other sources. In 1998, Mallet and Schaal also found that young school children (average age of 9.5 yr) were able to identify nonrelative peers. However, the authors were not interested in purely biological odors and did not control for any extraneous scents. The majority of other studies that have tackled human odor recognition have concentrated their efforts on the recognition of partners (Hold and Schleidt, 1977; Schleidt, 1980; Schleidt et al., 1981). These studies are insufficient to examine effects of familiarity because subjects were also influenced by sexual attraction. Weisfeld et al. (2003) might have examined nonrelatives other than partners; however, because they did not separate the two in their presentation, no conclusions can be drawn.

In the present study, we investigated the impact of familiarity and association on human odor recognition by testing the hypothesis that humans can recognize familiar nonrelatives by odor cues. As part of a program designed to engage students in scientific research, 53 General and Advanced Placement biology students (ages 15–18 yr) implemented a project to analyze the effect of odor on human recognition of, and preference for, friends, sex, and self. Thirty-seven students wore T-shirts for three consecutive nights. During that time, subjects were controlled for exposure to an extensive list of extraneous odors.

Methods and Materials

The study was approved by the Cornell University Committee on Human Subjects (Institutional Review Board) and the Geneva Central School District Administration for use of data collected for research purposes.

Subjects

A total of 37 Geneva High School students (Geneva, NY, USA; 26 females, 11 males), ages 15–18 yr ($\bar{X} = 16.77$), volunteered to participate in a study to examine human odor recognition of, and preference for friends, sex, and self. This population was diverse on both racial and socioeconomic levels. Students were asked to choose one friend to participate in the experiment with them. They were also asked to choose only friends with whom they had a platonic relationship. Of the pairings used for data analysis, 2 were male/male, 12 were female/female, and 7 were male/female.

Thirty-one students were members of classes participating in a university and National Science Foundation–sponsored program designed to engage students in scientific research. The two class instructors participated in the program and coordinated with S. Olsson to integrate inquiry research into their General and Advanced Placement Biology curriculum. As such, the entire class designed and implemented all aspects of this project as part of the curriculum and was informed of the testing procedures. The six students from other classes

were informed both by written and oral instructions from the student friend participating in the program. All class members were educated on the ethics of human subject research both for research training and to inform them of their rights as potential subjects. All information and case studies for the education were taken directly from The Cornell University Committee on Human Subjects Training Program for Researchers in the Use of Human Subjects, and The National Institutes of Health Human Participant Protections Education for Research Terms on-line tutorial. Students were assessed through in-class discussions of case studies for their understanding of autonomy, beneficence, and justice as regards the use of human subjects in research. Although participation in planning and data analysis was a mandatory aspect of the education, participation as experimental subjects was strictly voluntary. Students were informed that any assessment for grading purposes was based solely on participation in planning and analysis of the study. Furthermore, students who did not volunteer as subjects were identical to subjects in their participation in all other aspects of the project. Thus, only 31 of the 52 total class members and six friends from other classes chose to participate. All student information was kept confidential through the use of identification numbers, and students were presented only these numbers during the data analysis phase. Consequently, data records could not be traced to specific students.

Odor Collection and Storage

Subjects were provided with 100% cotton T-shirts to wear to bed on three consecutive nights with clean pajama or alternative bottoms. Before wearing the shirts, subjects were instructed to wash all bed sheets in an unscented laundry detergent provided to them. The 37 shirts as well as one unworn T-shirt used as a control were laundered together in the same detergent before wearing. Subjects were also presented with the same lightly scented shampoo, conditioner, and soap (Zoto International, Inc.) to use *each* night immediately before donning the shirt.

Subjects were instructed to use only the items provided to them, and avoid other soaps, lotions, deodorants, etc. Although they were not required to alter daytime activities, subjects were cautioned to avoid smoking, eating, and strenuous activity while wearing the shirts. Finally, they were instructed to wear the shirts at least 6 hr each night, and prevent other humans/pets from sleeping on or using the bed during the testing period. Each morning, subjects placed their shirts in a plastic zip-lock bag to store in the freezer during the day to minimize loss of odor. Upon collection, all sealed bags, including the unworn control in a sealed bag, were stored together in the same freezer at approximately -5°C .

Testing Procedure

Subjects were tested by S. Olsson on 3 consecutive days in January 2004, approximately 6–8 d after wearing the shirts. They were first asked to complete a questionnaire concerning the study, providing data such as age, sex, information about their friendships (length, number of days a week they met their friend, etc.), compliance with testing procedures, information about potential impediments to the study such as allergies or illness that could create olfactory deficits, and exposure to pets smoke and strong food odors that could facilitate identification of specific individuals.

Upon completion of the questionnaire, subjects were screened for olfactory deficits by identifying a sequence of three scents [orange, vanilla, and almond extracts (McCormick and Co., Inc., Hunt Valley, MD, USA)] diluted 50% in water, similar to screening methods described in another human odor recognition study (Kaitz et al., 1987). Subjects were asked

to identify the three correct scents from a list of 10 odor choices. Then, wearing latex or nitrile gloves, they were asked to open and smell the contents of five numbered zip-lock bags containing shirts worn by themselves, their friend, an unworn control, and two strangers from the opposite sex. The unworn control had been laundered and stored at -5°C with the worn T-shirts. The two “strangers” were subjects randomly chosen from classes other than the test subject. Strangers of the opposite sex were used to aid in testing odor recognition of sex. Bags and shirts were of similar size and color with no identifying marks. The bags were numbered 1–5 and presented in random order. Subjects were allowed to smell the contents as long as necessary and repeat if needed to complete a test.

In the first test, subjects rated the pleasantness of each shirt’s odor on a scale from 1–5 (1, very unpleasant; 2, somewhat unpleasant; 3, neutral; 4, somewhat pleasant; 5, very pleasant). They were then asked to determine the sex of each of the five shirt owners (including the unworn control shirt). Finally, each was asked to identify the two shirts belonging to self and friend.

Statistical Analyses

χ^2 Tests (StatXact-4 and StatXact-6, Cytel Software Corporation, 1998 and 2004) were used to compare pleasantness ratings for each type of shirt (Pearson’s with Monte Carlo estimates of exact P values using 10,000 samples) as well as compare frequencies of shirts chosen as self and friend to the 20% expected by chance (goodness-of-fit with exact P values). All χ^2 P values reported are exact. A Kolmogorov–Smirnov Z test was used to compare distributions of pleasantness ratings for the tested shirt types (SPSS Version 12.0, SPSS, Inc., 2001). For recognition of sex, a logistic model was fitted to the number of correct and incorrect selections for each individual using STATA (v.7, StataCorp, 2001) with a Huber–White robust variance estimator to obtain 95% confidence intervals, which accounted for positive correlation with individual. Finally, logistic regression (STATA v. 7, StataCorp, 2001) was used to determine the influence of a number of factors answered in the questionnaire (see testing procedure, above) on identification of self and friend.

Results

Olfactory Screening

Subjects were initially screened for olfactory deficits through identification of three odors: orange, vanilla, and almond. Only those who correctly identified at least two of three odorants were considered for further analysis. However, 20/37 subjects (54.0%) identified orange odor as lemon. Because of this, lemon was accepted as an alternate response to orange. Most of the subjects ($n = 31$, 83.8%) identified at least two of three odorants correctly, including those who chose lemon.

Human Odor Qualitative Comparisons

Each subject rated the pleasantness of five T-shirts (self, friend, male stranger, female stranger, and an unworn, blank shirt) on a scale of 1–5. Figure 1 depicts the distribution of pleasantness ratings for each of the five types of shirts. All distributions are similar with the exception of the unworn, blank shirt, which was considered neutral more frequently. According to a χ^2 test, the distribution of pleasantness ratings did not differ among the shirt

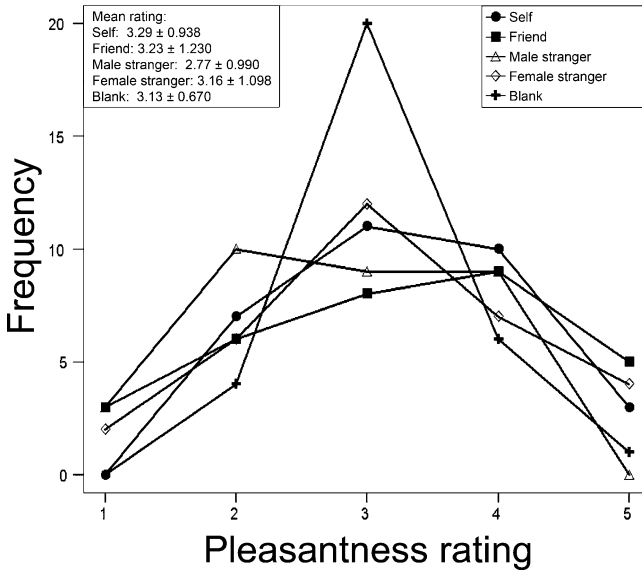


Fig. 1 Distributions of pleasantness for each of the five shirt types. Each shirt was rated 1–5, as follows: 1, very unpleasant; 2, somewhat unpleasant; 3, neutral; 4, somewhat pleasant; 5, very pleasant

types, ($df = 16, n = 155$) = 24.09, $P = 0.08$, nonsignificant (NS). These distributions were also similar when male and female subjects were analyzed separately [data not shown; males: χ^2 ($df = 16, n = 40$) = 22.81, $P = 0.110$, NS; females: χ^2 ($df = 16, n = 115$) = 23.28, $P = 0.109$, NS. A Kolmogorov–Smirnov Z test also did not find any difference between any paired rating distributions ($Z = 0.254 - 1.143, P > 0.1$).

Recognition of Sex via Odor Cues

Subjects were asked to identify the sex of the owner for each of the five shirts presented. A logistic model was fitted to these data with a robust variance estimator accounting for

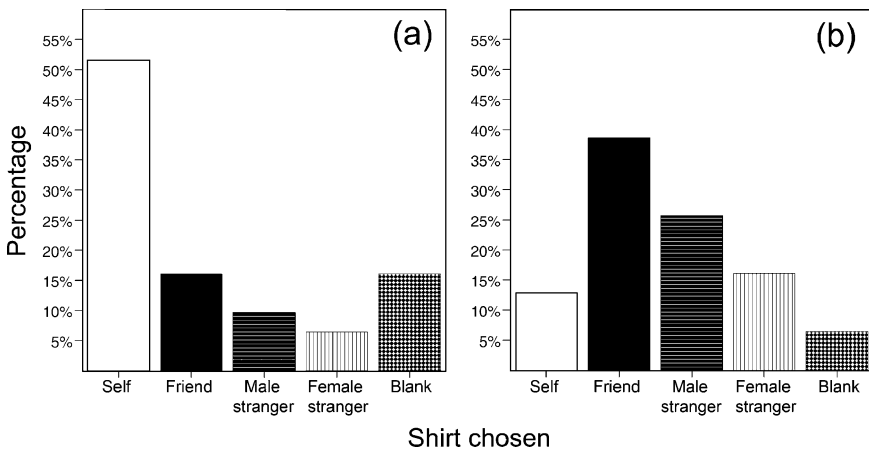


Fig. 2 Percentage of T-shirts chosen by the subjects as their (a) own shirt and (b) friend’s shirt ($n = 31$)

positive correlation with individual. On average, subjects identified $54.8 \pm 8\%$ shirts correctly (95% confidence interval), which does not differ significantly from chance. The unworn, blank shirt was identified male as frequently as it was identified female (15/31 or 48.4%, and 16/31 or 51.6%, respectively). However, males were 1.67 times more likely to identify the blank shirt as male (5:3), whereas females were 1.30 times more likely to identify it as female (13:10).

Self-Recognition via Odor Cues

Figure 2a shows the distribution of shirts identified by the subjects as their own. A total of 51.6% of the subjects identified their own shirt correctly, which is significantly greater than the 20% expected by chance, $\chi^2 (1, n = 31) = 19.36, P < 0.001$. Females identified their own shirt significantly greater than chance, $\chi^2 (1, n = 23) = 14.88, P < 0.001$, and males were marginally significant (MS), $\chi^2 (1, n = 8) = 4.5, P = 0.056, MS$. During testing, four shirts were identified by at least two to three subjects as having a “nonhuman” odor, such as food or cigarette smoke. Removal of these shirts from all aspects of the data pool (self, friend, and stranger) did not effect this significance, $\chi^2 (1, n = 14) = 12.07, P = 0.002$. Likewise, Table 1 shows that subjects could still identify their own shirt even when controlling for several factors that could have impacted result accuracy. This table was compiled from subjects’ questionnaire answers filled out the day of testing (see [Methods and Materials](#)). Subjects who answered “yes” to any factor such as allergies, sickness, or

Table 1 Recognition of self and friend odor cues as a function of specific conditions or situations

Condition controlled	No. subjects who answered “no” on questionnaire	Self-recognition		No. subjects whose friends answered “no” on questionnaire	Friend recognition	
		χ^2	<i>P</i> value		χ^2	<i>P</i> value
Pet owners	9	12.25	0.003	8	0.12	1.000, NS
Smokers ^a	24	10.01	0.004	25	4.00	0.074, NS
Allergies	23	14.88	<0.001	23 ^b	7.92	0.009 ^b
Strong foods consumed during testing	23	24.01	<0.001	24	7.04	0.013
Odorants used (i.e., perfumes, lotions, deodorant)	28	19.72	<0.001	27	4.90	0.033
Illness while wearing shirt or during olfactory trials	22	16.41	<0.001	22 ^c	3.68	0.063, MS ^c
Directions not followed (e.g., proper soaps not used, shirt not worn three nights, etc.)	20	15.31	<0.001	23	5.26	0.033

^a Subjects or friends with household members who smoke are also excluded.

^b Includes only subjects who answered “no” on questionnaire, but does not exclude any friends.

^c Includes only subjects who answered “no” to illness during trials and whose friends answered “no” to illness while wearing shirt.

exposure to smoke and strong food odors were removed from that analysis. A χ^2 test was performed using only those subjects who stated they were not affected by that factor. The table lists the number of remaining subjects for each condition considered (i.e., those who answered “no” on questionnaire). Additionally, logistic regression found no influence of any of these factors on choice of self ($P > 0.2$).

Recognition of Friends via Odor Cues

Based on questionnaire answers, most of the subjects (34/37, 91.9%) considered their chosen partner to be a friend rather than simply a classmate. Nevertheless, only 19/37 (51.4%) considered this person to be a close friend. All but one subject indicated that they met their friend at least three times a week, and 26/37 (70.3%) stated that they had known their friend at least 3 yrs.

A total of 38.7% of the subjects identified their friend’s shirt correctly, which is significantly greater than the 20% expected by chance, $\chi^2(1, n = 31) = 6.78, P = 0.014$ (Fig. 2b). This was only true for friends who knew each other at least 3 yr, $\chi^2(1, n = 21) = 6.86, P = 0.014$. Friends who knew each other less than 3 yr could not distinguish each other by odor, $\chi^2(1, n = 10) = 0.62, P = 0.698$, NS. However, logistic regression found no influence of friendship length on subjects’ ability to distinguish their friends’ odor ($P > 0.2$).

Removal of the four shirts identified two to three times as having a “nonhuman” odor from the data pool did affect the significance, $\chi^2(1, n = 14) = 2.16, P = 0.174$, NS. Nevertheless, Table 1 shows that subjects could still identify their friends even when their friends were controlled for most factors obtained from the questionnaire filled out the day of testing (i.e., only subjects whose friends stated they were not affected by that factor were included). The use of logistic regression found no influence of these factors on choice of friend ($P > 0.2$).

Finally, there was a sex difference in the ability of subjects to identify their friend’s shirt. Although females could identify their friends, $\chi^2(1, n = 23) = 11.13, P = 0.002$, males could not, $\chi^2(1, n = 8) = 0.28, P = 0.706$, NS. However, the low sample size could have contributed to the lack of significance among male subjects. Additionally, if subjects selected incorrectly, males were four times more likely to identify the female stranger as their friend than the male stranger (4:1), whereas females were seven times more likely to identify the male stranger (7:1). The propensity to identify a member of the opposite sex as the friend was not dependent on the pairing of the subjects (male/male, female/female, and male/female). Indeed, in the 11 cases where female/female pairs identified the incorrect shirt, subjects selected the male stranger in seven of the cases (64%). Likewise, in the three cases where male/male pairs identified friends incorrectly, they chose the female stranger once and their own shirt twice.

Discussion

Our results indicate that subjects, when presented with several different stimuli, could recognize both their own and their friend’s odor cue. However, subjects could not reliably distinguish the sex of the wearer nor did they prefer the scent of any particular individual (i.e., self, friend, stranger, or unworn). Apparently, the ability to recognize odor in this context did not require a preference for that scent. All shirt odors were rated similarly for

pleasantness, and although male stranger shirts were considered slightly less pleasant on average, this distinction was not significant.

Subjects were not only unable to distinguish sex via odor cues, but, as shown above, male and female odors were considered equally pleasant, indicating that neither sex possessed qualitative differences in odor. Furthermore, the unworn, blank control shirt was identified to be male as often as female, showing that subjects had no overall bias to choose one sex over the other. Nonetheless, both males from females were slightly more likely to choose their own sex for the unworn shirt. These results are in disagreement with previous studies (Hold and Schleidt, 1977; Schleidt, 1980; Schleidt et al., 1981). In these experiments, subjects (20–64%) were not only able to discriminate males from females, but many deemed female odor to be “more pleasant” than male. However, shirts were worn for a longer period of time (seven nights) and fewer controls were taken (i.e., subjects’ bed sheets not washed in same detergent, same shampoo not used by all subjects, shirts not stored under isolated, cold conditions, and ownership of pets not considered). Consequently, there was a greater potential for extraneous and unnatural odors to accumulate on the shirts and affect the results. Several studies reviewed in Doty (1981) also suggest the discrimination of sex via olfactory cues, and in these studies shirts/gauze pads were worn for 18–24 hr. In our study, subjects might have been able to distinguish sex had they worn the shirts for an extended period of time. It is also possible that the odor cues responsible for distinguishing men from women are less prominent during young adulthood than for adults used in the previous research. Lord and Kasprzak (1989) found that age was a significant factor in subjects’ ability to recognize self via olfaction. In fact, many of the subjects who failed the test were under 20 yr, similar to our subjects. Mallet and Schaal (1998) also found that 9-yr-old children were unable to determine the sex of their peers by smell alone. Finally, a study of volatiles emitted from subjects ages 26–75 yr found a positive correlation in the concentration of 2-nonenal with age, with detectable amounts only found in persons age 40 yr and above (Haze et al. 2001). Perhaps our subjects neither produce sufficient quantities of, nor are adequately familiar with the odor cues responsible for distinguishing sex.

In both recognition tests, our subjects were able to distinguish their own and their friend’s odor cue. Subjects could identify their own scent 51.6% of the time, and their friend’s 38.7%. The ability to recognize self via odor cues was not affected even when separately controlling for factors that might influence shirt odor, e.g., pets, smoking, allergies, strong foods eaten, odorants used, illness, and experimental error. The ability to recognize friends via odor cues was affected only when nonhuman-scented shirts, friends who were pet owners, or friends who were smokers were removed from the data set.

These results are in congruence with previous studies that confirm the ability of humans to recognize self via olfactory cues (Russell, 1976; Hold and Schleidt, 1977; Schleidt, 1980; Schleidt et al., 1981; Lord and Kasprzak, 1989; Mallet and Schaal, 1998; Platek et al., 2001). Furthermore, the percentage of subjects able to recognize their own scent (51.6%) falls well within values obtained in other studies [25% in Schleidt et al. (1981) to ~75% in Russell (1976) and Lord and Kasprzak (1989)].

Individuals were able to identify their friend’s odor although only half (51.4%) stated that their partner was a close friend. Furthermore, the result that only friends who knew each other longer than 3 yr could identify each other may indicate that a longer period of association is necessary to learn olfactory cues. However, because of the small sample size in relation to this question ($n = 10$ for friends <3 yr), and the lack of statistical relation to length of friendship, this conclusion must be made with caution. Additional studies with larger sample sizes may elucidate the validity of this claim.

Subjects who did not identify their friend's odor correctly were apt to choose the odor of a member of the opposite sex. This was true even for same-sex pairings, showing that individuals who chose incorrectly were not merely searching for odors of the same sex as their friend. This agrees with the conclusion that our subjects could not distinguish sex. Although subjects did not express a conscious preference for the odor of the opposite sex, this result indicates that they may consider the odor of the opposite sex to be a "friendly" odor. Implications of this on the role of odor in human mate preference are beyond the scope of this investigation and are not discussed here.

The ability to recognize friends via odor cues lends credence to the idea that association and familiarity are important aspects of conspecific olfactory recognition in humans. Further tests are necessary to determine the extent to which humans utilize association vs. phenotype matching for kin and conspecific recognition. Potentially, a study with adopted children and unknown relatives could address this concern. Adopted children, although lacking a coefficient of genetic relatedness, are kin in the human social context. Recognition of adopted children could occur purely through association. However, a recent study found that although mothers and siblings could identify odor of their biological children and siblings, they were unable to identify the odor of their stepchildren or stepsiblings (Weisfeld et al., 2003). Alternatively, relatives whom the subject has never met could only be identified through phenotype matching. These tests are essential to understand the mechanisms by which we use odor as a means for identification.

Although this examination uses indirect cues (i.e., T-shirts), there remains significant support for the recognition of nonrelatives via odor cues. Olfaction, particularly if operational *in utero*, may be important for identifying individuals early in life by supplementing newly developed senses. Furthermore, odor can function throughout life to enhance other visual, auditory, and tactile cues. Our sense of smell may have more impact on our life than we realize.

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How an Ant Manages to Display Individual and Colonial Signals by Using the Same Channel

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Abstract Cuticular hydrocarbons are used by some ants to discriminate nestmates from nonnestmates. Every member of the colony bears the same pattern because they are continuously exchanged among nestmates. The postpharyngeal gland (PPG) stores the blend of hydrocarbons and is involved in the distribution of this common mixture. However, some individuals might display individual information on the cuticle (such as a chemical signal of fertility) that must not be mixed within the common pool. We investigated how this paradox is solved in the ant *Pachycondyla goeldii* by analyzing the nature and localization of colonial and fertility signals. Workers in a queenless condition showed a dominance hierarchy that was correlated with ovarian development. Hydrocarbons from the cuticle and the PPG analyzed by gas chromatography (GC) and identified by GC-mass spectrometry showed a clear discrimination among colonies, supporting the involvement of the PPG in the colonial identity signal. We identified and selected 11 cuticular hydrocarbons that permitted us to discriminate ovarian development classes and that might function as a fertility signal. They allowed clear colony discrimination as well, which suggests that the two signals (the individual signal of fertility and the common signal of colony identity) can be conveyed by the same compounds. However, the hydrocarbons in the PPG did not discriminate among ovarian developmental classes, suggesting that the portion of variation in the cuticular hydrocarbons constituting the fertility signal is superimposed on the signal of colony identity.

Keywords *Pachycondyla* · Ants · Cuticular hydrocarbons · Colonial odor · Ovarian status · Fertility signaling

Introduction

In colonies of most social insect species, individual workers are able to discriminate between nestmates and nonnestmates. They are usually aggressive against nonnestmates, especially in the vicinity of the nest. They limit their altruistic behavior to members of their colony and protect it against intruders. According to Gestalt theory, each colony has a unique odor that is a blend of all individually produced odors (Crozier and Dix, 1979;

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Crozier, 1987), which allows for nestmate discrimination. In ants, this model implies the existence of a Gestalt organ where chemicals involved in nestmate discrimination are mixed before being displayed by the individual (Carlin and Hölldobler, 1986, 1987; Stuart, 1988; Crosland, 1989; Tsuji, 1990; Dahbi and Lenoir, 1998). This organ should contain chemicals from all colony members in order to develop a colonial odor.

In ants, it has been demonstrated that cuticular hydrocarbons are involved in the nestmate discrimination process (Bonavita-Cougourdan et al., 1987; Lahav et al., 1999; Thomas et al., 1999; Wagner et al., 2000). Cuticular hydrocarbons are synthesized in tissues from the abdomen (epidermis, fat body, oenocytes) (Soroker and Hefetz, 2000) and are likely transported by high-density lipophorin (an internal hydrocarbon carrier) through the hemolymph to the postpharyngeal gland (PPG) (Lucas et al., 2004). Hydrocarbons are exchanged between the PPG and the cuticle during self-grooming, and between nestmates during trophallaxis, allogrooming, and physical contact. This mutual redistribution of hydrocarbons between all colony members might allow the formation of a colonial odor (Lenoir et al., 1999; Soroker et al., 2003; Lucas et al., 2004). Thus, the PPG is considered as the Gestalt organ (Soroker et al., 1994; Lahav et al., 1999). Indeed, most of the hydrocarbons present on the cuticle are also present in this gland (Bagneres and Morgan, 1991; Do Nascimento et al., 1993; Lucas et al., 2004). In *Pachycondyla* ants, newly synthesized hydrocarbons are chiefly transported from the hemolymph to the basitarsal brushes of the front legs (Hefetz et al., 2001). These are licked during self-grooming, and cuticular hydrocarbons are transferred to the PPG. PPG hydrocarbons are then dispatched on the other parts of the same individual's cuticle or on its nestmates' cuticles during grooming (Meskali et al., 1995). Because *Pachycondyla* ants do not perform trophallaxis, hydrocarbons are only exchanged between nestmates during body contact and allogrooming.

All members of a colony bear a common odor, the colonial odor, but some members may display individual chemical signals on their cuticle that are not mixed in the common pool. For example, such signals are compounds yielding information about fertility, dominance status, or caste, in different social insects. Recent studies suggest that cuticular hydrocarbons are involved in fertility signaling in ants. In queenright ants, the queen displays the fertility signal (Liebig et al., 2000; Heinze et al., 2002). When several queens cooccur in the same colony, the cuticular hydrocarbons provide information on the degree of fecundity, and workers are able to discriminate among queens with different fecundities (Hannonen et al., 2002). In some species, the queen caste does not exist and workers (called gamergates) mate and reproduce (Peeters, 1991). The gamergate, often only one per colony, can be considered as a functional queen, and has been shown to bear a fertility signal (Monnin et al., 1998; Liebig et al., 2000; Cuvillier-Hot et al., 2004a). However, in queenless species, unmated egg layers tend to develop a fertility signal (Cuvillier-Hot et al., 2001, 2004b). In *Harpegnathos saltator*, where both queens and gamergates occur, the fertility signal is the same in both castes (Liebig et al., 2000). In many queenright species, the workers have retained the ability to lay unfertilized eggs that produce males. In these species, the loss of the queen is usually followed by a burst of worker reproduction. Moreover, workers compete to access reproduction, and form a dominance hierarchy (reviewed in Cuvillier-Hot et al., 2004a). Once the hierarchy is established, dominant workers may display a chemical signal of fertility and/or dominance that limits costly competitive interactions. Similarity between the fertility signal of queens and fertile workers has been shown (Heinze et al., 2002). Such dequeened systems are especially good models to study fertility signaling because morphological caste does not influence the signals studied. Some results suggest that the hydrocarbons involved in colony and fertility signaling belong to different classes (Dietemann et al., 2003).

The neotropical ant *Pachycondyla goeldii* (Forel) is an arboreal species that lives in ant gardens. Workers are aggressive to members of other colonies, indicating substantial society closure (D.D., personal observation). As in many poneromorphs, *P. goeldii* workers have retained the ability to lay unfertilized eggs that develop into males in the absence of the queen. This provides an opportunity to study the consequences of orphaning on reproductive conflict. The aim of our study was to investigate how ants manage to display at the same time a colonial signal that must be homogenized among nestmates and an individual signal of fertility that should not be transferred to nestmates. We first verified whether workers in orphaned groups display dominance hierarchy and whether it is related to reproductive activity. We then checked whether the cuticular and PPG hydrocarbon profiles are consistent with the Gestalt model. Then, we attempted to identify a fertility signal on the cuticle and determine how this signal is combined with the colonial odor.

Methods and Materials

Collection and Rearing of Colonies

Three entire queenright colonies of *P. goeldii* were collected in September 2002 near the dam of “Petit Saut,” French Guiana (colonies A, B, and C: 160, 225, and 198 workers, respectively). Mature colonies of this species usually contain about 200 workers. They were maintained in the laboratory in artificial nests made of plaster connected to an outside arena. Colonies were kept at $28 \pm 2^\circ\text{C}$ and $80 \pm 2\%$ RH. They were fed twice a week with crickets and diluted honey. Worker behavior was investigated in eight dequeened groups of 16 workers each (without brood). Three, two, and three dequeened groups were formed out of queenright colonies A, B, and C, respectively.

Behavioral and Physiological Observations

Ants were individually marked with dots of enamel paint on their gaster. To characterize dominance hierarchies, each dequeened group was observed at a rate of 10 hr/wk for several weeks, until more than 200 antennal boxing behaviors were observed (approximately 80 hr of observation). Antennal boxing is a ritualized behavior characteristic of dominance relationships between ants (Ito and Higashi, 1991; Heinze et al., 1996; Cuvillier-Hot et al., 2004a), during which a dominant worker makes a rapid lunge for a subordinate worker and aggressively pummels its head with the vibrating antenna. The frequency of antennal boxing varies considerably among groups, but is the most conspicuous and frequent dominance interaction in many ant species, and particularly in *P. goeldii*. It is the only quantifiable behavioral expression of dominance available to the observer. We recorded all antennal boxing behaviors (the only type of aggression observed), and workers were ranked according to the proportion of workers they dominated.

At the end of the period of behavioral observation, we dissected all surviving workers (102 ants). We noted the number of developing ovarioles for each individual. An ovariole was considered as developed if it contained at least one oocyte >0.5 mm. Pearson's correlation coefficient (see below) was computed between worker rank and the number of developing ovarioles for each of the eight dequeened groups. Individuals were assigned to three ovarian developmental classes: (1) undeveloped ovaries (no developing ovarioles); (2) moderately developed ovaries (between one and four developing ovarioles); (3) fully

developed ovaries (more than five developing ovarioles). Workers of *P. goeldii* normally have eight ovarioles.

Extraction and Analysis of Hydrocarbons from the Cuticle and Postpharyngeal Gland

Hydrocarbons extraction was performed on the two highest, the two lowest, and the two middle ranking workers in each group from colonies A and C, and three workers of each rank class in each group from colony B, giving a total of 54 individuals with 18 of each rank class. For each individual, the thorax and PPG were each soaked in 50 μl pentane with 1.5 μg *n*-nonadecane as an internal standard. Thoraces were removed after 10 min, and PPG were left in the tube. The relative proportions of cuticular hydrocarbons in ants can differ among body parts (Bonavita-Cougourdan et al., 1993). We chose the thorax instead of other body parts to characterize the individual odor because previous observations revealed that antennal boxing was elicited when the antennae of the focal individual touched the head or the thorax, but never the abdomen, of another ant. Furthermore, we needed the head to extract the PPG. Moreover, the abdomen is rich in chemical-producing glands that could have contaminated extracts of individual odor. Five μl of each sample were analyzed by gas chromatography with flame ionization detection (GC-FID) on a Varian 3900 (Varian, Palo Alto, CA, USA) instrument equipped with a split/splitless injector and a DB-5 fused silica capillary column (30 m \times 0.32 mm, 0.25 μm film thickness; J&W scientific column, Agilent Technologies, Palo Alto, CA, USA) with helium as carrier gas at a flow rate of 28.57 cm/sec. The temperature was maintained at 100°C for 5 min, with the split valve closed for the same interval. Next, the temperature was raised at 3°C/min to 300°C, and held constant for 10 min. In pilot tests, we could not detect additional compounds when the temperature was raised to 320°C instead of 300°C. The injector port was maintained at 200°C, and the FID was kept at 320°C. Peak areas were computed with Varian system control (version 6.20). Identification of hydrocarbons was performed on 30 workers from three orphaned colonies by GC/MS (VGM250Q) with a DB-5 fused silica capillary column (30 m \times 0.32 mm, 0.25 μm film thickness; J&W scientific column, Agilent). The temperature program was 120°C to 270°C at 5°C/min with a hold at 270°C for 30 min, and then heated to 295°C at 20°C/min with helium as the carrier gas at a flow rate of 28.57 cm/sec, the injector port was kept at 200°C. The mass spectrometer detector was a Fisons MD 800 (Foremost Equipment, Rochester, NY, USA) operated at 70 eV. The eluting hydrocarbons were identified by their fragmentation patterns.

Data Analysis

Compounds that were not present in all three colonies were excluded from the analysis. Four of the remaining compounds that were present on the cuticle but absent from the PPG were excluded: two of them were volatile (*n*-pentadecane and *x*-pentadecene), and two occurred irregularly (they were displayed by a few individuals and were not related to colony or ovarian development, suggesting they that were contaminants). Peak areas were transformed according to $Z = \ln[A_p/g(A_p)]$, where Z is the standardized peak area, A_p is the peak area, and $g(A_p)$ is the geometric mean of all peak areas in the respective individual (Aitchison, 1986), in order to correct for the high dependence of this type of data. However, the compounds were still too redundant to allow a discriminant analysis (DA) to be performed. We computed the correlation matrix and removed the compound that had the highest mean coefficient of correlation (*x,y*-tricosadiene; mean coefficient of correlation \pm SD: 0.49 \pm 0.2). This compound also had the highest number of significant correlations (17

out of the 19 coefficients of correlation computed). Thus, a total of 19 peaks were retained for the analysis.

Statistical analyses were performed with Statistica 6 software (Statsoft France 2003, Maisons-Alfort, Val-de-Marne, France), and standard DAs were performed on the standardized peak areas of hydrocarbons from cuticle and PPG to determine whether variation in hydrocarbons allows discrimination among individuals according to their colony of origin and ovarian development. For each compound, we computed the Pearson's correlation coefficient between standardized peak areas from cuticle and PPG profiles. A stepwise DA was performed on cuticular profiles to identify which combination of compounds allowed the best discrimination among ovarian development classes; at each step, the least discriminating compound was removed and a new DA was performed until only one compound remained. Thus, we obtained 18 DA models. Potential for discrimination was evaluated by the percentage of correct classifications under each model, and then, by their P value. The best model was selected. We then compared the values of

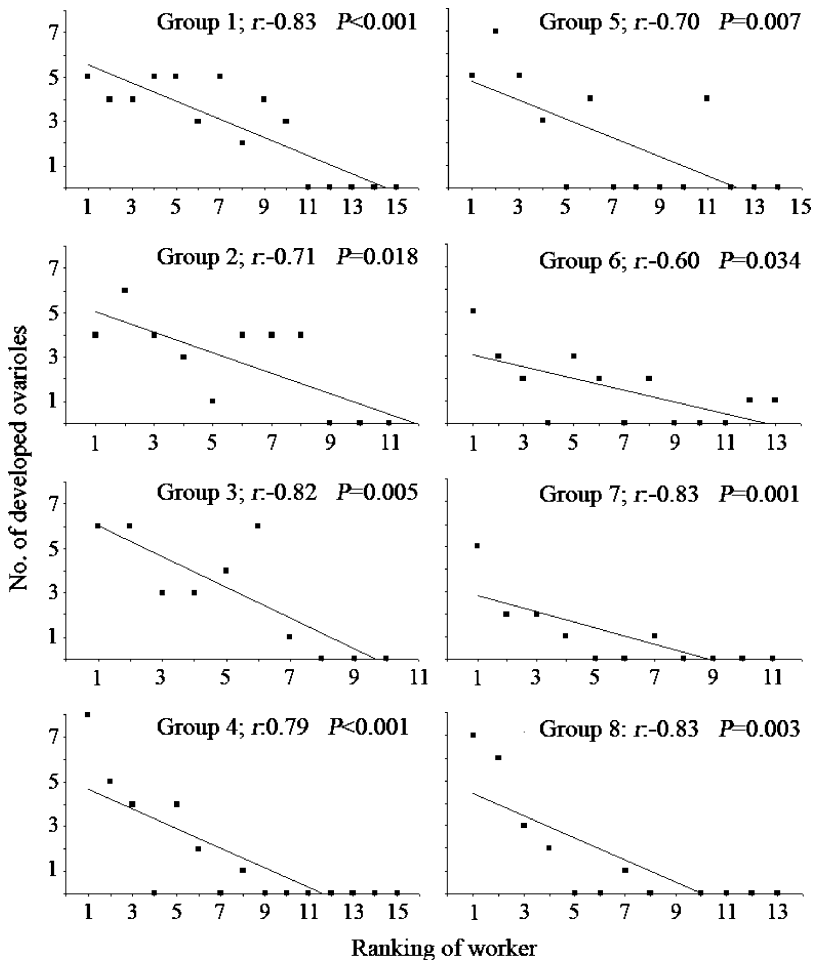


Fig. 1 Correlation between rank of worker and number of developed ovarioles in workers from each of eight queenless groups of *Pachycondyla goeldii*

the Pearson's correlation coefficient (see above) among the compounds included in and excluded from the model, by using a Mann-Whitney test. A new DA was performed with the compounds included in the model to see whether these compounds allowed us to discriminate colonies. Another DA was performed with these compounds in the PPG to test whether they allowed us to discriminate between ovarian classes in this gland. Finally, four principal component analyses (PCA) were computed with the cuticular compounds from the model: one with workers from all colonies to check whether these compounds differentiated colonies, and one with workers from each of the three colonies to test whether they differentiated ovarian development.

Results

Behavior and Ovarian Development

Each group displayed agonistic behaviors (mean \pm SE: 237.62 \pm 14.11 over 35–68 d) and showed a significant correlation between ovarian development and rank (Figure 1). High-ranking individuals had more developed ovarioles.

Table 1 Percentages of Thoracic and Postpharyngeal Gland (PPG) Hydrocarbons from Workers of Colonies A, B, and C^a

Hydrocarbon	Thoracic hydrocarbons			PPG hydrocarbons		
	Colony A	Colony B	Colony C	Colony A	Colony B	Colony C
<i>x</i> -Pentadecene	5.85 \pm 4.60	6.83 \pm 6.30	3.33 \pm 2.36	–	–	–
<i>n</i> -Pentadecane	1.85 \pm 1.22	1.69 \pm 1.72	0.86 \pm 0.60	–	–	–
<i>n</i> -Heneicosane	10.06 \pm 3.27	2.93 \pm 1.74	9.46 \pm 4.64	5.87 \pm 1.80	2.91 \pm 1.91	5.89 \pm 2.45
<i>n</i> -Docosane	1.81 \pm 0.35	0.69 \pm 0.20	1.57 \pm 0.22	1.13 \pm 0.33	0.68 \pm 0.35	0.85 \pm 0.21
<i>x,y</i> -Tricosadiene	4.02 \pm 3.38	0.48 \pm 0.73	2.23 \pm 1.57	4.34 \pm 4.47	0.84 \pm 0.63	2.71 \pm 2.23
<i>x</i> -Tricosene	16.86 \pm 9.27	3.84 \pm 4.86	16.21 \pm 10.74	29.13 \pm 18.53	6.44 \pm 4.58	36.11 \pm 18.76
<i>y</i> -Tricosene	0.38 \pm 0.12	0.62 \pm 0.30	1.91 \pm 0.85	0.31 \pm 0.24	1.06 \pm 0.39	3.00 \pm 2.16
<i>z</i> -Tricosene	0.03 \pm 0.06	7.44 \pm 3.41	0.03 \pm 0.04	0.01 \pm 0.03	5.63 \pm 3.52	0.17 \pm 0.06
<i>n</i> -Tricosane	28.57 \pm 6.30	13.46 \pm 4.60	30.46 \pm 4.43	17.99 \pm 10.85	11.8 \pm 8.08	11.83 \pm 3.83
11-Methyltricosane	2.19 \pm 2.55	tr	0.12 \pm 0.08	–	–	–
<i>x,y</i> -Tetracosadiene	0.55 \pm 0.16	0.79 \pm 0.29	1.07 \pm 0.59	0.94 \pm 0.49	1.48 \pm 0.64	2.00 \pm 1.43
<i>x</i> -Tetracosene	–	0.05 \pm 0.06	–	–	–	–
<i>n</i> -Tetracosane	0.29 \pm 0.12	0.34 \pm 0.11	0.51 \pm 0.17	0.15 \pm 0.12	0.21 \pm 0.19	0.14 \pm 0.07
<i>x,y</i> -Pentacosadiene	5.40 \pm 2.90	6.52 \pm 3.00	2.61 \pm 1.81	7.79 \pm 7.37	2.24 \pm 3.36	1.60 \pm 1.70
<i>x</i> -Pentacosene	13.68 \pm 6.54	35.67 \pm 13.25	13.29 \pm 9.86	26.36 \pm 13.09	60.94 \pm 20.64	32.85 \pm 23.87
<i>y</i> -Pentacosene	–	2.12 \pm 1.31	–	–	–	–
<i>n</i> -Pentacosane	3.28 \pm 1.18	4.63 \pm 1.82	7.13 \pm 2.46	1.60 \pm 1.40	2.17 \pm 2.30	1.36 \pm 0.77
Hexacosadiene	1.51 \pm 2.11	0.01 \pm 0.02	0.03 \pm 0.05	1.95 \pm 2.23	0.01 \pm 0.02	0.02 \pm 0.03
<i>x</i> -Hexacosene	–	0.25 \pm 0.14	0.56 \pm 0.46	–	–	–
<i>n</i> -Hexacosane	0.11 \pm 0.15	0.23 \pm 0.12	0.34 \pm 0.13	0.06 \pm 0.07	0.07 \pm 0.09	0.04 \pm 0.02
<i>x,y</i> -Heptacosadiene	0.08 \pm 0.12	0.02 \pm 0.03	0.01 \pm 0.02	0.09 \pm 0.16	0.02 \pm 0.02	0.02 \pm 0.02
<i>x</i> -Heptacosene	0.02 \pm 0.07	0.41 \pm 0.22	0.16 \pm 0.17	0.02 \pm 0.04	tr	0.01 \pm 0.02
<i>y</i> -Heptacosene	0.09 \pm 0.40	1.48 \pm 1.07	–	–	0.41 \pm 0.55	–
<i>n</i> -Heptacosane	1.53 \pm 0.71	3.07 \pm 2.39	3.81 \pm 1.16	0.66 \pm 0.77	0.78 \pm 0.87	0.49 \pm 0.31
3-Methylheptacosane	0.08 \pm 0.16	tr	0.02 \pm 0.02	–	–	–
<i>n</i> -Octacosane	0.17 \pm 0.18	0.50 \pm 0.36	0.38 \pm 0.14	0.09 \pm 0.10	0.14 \pm 0.08	0.07 \pm 0.10
<i>x,y</i> -Nonacosadiene	0.03 \pm 0.06	0.37 \pm 0.25	0.28 \pm 0.11	0.10 \pm 0.12	0.31 \pm 0.27	0.05 \pm 0.05
<i>x</i> -Nonacosene	–	0.61 \pm 0.69	–	–	0.09 \pm 0.16	–
<i>n</i> -Nonacosane	1.57 \pm 0.51	4.93 \pm 4.06	3.63 \pm 1.69	1.42 \pm 1.72	1.78 \pm 2.22	0.80 \pm 0.64

^aMean values \pm SD; *N*=18 for each colony.

–: Compound absent from the colony; tr: trace (<0.01% of total hydrocarbons). *x*, *y*, and *z* indicate different and undetermined positions of the double bond.

Hydrocarbon Analysis

The hydrocarbons identified from the three colonies ranged from C₁₅ to C₂₉ in chain length and were *n*-alkanes, alkenes, and alkadienes (Table 1). Cuticular and PPG hydrocarbon profiles of *P. goeldii* lacked methyl branched alkanes. Among the hydrocarbons, the relative quantities of linear *n*-alkanes showed less correlation between the thoracic cuticle and PPG (Table 2). Moreover, for each colony, we found that each linear *n*-alkane that we identified was present in a higher proportion on the thoracic cuticle than in the PPG (Table 1).

Chemical analysis of the thoraces revealed that the hydrocarbon phenotypes of the colonies were qualitatively similar: 82.75% (*N* = 29) of the hydrocarbons detected were present in all three colonies. Only five compounds did not occur in all three colonies. *x*-Tetracosene, *y*-pentacosene, *y*-heptacosene, and *x*-nonacosene were detected only in colony B, whereas *x*-hexacosene was detected in colonies B and C. Those compounds may have been present in the other colonies, but our analysis did not detect them. For each colony, the cumulative relative proportion of these five hydrocarbons was small (mean % over workers ± SD: 0.00 ± 0.00, 4.52 ± 2.99, and 0.56 ± 0.46 for colony A, B, and C, respectively). Two compounds were abundant on thoracic cuticle: *n*-tricosane and *x*-pentacosene represented more than 40% of total hydrocarbon (Table 1).

The qualitative hydrocarbon content of the PPG was similar among colonies: 90.90% (*N* = 22) of the compounds were present in all three colonies. *y*-Heptacosene and *x*-nonacosene occurred only in PPG from colony B but represented only 0.50 ± 0.62% (mean ± SD) of the total amount of hydrocarbons. All compounds from the PPG were also detected on the cuticle. The two most abundant hydrocarbons from the cuticle, *n*-tricosane and *x*-pentacosene, were also the most abundant in the PPG (they also represented more than 40% of the total).

Table 2 Correlations Between Cuticular and Postpharyngeal Gland (PPG) Hydrocarbons from 54 Workers of *Pachycondyla Goeldii*^a

Hydrocarbon	Pearson's correlation coefficient	<i>P</i> value
<i>x,y</i> -Hexacosadiene hexacosadiene	0.98	<0.001
<i>z</i> -Tricosene	0.91	<0.001
<i>n</i> -Heneicosane	0.88	<0.001
<i>x</i> -Tricosene	0.85	<0.001
<i>y</i> -Tricosene	0.81	<0.001
<i>x,y</i> -Tetracosadiene	0.81	<0.001
<i>x</i> -Pentacosene	0.80	<0.001
<i>n</i> -Docosane	0.60	<0.001
<i>x,y</i> -Heptacosadiene	0.53	0.004
<i>x,y</i> -Nonacosadiene	0.42	0.008
<i>n</i> -Tricosane	0.36	0.005
<i>n</i> -Nonacosane	0.29	0.044
<i>n</i> -Heptacosane	0.27	0.036
<i>n</i> -Pentacosane	0.23	>0.05
<i>n</i> -Hexacosane	0.23	>0.05
<i>x,y</i> -Pentacosadiene	0.21	>0.05
<i>n</i> -Octacosane	0.12	>0.05
<i>n</i> -Tetracosane	-0.04	>0.05
<i>o</i> -Heptacosene	-0.1	>0.05

^aHydrocarbon names in italics represents compounds that when included in the model allowed the best discrimination among ovarian development classes.

Discriminant Analysis

Discriminant analyses were performed on all compounds of cuticular and PPG hydrocarbon profiles (19 variables; Figure 2, Table 2). They showed a clear discrimination among colonies on cuticular (Figure 3A; $F_{(38,66)} = 83$, $P < 0.001$; 100% of all the individuals are correctly classified) and PPG profiles (Figure 3B; $F_{(38,66)} = 4$, $P < 0.001$; 91% of all the individuals are correctly classified). However, DAs on all variables did not discriminate

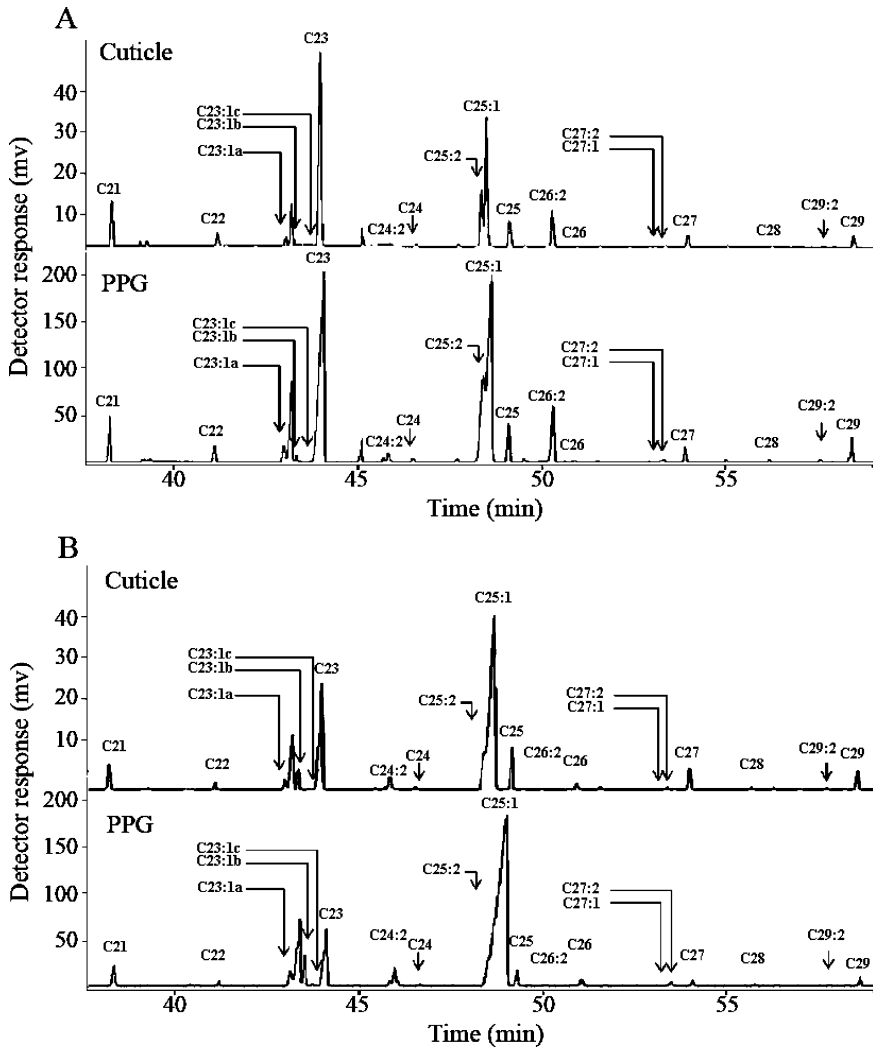


Fig. 2 Total ion chromatograms of cuticular and PPG hydrocarbons extracted from a worker of *P. goeldii* with fully developed (A) and undeveloped (B) ovaries. C21 = *n*-heneicosane; C22 = *n*-docosane; C23:1a = *x*-tricosene; C23:1b = *y*-tricosene; C23:1c = *z*-tricosene; C23 = *n*-tricosane; C24:2 = *x,y*-tetracosadiene; C24 = *n*-tetracosane; C25:2 = *x,y*-pentacosadiene; C25:1a = *x*-pentacosene; C25 = *n*-pentacosane; C26:2 = *x,y*-hexacosadiene; C26 = *n*-hexacosane; C27:1 = *x*-heptacosene; C27:2 = *x,y*-heptacosadiene; C27 = *n*-heptacosane; C28 = *n*-octacosane; C29:2 = *x,y*-nonacosadiene; C29 = *n*-nonacosane. The prefixes *x*, *y*, and *z* indicate different and undetermined positions of the double bond

among classes of ovarian development (cuticle: $F_{(38,66)} = 1.5$, $P = 0.063$; PPG: $F_{(38,66)} = 1$, $P > 0.05$), indicating that the fertility signal is likely encoded in only a subset of compounds. Thirteen compounds out of 19 were significantly correlated between cuticle and PPG (Table 2).

To identify the substances with the highest discriminatory power, we conducted a stepwise DA on cuticular profiles. Out of the 18 models, the one containing 11 compounds was the best for discriminating among the classes of ovarian development (Table 3). The 11 compounds retained in the model have correlation coefficients between cuticle and PPG that are significantly greater than the eight compounds excluded from the model (Table 2, $U = 20$, $P = 0.047$), suggesting that these 11 compounds are also involved in the colony identity signal. Standard DA on cuticular profiles of these 11 compounds allowed us to discriminate among classes of ovarian development ($F_{(22,82)} = 2.9$, $P < 0.001$; 78% of all the individuals are correctly classified). The three classes of ovarian development were segregated (Figure 4; 1: undeveloped ovaries, 2: moderately developed ovaries, 3: fully developed ovaries; Mahalanobis distances, 1–3 = 7.2, $F_{(11,42)} = 4.2$, $P < 0.001$; 1–2 = 3, $F_{(11,42)} = 2.3$, $P = 0.03$; 2–3 = 5.1, $F_{(11,42)} = 2.6$, $P = 0.01$). Moreover, this DA model correctly classified 92% of the individuals with undeveloped ovaries, 59% of the individuals with moderately developed ovaries, and 75% of the individuals with fully developed ovaries. The “moderately developed ovaries” class is intermediate between the two other classes, and this explains this class’s relatively low percentage of correct classification. The standard DA on the 11 compounds discriminated among colonies as well ($F_{(22,88)} = 88$, $P < 0.001$; 100% of all the individuals are correctly classified). However, the standard DA on the 11 compounds in the PPG did not discriminate among classes of ovarian development ($F_{(22,82)} = 1.2$, $P > 0.05$).

Principal Component Analyses

A PCA on the 11 cuticular hydrocarbons of workers from all colonies showed a distinction among the three colonies (Figure 5A). The first two factors explained a high proportion of the variance (60.29%). Factor 1 differentiated colonies A and C from colony B, and factor 2 differentiated colonies A and B from colony C. Principal component analyses on each colony illustrated the differentiation between workers with fully developed and undevel-

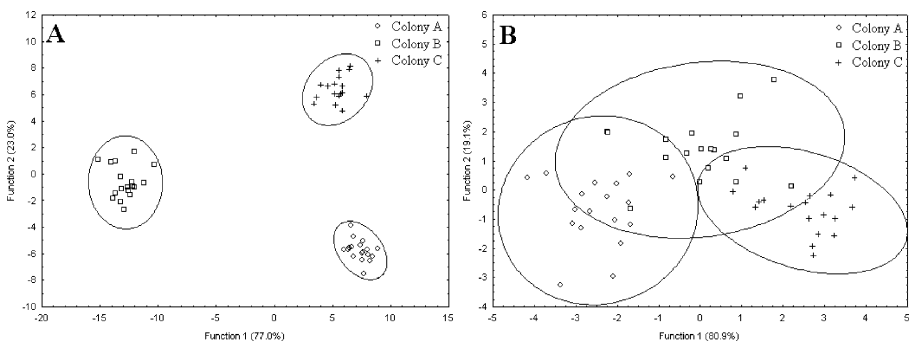


Fig. 3 Discriminant analysis of the cuticular (A) and PPG (B) hydrocarbon profiles from three colonies of *Pachycondyla goeldii* ($N = 18$ for each colony). Ellipses represent 95% confidence limits. The percentages of variance explained by each of the two discriminant functions are provided parenthetically on the axis labels

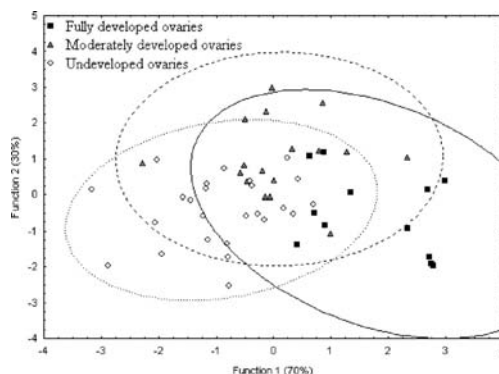
Table 3 Stepwise Discriminant Analysis of Cuticular Hydrocarbon Profiles from 54 Workers of *Pachycondyla Goeldii* Classified into Three Categories of Ovarian Development^a

Step	Hydrocarbons removed	Nb of Hc excluded from the model	Nb of Hc included in the model	Correct classification (%)	<i>P</i> value
1	None	0	19	75.92	>0.05
2	<i>x</i> -Heptacosene	1	18	75.92	0.03
3	<i>x,y</i> -Nonacosadiene	2	17	77.77	0.02
4	<i>n</i> -Tetracosane	3	16	75.92	0.01
5	<i>n</i> -Docosane	4	15	77.77	0.004
6	<i>y</i> -Tricosene	5	14	77.77	0.002
7	<i>n</i> -Hexacosane	6	13	77.77	0.001
8	<i>n</i> -Nonacosane	7	12	77.77	<0.001
9	<i>n</i>-octacosane	8	11	77.77	<0.001
10	<i>x,y</i> -Pentacosadiene	9	10	75.92	<0.001
11	<i>n</i> -Tricosane	10	9	74.07	<0.001
12	<i>n</i> -Heneicosane	11	8	72.22	<0.001
13	<i>x,y</i> -Tetracosadiene	12	7	72.22	<0.001
14	<i>n</i> -Heptacosane	13	6	70.37	<0.001
15	<i>x</i> -Tricosene	14	5	68.51	<0.001
16	<i>z</i> -Tricosene	15	4	66.66	<0.001
17	<i>x,y</i> -Heptacosadiene	16	3	57.40	0.003
18	<i>x</i> -Pentacosene	17	2	48.14	0.003
19	<i>n</i> -Pentacosane	18	1	53.70	>0.05
20	<i>x,y</i> -Hexacosadiene	19	0	–	–

^aThe ninth step (bold font) showed both the best percentage of correct classification and the most significant *P* value (*P* = 0.00023).

oped ovaries, despite the low number of workers (*N* = 18) in each analysis. Workers with moderately developed ovaries were less clearly segregated and largely overlapped the undeveloped ovaries class. The fully developed and undeveloped ovaries classes were separated along the axis of the first factor alone for colonies A and B (Figure 5B and C, respectively), and along the axis of the second factor alone for colony C (Figure 5D). For each PCA, the first two factors accounted for more than 65% of the variance.

Fig. 4 Discriminant analysis of 54 workers of *P. goeldii* based on the 11 cuticular hydrocarbons retained in the best discriminant model of the stepwise discriminant analysis, showing discrimination among three classes of ovarian development. Ellipses represent 95% confidence limits. The percentages of variance explained by each of the two discriminant functions are provided parenthetically on the axis labels



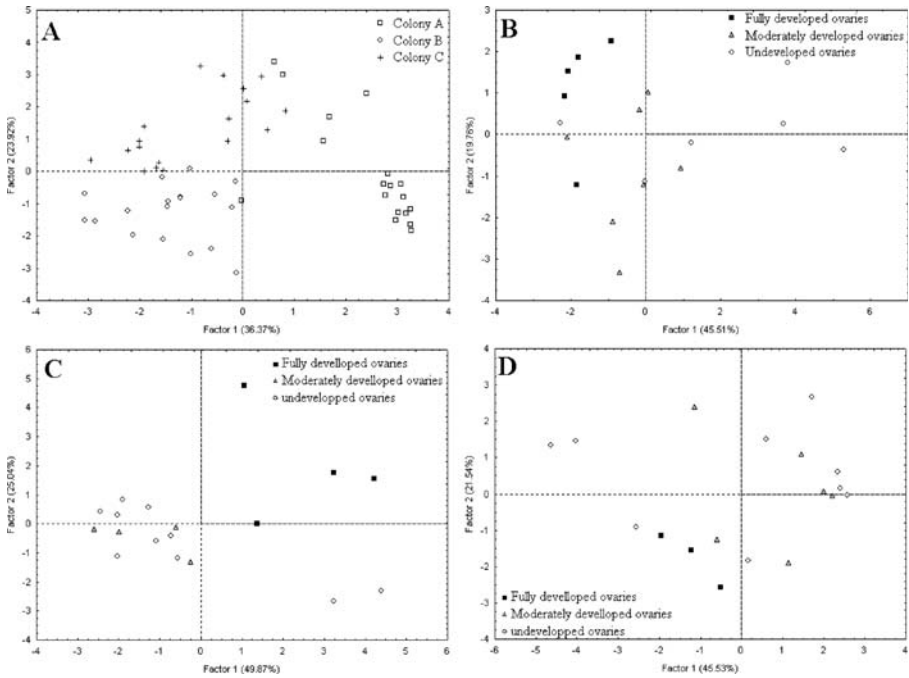


Fig. 5 Principal component analysis of *P. goeldii* workers based on the 11 cuticular hydrocarbons retained in the best discriminant model of the stepwise discriminant analysis. (A) Fifty-four workers from three colonies; (B), (C), and (D) 18 workers each from colonies A, B, and C, respectively, by classes of ovarian development. The percentages of variance explained by each of the two factors are provided parenthetically

Discussion

In the ant *P. goeldii*, the patterns of cuticular and PPG hydrocarbons vary strongly among colonies, and many compounds are correlated between the cuticle and the PPG. However, ovarian development in workers from queenless groups were best discriminated on the basis of 11 cuticular hydrocarbons. These compounds still allow a clear discrimination between colonies. Moreover, these compounds are among the most correlated between cuticle and PPG. Thus, workers bear two signals—colonial identity and reproductive state—on their cuticle. A striking result is that the two signals can be potentially conveyed by the same set of compounds. Although this insights may lead to a new understanding of ant communication, our results are purely correlational at this point. Indeed, we still need to prove that the ants actually perceive and react to these signals.

Numerous studies have suggested the importance of hydrocarbons in nestmate recognition (Vander Meer and Morel, 1998; Lenoir et al., 1999; Ozaki et al., 2005). They have shown that workers from the same colony present a similar hydrocarbon pattern on their cuticle, which is considered to be a colonial signature (Bonavita-Cougourdan et al., 1987; Vander Meer and Morel, 1998). According to Gestalt theory (Crozier and Dix, 1979), nestmates blend their hydrocarbons to form a common colonial odor. The hydrocarbons involved in nestmate discrimination appear to be mixed in the PPG (Meskali et al., 1995; Lenoir et al., 1999; Soroker et al., 2003; Lucas et al., 2004), consequently the hydrocarbon

content of the PPG should more or less match the cuticular profile. In our focal species, we observed that two-thirds of the compounds have relative proportions that are significantly correlated between PPG and cuticle. Moreover, colonies could be discriminated on the basis of all hydrocarbons from both cuticle and PPG, providing arguments in favor of the Gestalt model of nestmate discrimination in *P. goeldii*. However, one-third of the compounds was not correlated between PPG and cuticle. A probable reason is that they are involved in preventing desiccation, the primary function of the cuticular hydrocarbons in insects. The higher the melting point of a hydrocarbon, the better its capacity to prevent water loss. At a given chain length, linear *n*-alkanes are better candidates for this function than alkenes (Hadley, 1994). We observed in our study that linear *n*-alkanes tended to have a low correlation between PPG and cuticle. A surprising result was the absence of methyl branched alkanes in the *P. goeldii* profiles. This class of hydrocarbons is common on the cuticle of other ants, and we do not know why they do not occur in our focal species.

In addition to conveying information about colonial identity, the cuticular hydrocarbons of queenless *P. goeldii* workers bear another signal: 11 compounds allow discrimination of ovarian development classes, suggesting a chemical signal of fertility that could allow subordinates to recognize breeders. Several studies have shown that fertility signals in social Hymenoptera can be conveyed by cuticular hydrocarbons. However, the quality and quantities of compounds potentially involved vary across species. In some species, the signal can be composed of few compounds. This is the case in the ants *Dinoponera quadriceps* (Monnin et al., 1998; Peeters et al., 1999), *H. saltator* (Liebig et al., 2000), *Myrmecia gulosa* (Dietemann et al., 2003), and *Pachycondylainversa* (Heinze et al., 2002). In contrast, in the ants *Diacamma ceylonense* (Cuvillier-Hot et al., 2001), *Streblognathus peetersi* (Cuvillier-Hot et al., 2004a), and *Formica fusca* (Hannonen et al., 2002), the signal varied in multiple hydrocarbons. In *P. goeldii*, the optimal signal is composed of 11 compounds. Thus, even within the same genus, species may display different signals. Our data suggest that this signal has a different nature in *P. goeldii* than in the other species studied to date. We could not identify a single or a few compounds that were simply more abundant on individuals with more developed ovaries, but we identified a group of compounds whose relative proportions were characteristic of the degree of ovarian development. The fertility signal could thus be potentially more complex than previously thought. These studies draw some correlation between cuticular hydrocarbons and fertility. However, we do not know, except in a few cases (Dietemann et al., 2003; D'ettore et al., 2004), whether the ants perceive these compounds, and, assuming they do, whether they use the entire signal. Future investigations should address these possibilities.

Cuticular hydrocarbons are known to vary according to colony, reproductive status, age, and caste (Hölldobler and Wilson 1990; Peeters et al., 1999; Kaib et al., 2000; Cuvillier-Hot et al., 2001). Thus, the same channel can convey various types of information. It is unknown, however, whether the same chemicals are involved in the different signals. As the cuticle bears many compounds, we might expect different signals to be conveyed by different hydrocarbons. However, we found that the cuticular hydrocarbons responsible for the best discrimination among ovarian development classes also yielded a clear discrimination among colonies and had significantly higher correlation coefficients between cuticle and PPG than the other hydrocarbons. This suggests that these compounds are among the most important for colonial identity. Our interpretation is that the two signals, colony identity and reproductive status, can be potentially conveyed by the same compounds. Moreover, in addition to a sharp difference between colonies, there is a fine-tuned variation accounting for ovarian development. Two facts explain the high correlation between the relative proportions of compounds in the PPG and in the cuticle: the PPG is

involved in the formation of the colony identity signal displayed on the cuticle, and this signal is different among colonies. Variations in cuticular hydrocarbons related to fertility are much weaker. Thus, even if they do not occur in the PPG, these variations have little influence on the correlation between PPG and cuticle.

If colony identity and fertility signals are conveyed through the same compounds, one signal must be shared between nestmates and the other must remain individual. Body contacts and nestmates grooming (and trophallaxis in some species) lead to the homogenization of the cuticular and PPG hydrocarbons within the colony (Soroker et al., 1994, 1998). The presumed function of the PPG is to store a blend of hydrocarbons from all the nestmates that can be more easily spread on the cuticle. To remain individual-specific, the fertility signal must not be involved in this circulation scheme. Therefore, the PPG should not contain this signal. Indeed, our analysis of the PPG showed that the 11 compounds that allow for discrimination of reproductive status on the cuticle do not do so in the PPG. In contrast, Dietemann et al. (2003) showed that workers of the ant *M. gulosa* discriminate between the PPG hydrocarbons of reproductive and nonreproductive workers. Their results suggest that the fertility signal follows the same pathway as the colony identity signal, i.e., through the PPG. The two signals should then be made of different compounds. The model we propose in *P. goeldii* is not a general one, and more information is needed in other species to draw broader conclusions. How is the fertility signal of *P. goeldii* prevented from being in the PPG? Our results suggest that the hydrocarbons are transferred from the PPG to the cuticle and not in the opposite direction. How does the fertility signal persist after the spread of compounds from the PPG then? The fine-tuned variation responsible for the discrimination between ovarian development classes seems to be superimposed on the colony identity signal on the cuticle. The hydrocarbons displayed on the cuticle would result from two different regulating pathways: one responsible for the colonial identity signal involving the PPG, and another responsible for the fertility signal, perhaps involving the gaster.

One way the hydrocarbon pathways of the colony identity and the fertility signal could be kept separate is by lipophorin, the internal hydrocarbon carrier. This molecule is known to bind hydrocarbons relatively unselectively, but the unloading mechanism might depend on body tissue and on the hydrocarbon transported, and thus provide target tissue specificity (Schal et al., 1998, 2001). The unloading of the compounds that are involved in colony identity and the fertility signal could be slightly different between the PPG and the cuticle. Thus, because of differences in biosynthesis, the relative proportions of these compounds would be roughly similar between PPG and cuticle, accounting for the colony identity signal, but the slightly different unloading specificity close to the cuticle would account for the fertility signal, only present on the cuticle. The frequent hydrocarbon exchanges between PPG and cuticle and between nestmates would dilute the fertility signal, which would, therefore, have to be updated continuously. This could be achieved through the tissue-specific loading/unloading mechanism of hydrocarbons on the lipophorin. A strongly marked difference between PPG and cuticle in the unloading mechanism of certain hydrocarbons could explain the fact that some of them have relative proportions that are not correlated between these two body sites. Hydrocarbons whose function is to prevent desiccation would thus only be displayed on the cuticle.

Our results suggest that there is a functional relationship between the PPG and the cuticular hydrocarbons involved in the colonial odor, but that the fertility signal remains independent of the PPG. Each ant can display two signals at the same time and with the same compounds, a rough signal of colony identity that is common to all nestmates, and a fine-tuned individual signal of fertility. However, it remains to be seen whether the fine-

tuned variation that allowed us to discriminate between classes of ovarian development is perceived as a fertility signal by ants.

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Sex Pheromone of the Cranberry Root Grub *Lichnanthe vulpina*

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Abstract The cranberry root grub *Lichnanthe vulpina* (Hentz) (Coleoptera: Glaphyridae) is a pest of cranberries in Massachusetts, reducing yield and vine density. (*Z*)-7-Hexadecenol and (*Z*)-7-hexadecenal were identified from the female effluvia collection by gas chromatographic–electroantennographic detection and gas chromatography–mass spectrometry. The double-bond position was confirmed by dimethyl disulfide derivatization. Both compounds were tested in the field, each alone and as blends of the two. Each compound alone captured males; however, (*Z*)-7-hexadecenol alone captured significantly more males than did (*Z*)-7-hexadecenal alone. The addition of varying amounts of (*Z*)-7-hexadecenal to (*Z*)-7-hexadecenol did not statistically affect male capture. Flight activity of the cranberry root grub may be monitored with traps baited with rubber septa containing 300 µg of (*Z*)-7-hexadecenol. A test of trap vane colors indicated that traps with green or black vanes maximized target male catch while minimizing nontarget catch of important cranberry pollinators.

Keywords (*Z*)-7-Hexadecenol · (*Z*)-7-Hexadecenal · Gas chromatography–mass spectrometry · Electroantennogram · Scarab beetle · Glaphyridae

*Deceased May 15, 2001. He is dearly missed by his family, friends, and colleagues.

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Introduction

The cranberry root grub (CRG) *Lichnanthe vulpina* (Hentz) (Coleoptera: Glaphyridae) is a native scarab beetle species whose immature stages have a long history as root-feeding pests of cranberry beds in Massachusetts, first being reported in 1911 (Franklin, 1950). Dunn and Averill (1996) found that of the 38 Massachusetts cranberry beds sampled, 61% were infested with CRG larvae. Currently, the only control strategy for the CRG is renovation. Bog renovation, the removal and disposal of the top 25–30 cm of the bog (including the cranberry plants and associated CRG larvae), is an extreme and expensive solution for a problem that may eventually recur. The identification and commercial availability of the CRG sex pheromone of this species could provide a useful tool for monitoring or managing this pest.

The objective of this study was to identify the sex pheromone of *L. vulpina* as well as to determine a trap color that would maximize target capture while minimizing capture of honeybees and bumblebees, important pollinators of cranberries.

For more information on the taxonomy, behavior, and distribution of the *Lichnanthe*, see Westcott (1976), Carlson (1980), and O'Donnell (1996).

Methods and Materials

Pheromone Collections

Third instar larvae of CRG were collected in mid-April by digging them from the soil in an infested cranberry bog in Carver, MA. Recovered larvae were kept individually in ~30-ml plastic cups in a 3:1 mix of greenhouse sand and screened peat moss raised to ca. 12% moisture. They were housed in a controlled environment room at 25°C during the 16-hr photophase and 20°C during the 8-hr scotophase. After pupation and adult emergence, individuals were separated by sex, and up to 15 females were placed together in an all-glass collection vessel during the photophase because the species is diurnal (Zhang et al., 1994; O'Donnell, 1996). Twelve female collections were made during the course of the study. During the photophase, pump-drawn air was filtered through charcoal, bubbled through distilled water, passed over and among the females, and finally through a glass tube filled with adsorbent Super Q polymer material (Alltech, Deerfield, IL, USA). Volatiles were eluted from the Super Q using ca. 2 ml of dichloromethane before condensing under a nitrogen stream to a volume of ca. 20 μ l.

Instrumentation

The coupled gas chromatograph–electroantennogram detector (GC-EAD) system used was as previously described in Zhang et al. (1997, 1999, 2003). A Hewlett Packard (HP) 5880 gas chromatograph equipped with a 30 m \times 0.25 mm ID, 0.25- μ m film-thickness nonpolar SE-30 capillary column (Alltech Associates) in the splitless mode with nitrogen as carrier was used for GC-EAD analysis (150°C for 2 min, then programmed to 250°C at 10°C/min and held for 25 min) or a 30 m \times 0.25 mm ID, 0.25- μ m film-thickness polar Stabilwax capillary column (Restek Corp., 150°C for 2 min, then programmed to 220°C at 10°C/min and held for 25 min). The capillary column effluent and nitrogen makeup gas (10 ml/min) were split (~1:1) by a Y GlasSeal capillary column connector (Supelco, Inc.) to the flame ionization detector (FID) and EAD. After removing an antenna from the beetle, one lamella

tip and the scape were positioned between two gold wire electrodes, which were immersed in saline-filled (0.9% NaCl) wells in a small acrylic plastic holder. This holder held the antennal club open, exposing the sensilla to the airstream (see photo in Robbins et al., 2003). The output recording electrodes were connected to a high-impedance 1:100 amplifier with automatic baseline drift compensation. The airstream flowing over the antennae (about 500 ml/min) was humidified by bubbling through distilled water before entering the EAD interface. The antennal preparation was cooled to ~5°C inside a condenser by circulating near 0°C water from a bench-top refrigeration unit (RTE-100, NESLAB instruments, Inc., Portsmouth, NH, USA) through the insulation layer of the modified condenser containing the acrylic plastic holder mounted on top of the GC. An HP 3390A integrator was used for EAD recording.

Electronic impact gas chromatography–mass spectrometry (GC-MS) was conducted on an HP 5890 GC coupled to an HP 5970B mass selective detector using an identical SE-30 capillary column [150°C for 2 min, then programmed to 250°C at 10°C/min and held for 25 min for regular analysis; 180°C for 2 min, then programmed to 230°C at 15°C/min and held for 50 min for analysis of dimethyl disulfide (DMDS) adducts] or a DB-5 capillary column (60 m × 0.25 mm ID, 0.25- μ m film thickness, J&W Scientific Inc.; 50°C for 2 min, then programmed to 300°C at 15°C/min and held for 50 min) but with helium as carrier gas. A 70-eV electron beam was employed for sample ionization.

Chemicals

Synthetic (*Z*)-7-hexadecenol (Z7-16:OH) and (*Z*)-7-hexadecenal (Z7-16:Ald) were purchased from Pheromone Bank, Wageningen, The Netherlands. Purities of the chemicals, as determined on 30-m polar Stabilwax and nonpolar SE-30 GC capillary columns, were > 98%.

Microderivatization

Dimethyl disulfide derivatives of extracts and synthetic standards were prepared according to standard procedures (Buser et al., 1983; Dunkelblum et al., 1985). Dichloromethane solutions of effluvia extracts or hexane solutions of synthetic monounsaturated standards (10 μ l, 20 ng/ μ l) were treated with 50 μ l of DMDS (Aldrich Chem. Co., 99+%) and one drop of an iodine solution (60 mg/ml diethyl ether). The mixtures were kept at 60°C for 4 hr. After cooling to room temperature, one drop of 5% aqueous sodium thiosulfate (Na₂S₂O₃) was added, and the solutions were shaken vigorously to reduce the iodine. The organic phase was removed, and the aqueous phase was extracted with 100 μ l hexane. The combined extracts were then dried over anhydrous sodium sulfate (Na₂SO₄) and concentrated to ~20 μ l under nitrogen for GC-MS analysis.

The effluvia collection, synthetic Z7-16:OH, and synthetic Z7-16:Ald (30 ng in hexane) were each treated separately in a conical glass vial with 5 μ l of acetic anhydride–pyridine (10:1, v/v), sealed with a Teflon lined screw cap, and heated at 40°C in a GC oven for 30 min. After 1 μ l water was added to destroy the excess anhydride, the organic layer was removed for GC-MS analysis.

Field Evaluation of Synthetic Lures

The following protocol was used for field testing in all years. Lures were formulated by dissolving Z7-16:OH and Z7-16:Ald in hexane, dispensing appropriate amounts into

5-mm rubber stopper septa (Thomas Scientific, Swedesboro, NJ, USA), and allowing the hexane to evaporate in a fume hood. Lures were deployed in the field in cross-vane traps. Traps were placed ca. 20 m apart along the cranberry bog edge and were randomized at deployment. The bottom of the trap was hung ca. 60 cm from the ground. Dates in parentheses at the end of each section indicate the dates that the traps were checked and rerandomized.

Effect of Different Proportions of the Pheromone Constituents on CRG Trap Catches

In 1999, treatments tested in the field included eight blends of Z7-16:OH and Z7-16:Ald at a dose of 1000 $\mu\text{g}/\text{septa}$ in the ratios of 100:0, 90:10, 80:20, 60:40, 40:60, 20:80, 10:90, and 0:100 and a solvent-only control treatment. One set of the nine treatments was deployed at each of three Massachusetts cranberry bogs during the flight period in July (July 6–13, 15, 16, and 19).

Effect of Different Doses of a 90:10 Blend of the Alcohol and Aldehyde on Trap Catches

In 2000, a test was deployed to compare doses of a 90:10 blend of Z7-16:OH and Z7-16:Ald. The doses included 100, 300, 600, and 1000 $\mu\text{g}/\text{septa}$ and a solvent-only control septa. One set of five treatments was deployed at each of four Massachusetts cranberry bogs during the flight period in July (July 3–9, 14, 18, and 25).

Effect of Trap Vane Color on CRG and Nontarget Insect Catches

A test was deployed in 2000 to test the effect of vane color on CRG adult male catch and honeybee and bumblebee catches. Honeybee and bumblebee catches are of concern to growers because they are essential for crop pollination. This test was conducted to determine a color that would maximize CRG male catches while reducing bee catches. All traps were baited with lures consisting of a 90:10 blend of Z7-16:OH and Z7-16:Ald at a dose of 1000 $\mu\text{g}/\text{septa}$. Vanes for the lab-constructed cross-vane traps were fabricated from 4-mm corrugated plastic of six different colors (white, red, yellow, blue, black, and green) purchased from KIVA Container Corporation, Taylors, SC, USA. One set of six treatments was deployed at each of four Massachusetts cranberry bogs during the flight period in July (July 3–9, 14, 18, and 25).

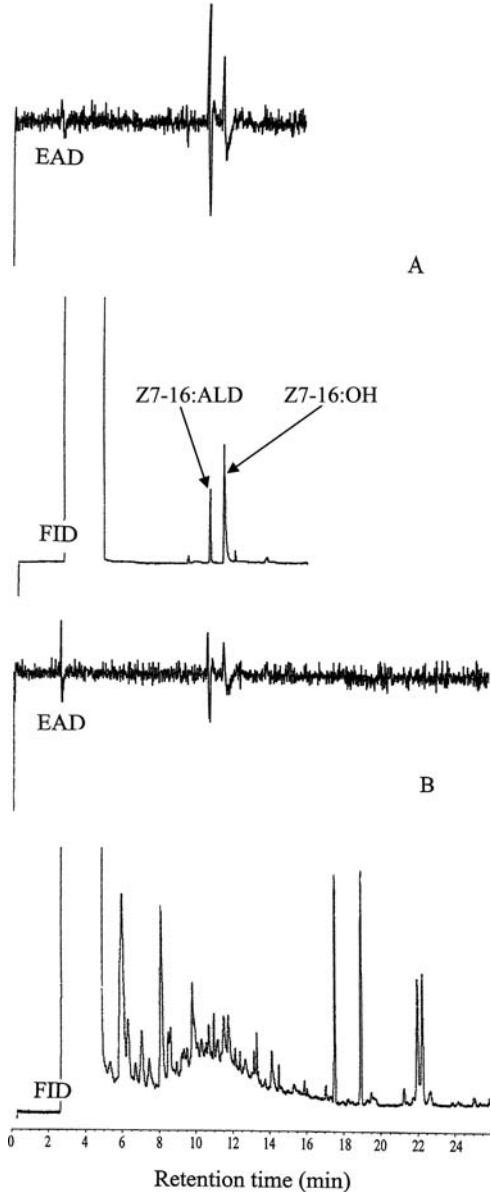
Effect of the Presence of Different Amounts of the Aldehyde on the Optimum Dose of the Alcohol on Male Catches

In 2004, a test was deployed to compare male CRG capture when various amounts of Z7-16:Ald were added to 1000- μg doses of Z7-16:OH. This test was initiated because the results of the 1999 test (see Fig. 2) did not resolve the role of Z7-16:Ald in male capture. The six treatments tested included 1000 μg of Z7-16:OH alone, 1000 μg of Z7-16:OH + 10% (111 μg) of Z7-16:Ald, 1000 μg of Z7-16:OH + 20% (250 μg) of Z7-16:Ald, 1000 μg of Z7-16:OH + 40% (666 μg) of Z7-16:Ald, 1000 μg of Z7-16:Ald, and a solvent-only control. One set of the six treatments was deployed at each of two Massachusetts cranberry bogs, and two sets were deployed at separate locations on a third bog during the flight period (June 28, July 1, 5, and 8).

In 2005, a test was deployed to compare male CRG capture when various amounts of Z7-16:Ald were added to 300- μg doses of Z7-16:OH. In this test, the dose of Z7-16:OH was

reduced from 1000 $\mu\text{g/septa}$ (see 2004 test above) to 300 $\mu\text{g/septa}$ to provide a more sensitive assay of male CRG response to added amounts of the Z7-16:Ald. The three treatments tested included 300 μg of Z7-16:OH alone, 300 μg of Z7-16:OH + 10% (33 μg) of Z7-16:Ald, and 300 μg of Z7-16:OH + 20% (75 μg) of Z7-16:Ald. Four sets of the three treatments were deployed at separate locations on a large Massachusetts cranberry bog during the flight period (July 3, 5, 8, 11, 16, and 19).

Fig. 1 Simultaneous EAD and FID responses of a male CRG antenna to (A) synthetic Z7-16:Ald and Z7-16:OH (10 ng, 1:3 ratio, v/v); (B) effluvia trapped from 15 virgin female CRG (3–13 days old) on an SE-30 capillary column



Statistics

Using the Levene test for homogeneity of variance, data sets were tested for homogeneity of variance and log-transformed ($x + 1$) as necessary. Data were analyzed using a one-way analysis of variance, F at $P < 0.05$, with *post hoc* comparisons using Fisher's least significant difference (LSD) test.

Results and Discussion

Pheromone Identification

Coupled GC-EAD analyses of female effluvia extracts demonstrated that male beetle antenna consistently responded to two compounds (Fig. 1B). Two EAD-active peaks from 12 female effluvia collections were observed at 10.39 and 11.14 min on a 30-m SE-30 capillary column and at 10.82 and 14.27 min on a 30-m Stabilwax column at about a 1:3 ratio. The MS of the active component corresponding to the later EAD response in the effluvia extracts exhibited a comparatively strong ion at m/z 222 (5%) as the highest mass fragment and matched spectra of monounsaturated C_{16} derivatives retrieved from the Wiley 275 mass spectral database. In addition, the MS of the earlier component showed the largest fragment at m/z 220 (3%), 2 amu less than the later compound, indicating that it could possess an additional unsaturation.

The identity of the EAD-active compounds was determined by capillary GC-MS analysis of DMDS derivatives of the female effluvia extracts. Pairs of diagnostic sulfide fragments were observed at m/z 161 (81%) and 173 (100%) with the molecular ion at m/z 334 (52%). Pairs of diagnostic fragments were also observed at m/z 159 (18%) and 173 (100%) with molecular ion at m/z 332 (44%). These observations indicated that $\Delta 7$ -16:OH and $\Delta 7$ -16:Ald, respectively, were likely candidates for the natural pheromone. The $\Delta 7$ -16:OH was also verified by a pair of diagnostic sulfide fragments observed at m/z 173 (94%)

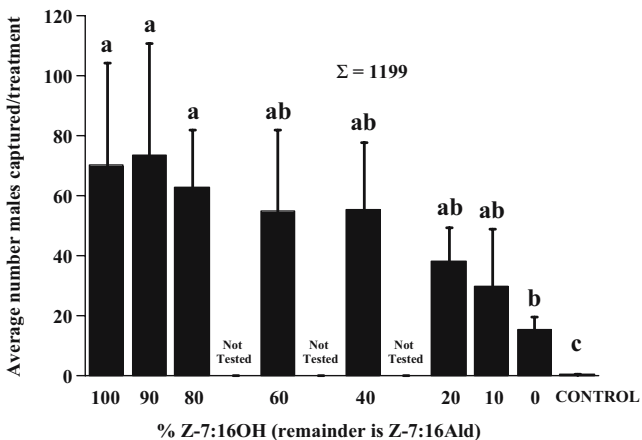


Fig. 2 Average capture/treatment (mean \pm SE) of male CRG beetles in traps baited with various 1000- μ g blends of Z-7:16:OH and Z-7:16:Ald, 1999. Data were transformed using $\log(x + 1)$ before analysis. Bars with the same letter are not significantly different ($P < 0.05$, Fisher's LSD test)

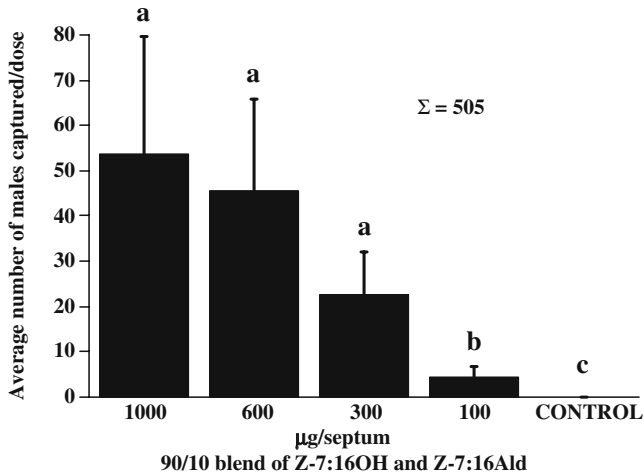


Fig. 3 Average capture/treatment (mean ± SE) of male CRG beetles in traps baited with various doses of a 90:10 blend of Z7-16:OH and Z7-16:Ald, 2000. Data were transformed using $\log(x + 1)$ before analysis. Bars with the same letter are not significantly different ($P < 0.05$, Fisher’s LSD test)

and 203 (75%) with the molecular ion at m/z 376 (30%) from the microacetylation preparation of the female effluvia collection. To confirm the above conclusion, and to determine pheromone geometry, synthetic standards of the (*E*) and (*Z*) isomers of Δ 7-16:OH, Δ 7-16:Ald, and Δ 7-16:Ac were then subjected to the corresponding analysis. The MS spectra and GC retention times of synthetic Z7-16:OH, Z7-16:Ald, Z7-16:Ac, and their DMDS adducts were indistinguishable from those of natural products on both SE-30 and Stabilwax capillary columns. The natural pheromone in each case corresponded to the later-eluting isomers (monounsaturated C_{16} derivatives) with an SE-30 capillary column and earlier-eluting isomers (DMDS adducts) on a Stabilwax capillary column, which established

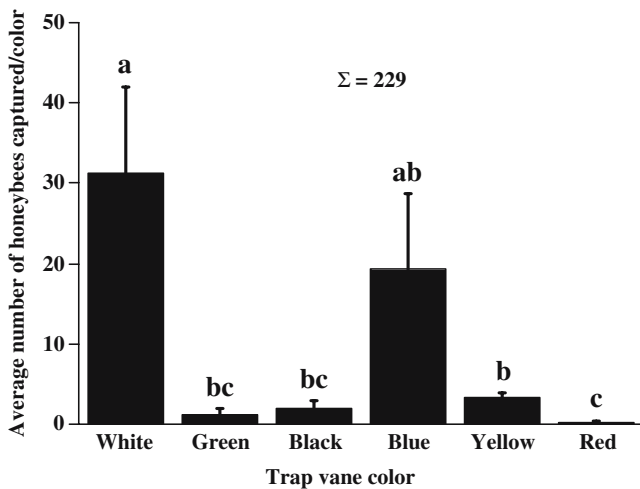


Fig. 4 Average capture/treatment (mean ± SE) of honeybees in traps of various vane colors, 2000. Bars with the same letter are not significantly different ($P < 0.05$, Fisher’s LSD test)

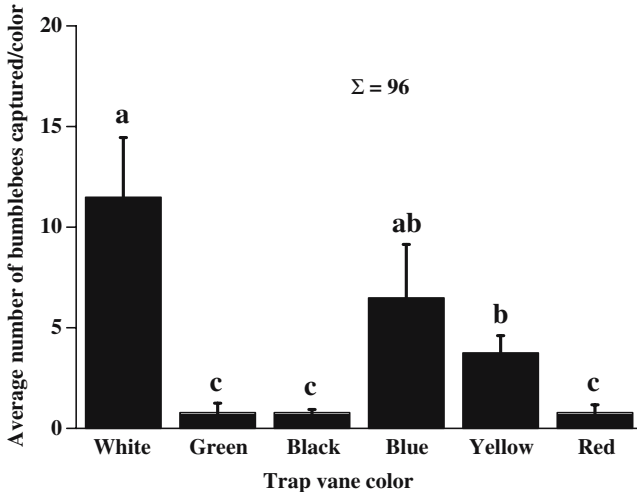


Fig. 5 Average capture/treatment (mean \pm SE) of bumblebees in traps of various vane colors, 2000. Data were transformed using $\log(x + 1)$ before analysis. Bars with the same letter are not significantly different ($P < 0.05$, Fisher's LSD test).

these components to be the (Z) isomers. The strong antennal responses to Z7-16:OH and Z7-16:Ald were confirmed with authentic standards (Fig. 1A).

Effect of Different Proportions of the Pheromone Constituents on CRG Trap Catches

Traps baited with Z7-16:OH only captured significantly more males than did traps baited with Z7-16:Ald. Male captures decreased steadily in response to increasing amounts of Z7-16:Ald relative to the Z7-16:OH, but in all cases, baited treatments captured more males than the control ($F_{8,18} = 8.18$; $P < 0.001$; Fig. 2).

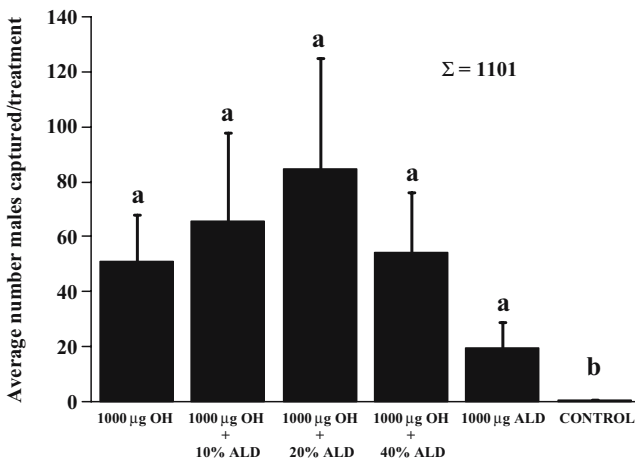


Fig. 6 Average capture/treatment (mean \pm SE) of male CRG beetles in traps baited with blends of Z7-16:OH and Z7-16:Ald, 2004. In treatments containing Z7-16:OH, the Z7-16:OH was held constant at 1000 µg/septum. Data were transformed using $\log(x + 1)$ before analysis. Bars with the same letter are not significantly different ($P < 0.05$, Fisher's LSD test).

Effect of Different Doses of a 90:10 Blend of the Alcohol and Aldehyde on Trap Catches

Traps baited with the 100- μg dose captured a smaller number of males than did traps baited with the 300, 600, or 1000- μg doses ($F_{4,15} = 13.32$; $P < 0.001$; Fig. 3).

Effect of Trap Vane Color on CRG and Nontarget Insect Catches

In the 2000 vane color trial, color of the trap vanes did not affect the number of males captured when traps were baited with lures loaded with 1000 μg of a 90:10 ratio of Z7-16:OH and Z7-16:Ald ($F_{5,18} = 0.35$; $P = 0.87$; data not shown). However, the numbers of honeybees ($F_{5,18} = 4.67$, $P < 0.05$; Fig. 4) and bumblebees ($F_{5,18} = 11.64$, $P < 0.001$; Fig. 5) caught were affected by trap color, being captured most frequently in traps with white or blue vanes. These findings are similar to those reported for *Hoplia equina* LeConte, another scarab pest of cranberry (Weber et al., 2005). Based on the findings of these two studies, it is recommended that green or black vanes be used, thereby ensuring effective male capture while reducing the negative impact on beneficial insects.

Effect of the Presence of Different Amounts of the Aldehyde on the Optimum Dose of the Alcohol on Male Catches

In 2004, there were no significant differences among baited treatments ($F_{5,18} = 5.98$, $P < 0.05$; Fig. 6). The high averages in the treatments testing 10% Z7-16:Ald and 20% Z7-16:Ald were caused by two large capture events that grossly inflated the averages in those treatments. In 2005, there were no significant differences among the treatments ($F_{2,11} = 0.096$, $P = 0.91$; data not shown).

Although the female-produced sex pheromone of the CRG contains both Z7-16:OH and Z7-16:Ald, and each compound alone captures males, the Z7-16:Ald, when combined in various ratios with the Z7-16:OH, did not increase male capture. We conclude that although both compounds can be classified as pheromone components, Z7-16:OH alone is sufficient for monitoring or management programs.

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(1*S*)-1-Ethyl-2-Methylpropyl 3,13-Dimethylpentadecanoate: Major Sex Pheromone Component of Paulownia Bagworm, *Clania variegata*

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Abstract The Paulownia bagworm, *Clania variegata* Snell. (Lepidoptera: Psychidae), is one of the most significant forest defoliators in China. In gas chromatographic (GC)–electroantennographic detection analyses of pheromone gland extracts of female *C. variegata* on three GC columns (DB-5, DB-23, DB-210), two compounds (**A** and **B**) elicited strong responses from male antennae. The more abundant component **B** was isolated by high-performance liquid chromatography and identified as 1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate by transesterification, GC-mass spectrometry (MS), and comparison of its spectral and GC retention characteristics with those of synthetic compounds. In field trapping experiments in China, racemic and (1*S*)-1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate [but not the (1*R*)-stereoisomer] attracted male *C. variegata*. The absolute configuration of **B** (a molecule with three chiral centers) and the structure of component **A** remain to be determined.

Keywords Paulownia bagworm · *Clania variegata* · Psychidae · Sex pheromone · 1-Ethyl-2-methylpropyl 3,13-dimethylpentadecanoate

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Introduction

The Paulownia bagworm, *Clania variegata* Snell. (Lepidoptera: Psychidae), occurs in China, Japan, Korea, Vietnam, Thailand, and India (Zhang, 1997). In China, *C. variegata* is one of the most important pest insects in managed forests. The polyphagous larvae defoliate mainly empress trees, *Paulownia* spp., and false acacia trees, *Robinia pseudacacia*, but also feed on a variety of other tree species (e.g., walnut, chestnut, ginkgo, elm, oak, mulberry, pear, peach, apple), and agricultural crops (e.g., maize, cotton, peanuts, soybean; Zhang, 1997).

Like other bagworms, *C. variegata* has an unusual life history. Upon encountering a suitable host, wind-dispersed neonate larvae envelop themselves in a self-constructed bag, which they enlarge throughout their development. Following eclosion, vermiform apterous females stay within their pupal case and await the arrival of winged males. To attract males, females release sex pheromone from a gland in the dorsal mesothorax, and expel pheromone-impregnated scales (hairs) out of the pupal case into the lower part of the bag (Zhao, 1981, 1984). Conspecific males attracted by the pheromones alight on the bag, insert their extensible abdomen through the bag into the female pupal case, and mate with the adult female.

Despite this intriguing system of sexual communication and mating, and the ecological and economic significance of bagworms, the sex pheromones of only three species of bagworms have been identified. (1*R*)-1-Methylbutyl decanoate is the major sex pheromone component of both *Thyridopteryx ephemeraeformis* (Leonhardt et al., 1983) and *Oiketicus kirbyi* (Rhainds et al., 1994). In *O. kirbyi*, the *R* but not the *S* enantiomers of 2-pentyl octanoate, 2-pentyl nonanoate, 2-hexyl decanoate, and 2-pentyl dodecanoate enhance the attractiveness of the major pheromone component (Rhainds et al., 1994). 1-Methylethyl octanoate is the main sex pheromone component of *Megalophanes viciella* in Bulgaria and Romania (Subchev et al., 2000).

Previous analyses of the major sex pheromone component of *C. variegata* by gas chromatography (GC), GC–mass spectrometry, and GC–infrared spectroscopy suggested that it was an ester with the molecular formula $C_{16}H_{33}COOC_6H_{13}$, and with a methyl branch in both the acyl and the alcohol portions (Tan et al., 1987). Here we report the identification of (1*S*)-1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate as the major component of the sex pheromone of *C. variegata*.

Methods and Materials

Experimental Insects

Late instar larvae or pupae were collected in a forest near the city of Qidong (Jiangsu Province, P. R. China) and sent under import permit from Agriculture and Agri-Food Canada (Food Production and Inspection Branch) to Simon Fraser University. They were kept in mesh cages at 24°C under a photoperiod of 13L:11D. Eclosed males were transferred to and kept individually in filter-paper-lined Petri dishes. Females were removed from their bag and pupal case, and their mesothoracic pheromone gland (plus surrounding tissue) was excised with a razor blade and extracted with hexane for 15 min.

Aliquots of one female equivalent (FE) of this extract, and other pheromone extracts previously prepared in China, were analyzed by coupled gas chromatography–electro-

antennographic detection (GC-EAD) and GC–mass spectrometry (Saturn 2000 Ion trap GC-MS), with procedures and equipment previously described in detail (Arn et al., 1975; Gries et al., 2002). To isolate the major candidate pheromone component, crude pheromone extract was fractionated by high-performance liquid chromatography (HPLC), employing a Waters LC 625 chromatograph equipped with a Waters 486 variable-wavelength UV–visible detector set at 210 nm, HP Chemstation software (Rev.A.07.01), and a reverse-phase Nova Pak[®] C₁₈ (3.9 × 300 mm) column (Waters) eluted with acetonitrile (1 ml/min).

To isolate **B** for transesterification, a pheromone gland extract from 600 female moths was evaporated to dryness, 50 μl of acetonitrile were added, and the solution was injected into the HPLC column. Eighty 0.2-min (200 μl) fractions were collected between 20 and 36 min [the elution time range (± 6 min) of synthetic esters referred to below], concentrated and analyzed by GC-MS. The three HPLC fractions (retention times: 26.8–27.4 min) containing **B** (as determined by GC-MS) were combined, extracted with pentane (500 μl), and evaporated to dryness.

Component **B** was then transesterified by adding a solution of 2 mg KOH in 100 μl methanol and keeping the sample at 40°C for 3 hr.

Syntheses

13-Methyl-1-tetradecene (1a, Fig. 1, Scheme 1). CuI (1.22 g, 6.4 mmol) in THF (150 ml) was added to a freshly prepared and cooled (−23°C) solution of isobutylmagnesium bromide [from 7.0 ml (64 mmol) of isobutyl bromide and activated Mg (3.10 g, 128 mmol)]. After 10 min stirring, 11-bromo-1-undecene (4.71 g, 20.2 mmol; Aldrich) in THF (50 ml) was slowly added. The mixture was stirred at −20°C for 30 min, then warmed to room temperature, quenched with aqueous, saturated NH₄Cl, extracted with hexane (3 × 75 ml), washed with water and brine, and dried (MgSO₄). Solvents were removed *in vacuo*. Purification by flash chromatography (hexane) afforded 13-methyl-1-tetradecene (**1a**, 3.1 g, 14.7 mmol, 73%), which had been previously characterized (Trivedi and Mamdapur, 1986).

12-Methyl-1-tetradecene (1b, Fig. 1, Scheme 1). Compound **1b** was synthesized by the same procedure (see **1a**), starting with 2-bromobutane (10.5 ml, 96 mmol), Mg (4.65 g, 191 mmol), CuI (1.82 g, 9.6 mmol) and 11-bromo-1-undecene (4.71 g, 20.2 mmol). The yield of **1b** was 3.40 g (16.2 mmol, 80%). ¹H NMR (CDCl₃): δ 0.84 (d, 3H, *J* = 6.7 Hz), 0.86 (t, 3H, *J* = 7.3 Hz), 1.19–1.42 (m, 19H), 2.04 (m, 2H), 4.93 (tdd, 1H, *J* = 1.2, 2.3, 10.2 Hz), 5.00 (tdd, 1H, *J* = 1.6, 2.3, 17.2 Hz), 5.82 (tdd, 1H, *J* = 6.7, 10.2, 17.1 Hz). ¹³C NMR (CDCl₃): δ 11.40, 19.23, 27.13, 28.98, 29.18, 29.52, 29.54, 29.65, 29.73, 30.05, 33.84, 34.43, 36.67, 114.06, 139.24. Anal. calcd. for C₁₅H₃₀ (%): C, 85.63; H, 14.37; found C, 85.82; H, 14.10.

11-Methyl-1-tetradecene (1c, Fig. 1, Scheme 1). Compound **1c** was synthesized by the same procedure (see **1a**), starting with 2-bromopentane (10 g, 66 mmol), Mg (3.17 g, 130 mmol), CuI (1.25 g, 6.6 mmol) and 10-iodo-1-decene (6.65 g, 25 mmol). The 10-iodo-1-decene was obtained in 95% yield by mesylation of 9-decen-1-ol (Aldrich) with methanesulfonyl chloride and triethylamine in dichloromethane at room temperature, and subsequent replacement of the methanesulfonate group by iodide (7 hr stirring with 5-fold excess of NaI in DMSO at 50°C). Yield of **1c** was 4.10 g (19.5 mmol, 78%). After chromatographic purification, the by-product 4,5-dimethyloctane was removed by vacuum distillation (room temperature, 1–2 mm Hg, 3 hr). ¹H NMR (CDCl₃): δ 0.84 (d, 3H, *J* = 6.6 Hz), 0.88 (t, 3H, *J* = 7.2 Hz), 1.20–1.42 (m, 19H), 2.05 (m, 2H), 4.93 (tdd, 1H, *J* = 1.2, 2.4, 10.2 Hz), 4.99 (tdd, 1H, *J* = 1.6, 2.4, 17.0 Hz), 5.82 (tdd, 1H, *J* = 6.7, 10.2, 17.0 Hz). ¹³C NMR (CDCl₃): δ 14.42, 19.67, 20.15, 27.09, 28.97, 29.19, 29.54, 29.68, 30.03, 32.50,

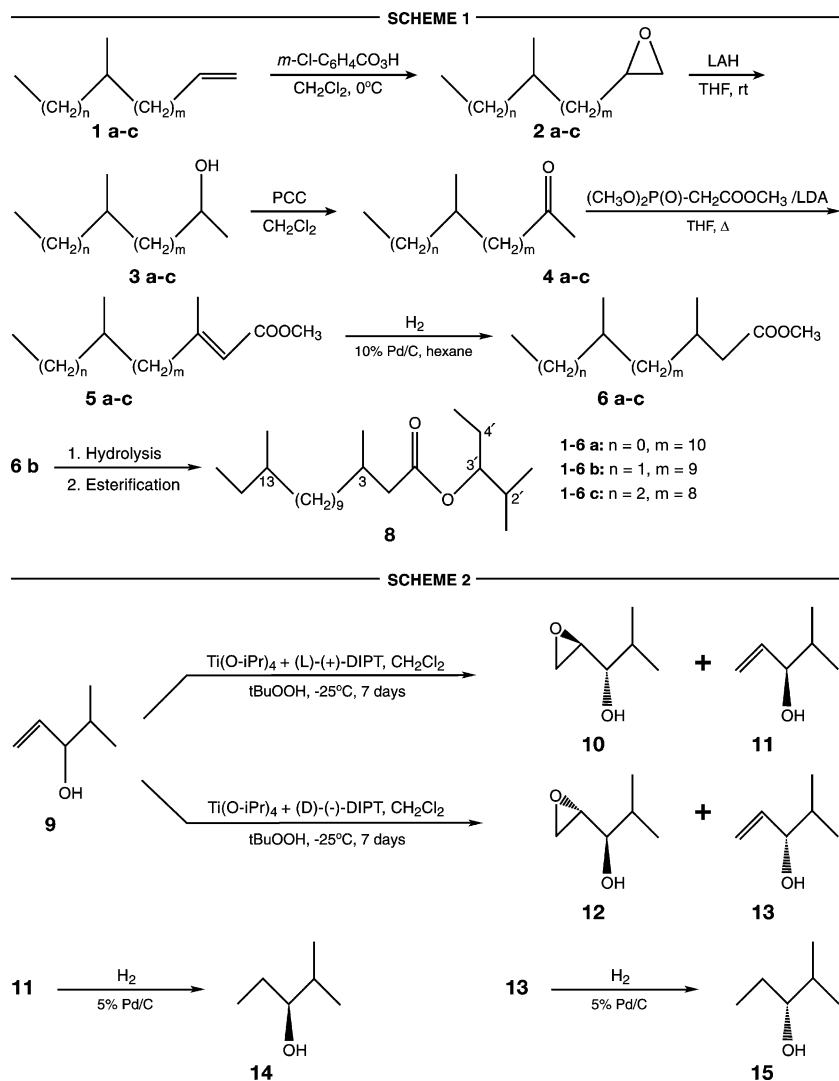


Fig. 1 Schemes for the syntheses of 3,12-, 3,13-, and 3,14-dimethylpentadecanoic acid methyl esters (**6a–c**), 1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate (**8**), and for the syntheses of (3*S*)-2-methyl-3-pentanol (**14**) and (3*R*)-2-methyl-3-pentanol (**15**)

33.85, 37.10, 39.44, 114.07, 139.25. Anal. calcd. for $C_{15}H_{30}$ (%): C, 85.63; H, 14.37; found C, 85.44; H, 13.99.

1,2-Epoxy-13-methyltetradecane (**2a**, Fig. 1, Scheme 1). 3-Chloroperoxybenzoic acid (12.0 g, 52 mmol, 75% pure; Aldrich) was added at 0°C in two portions to **1a** (3.10 g, 14.7 mmol) in dichloromethane (100 ml). The reaction mixture was stirred for 4 hr, warmed to room temperature, and quenched with 150 ml of 2N NaOH. The mixture was extracted with hexane (2 × 100 ml). Extracts were washed twice with water and brine, and dried ($MgSO_4$). After filtration and removal of solvents *in vacuo*, flash chromatography afforded

2.25 g (9.9 mmol, 67% yield) of 1,2-epoxy-13-methyltetradecane (**2a**) that had been previously characterized (Trivedi and Mamdapur, 1986).

1,2-Epoxy-12-methyltetradecane (2b, Fig. 1, Scheme 1). Compound **2b** was synthesized by oxidation of **1b** (3.30 g, 15.8 mmol) with 3-chloroperoxybenzoic acid (6.0 g, 26 mmol, 75% pure) under the same conditions as for **2a**. After work-up and flash chromatography, 3.55 g (99% yield) of **2b** was obtained. $^1\text{H NMR}$ (CDCl_3): δ 0.83 (d, 3H, $J = 6.5$ Hz), 0.85 (t, 3H, $J = 7.3$ Hz), 1.19–1.36 (m, 16H), 1.38–1.48 (m, 3H), 1.52 (m, 2H), 2.45 (dd, 1H, $J = 2.8, 5.1$ Hz), 2.74 (dd, 1H, $J = 4.0, 5.1$ Hz), 2.90 (ddt, 1H, $J = 2.8, 4.0, 5.7$ Hz). $^{13}\text{C NMR}$ (CDCl_3): δ 11.39, 19.20, 25.96, 27.08, 29.43, 29.47, 29.55 (2), 29.66, 29.49, 32.48, 34.37, 36.61, 47.10, 52.37. Anal. calcd. for $\text{C}_{15}\text{H}_{30}\text{O}$ (%): C, 79.58; H, 13.36; found C, 79.50; H, 13.00.

1,2-Epoxy-11-methyltetradecane (2c, Fig. 1, Scheme 1). Compound **2c** was synthesized by oxidation of **1c** (4.00 g, 19.0 mmol) with a 1.5 molar excess of 3-chloroperoxybenzoic acid under the same conditions as for **2a**. Yield of **2c** was 4.20 g (18.6 mmol, 98%). $^1\text{H NMR}$ (CDCl_3): δ 0.83 (d, 3H, $J = 6.6$ Hz), 0.87 (t, 3H, $J = 7.4$ Hz), 1.19–1.36 (m, 16H), 1.39–1.48 (m, 3H), 1.50 (m, 2H), 2.46 (dd, 1H, $J = 2.8, 5.1$ Hz), 2.74 (dd, 1H, $J = 4.0, 5.1$ Hz), 2.90 (ddt, 1H, $J = 2.8, 4.0, 5.7$ Hz). $^{13}\text{C NMR}$ (CDCl_3): δ 14.39, 19.64, 20.19, 25.96, 27.03, 29.44, 29.55, 29.58, 29.96, 32.45, 32.48, 37.09, 39.39, 47.12, 52.41. Anal. calcd. for $\text{C}_{15}\text{H}_{30}\text{O}$ (%): C, 79.58; H, 13.36; found C, 79.81; H, 12.99.

13-Methyl-2-tetradecanol (3a, Fig. 1, Scheme 1). Epoxide **2a** (2.30 g, 10.2 mmol) was slowly added to a stirred suspension of lithium aluminum hydride (LAH; 2.38 g, 70.0 mmol) in THF (150 ml). After 45 min at room temperature, the mixture, with vigorous stirring, was slowly quenched with 2N NaOH until a white precipitate formed on the flask bottom. The organic layer was decanted, the solids were washed with ether (2 \times 75 ml), and the wash was combined with the decanted solution. The mixture was stirred with anhydrous MgSO_4 , filtered, and concentrated to afford 1.70 g (7.40 mmol, 74% yield) of pure **3a**. $^1\text{H NMR}$ (CDCl_3): δ 0.85 (d, 6H, $J = 6.4$ Hz), 1.17 (d, 3H, $J = 6.4$ Hz), 1.22–1.31 (m, 16H), 1.37–1.46 (m, 4H), 1.50 (m, 1H), 3.78 (m, 1H). $^{13}\text{C NMR}$ (CDCl_3): δ 22.63 (2), 23.45, 25.76, 27.40, 27.95, 29.59, 29.61, 29.65 (2), 29.69, 29.92, 39.05, 39.38, 68.14. Anal. calcd. for $\text{C}_{15}\text{H}_{32}\text{O}$ (%): C, 78.87; H, 14.12; found C, 79.12; H, 13.96.

12-Methyl-2-tetradecanol (3b, Fig. 1, Scheme 1). Compound **3b** was obtained from **2b** (3.50 g, 15.5 mmol) by reduction with LAH (3.7 g, 109 mmol) under the same conditions as **3a**. Yield of **3b** was 2.75 g (12.0 mmol, 77%). $^1\text{H NMR}$ (CDCl_3): δ 0.82 (d, 3H, $J = 6.4$ Hz), 0.84 (t, 3H, $J = 7.3$ Hz), 1.17 (d, 3H, $J = 6.2$ Hz), 1.19–1.34 (m, 16H), 1.35–1.46 (m, 4H), 1.51 (m, 1H), 3.77 (m, 1H). $^{13}\text{C NMR}$ (CDCl_3): δ 11.38, 19.19, 23.45, 25.76, 27.08, 29.46, 29.59, 29.61, 29.63, 29.68, 29.99, 34.36, 36.61, 39.35, 68.11. Anal. calcd. for $\text{C}_{15}\text{H}_{32}\text{O}$ (%): C, 78.87; H, 14.12; found C, 78.48; H, 13.79.

11-Methyl-2-tetradecanol (3c, Fig. 1, Scheme 1). Compound **3c** was obtained from **2c** (4.10 g, 18.1 mmol) by reduction with LAH (4.0 g, 126 mmol) under the same conditions as **3a**. Yield of **3c** was 3.61 g (15.8 mmol, 87%). $^1\text{H NMR}$ (CDCl_3): δ 0.83 (d, 3H, $J = 6.6$ Hz), 0.86 (t, 3H, $J = 7.2$ Hz), 1.17 (d, 3H, $J = 6.2$ Hz), 1.19–1.48 (m, 21H), 3.78 (m, 1H). $^{13}\text{C NMR}$ (CDCl_3): δ 14.39, 19.64, 20.12, 23.45, 25.77, 27.04, 29.62, 29.63, 29.65, 29.99, 32.45, 37.06, 39.36, 39.40, 68.14. Anal. calcd. for $\text{C}_{15}\text{H}_{32}\text{O}$ (%): C, 78.87; H, 14.12; found C, 78.83; H, 13.90.

13-Methyl-2-tetradecanone (4a, Fig. 1, Scheme 1). Pyridinium chlorochromate (PCC; 2.13 g, 9.88 mmol) was added in one portion to a stirred solution of **3a** (1.50 g, 6.57 mmol) in dichloromethane (15 ml). The reaction mixture was stirred for 1 hr, then 30 ml of hexane/ether (1:1) were added. Filtering the mixture through silica (15 g) and washing the silica with hexane/ether (100 ml) afforded a clear solution of **4a**. After removal of solvents *in*

vacuo, the yield of **4a** was 1.45 g (6.40 mmol, 97%). ^1H NMR (CDCl_3): δ 0.85 (d, 6H, $J = 6.4$ Hz), 1.21–1.30 (m, 16H), 1.50 (m, 1H), 1.56 (m, 2H), 2.12 (s, 3H), 2.41 (t, 2H, $J = 7.6$ Hz). ^{13}C NMR (CDCl_3): δ 22.64 (2), 23.88, 27.39, 29.18, 29.39, 29.46, 29.59, 29.66, 29.80, 29.91, 39.05, 43.81, 209.24. Anal. calcd. for $\text{C}_{15}\text{H}_{30}\text{O}$ (%): C, 79.58; H, 13.36; found C, 79.84; H, 13.09.

12-Methyl-2-tetradecanone (4b). Compound **4b** was synthesized from **3b** (2.60 g, 11.4 mmol) and PCC (3.69 g, 17.1 mmol). Yield of **4b** was 2.51 g (11.1 mmol, 97%). ^1H NMR (CDCl_3): δ 0.82 (d, 3H, $J = 6.4$ Hz), 0.84 (t, 3H, $J = 7.3$ Hz), 1.19–1.40 (m, 17H), 1.55 (m, 2H), 2.12 (s, 3H), 2.40 (t, 2H, $J = 7.5$ Hz). ^{13}C NMR (CDCl_3): δ 11.38, 19.19, 23.84, 27.06, 29.16, 29.38, 29.45, 29.46, 29.62, 29.81, 29.96, 34.36, 36.60, 43.79, 209.32. Anal. calcd. for $\text{C}_{15}\text{H}_{30}\text{O}$ (%): C, 79.58; H, 13.36; found C, 79.89; H, 13.19.

11-Methyl-2-tetradecanone (4c, Fig. 1, Scheme 1). Compound **4c** was synthesized from **3c** (3.40 g, 14.9 mmol) and PCC (4.83 g, 22.4 mmol). The yield of **4c** was 3.30 g (14.5 mmol, 97%). ^1H NMR (CDCl_3): δ 0.82 (d, 3H, $J = 6.6$ Hz), 0.86 (t, 3H, $J = 7.2$ Hz), 1.19–1.40 (m, 17H), 1.56 (m, 2H), 2.12 (s, 3H), 2.41 (t, 2H, $J = 7.5$ Hz). ^{13}C NMR (CDCl_3): δ 14.39, 19.63, 20.11, 23.85, 27.01, 29.17, 29.39, 29.49, 29.82, 29.92, 32.44, 37.03, 39.38, 43.80, 209.35. Anal. calcd. for $\text{C}_{15}\text{H}_{30}\text{O}$ (%): C, 79.58; H, 13.36; found C, 79.31; H, 13.28.

Methyl 3,14-dimethylpentadecanoate (6a, Fig. 1, Scheme 1). Trimethyl phosphonoacetate (1.10 ml, 7.0 mmol; Aldrich) was added in one portion under argon to a stirred solution (-25°C) of lithium diisopropylamide (LDA; 8.0 mmol; Aldrich) in dry THF (120 ml). The solution was stirred 1 hr, warmed to room temperature, and **4a** (1.40 g, 6.18 mmol) in THF (10 ml) was added. The reaction mixture was refluxed for 4 hr, cooled, quenched with water (150 ml), and extracted (3×75 ml) with hexane/ether (1:1). The combined extracts were washed with water and brine, dried (MgSO_4), and concentrated. The crude mixture (**5a**, 1.3 g), containing >95% unsaturated esters ($E/Z = 3:1$, GC), was hydrogenated for 3 hr with 10% Pd/C catalyst (0.40 g) in hexane under H_2 atmosphere. Filtering and flash chromatography (60 g SiO_2 , 5% ether in hexane as eluent) afforded **6a** (1.20 g, 4.22 mmol, 68% yield based on **4a**). The overall yield of **6a** was 24%. ^1H NMR (CDCl_3): δ 0.85 (d, 6H, $J = 6.6$ Hz), 0.92 (d, 3H, $J = 6.7$ Hz), 1.21–1.32 (m, 20H), 1.51 (m, 1H), 1.94 (m, 1H), 2.10 (dd, 1H, $J = 8.2$, 14.7 Hz), 2.30 (dd, 1H, $J = 6.0$, 14.7 Hz), 3.66 (s, 3H). ^{13}C NMR (CDCl_3): δ 19.72, 22.64 (2), 26.90, 27.41, 27.96, 29.61, 29.63, 29.67, 29.70, 29.74, 29.93, 30.34, 36.72, 39.04, 41.66, 51.31, 173.80. Anal. calcd. for $\text{C}_{18}\text{H}_{36}\text{O}_2$ (%): C, 76.00; H, 12.76; found C, 75.64; H, 12.37.

Methyl 3,13-dimethylpentadecanoate (6b, Fig. 1, Scheme 1). Compound **6b** was synthesized analogous to **6a**, starting with LDA (12 mmol), trimethyl phosphonoacetate (1.45 ml, 10 mmol), and **4b** (1.90 g, 8.40 mmol). The resulting mixture of isomers was hydrogenated with 10% Pd/C catalyst (0.40 g). Yield of **6b** was 1.21 g (4.25 mmol, 51% based on **4b**). Overall yield was 30%. ^1H NMR (CDCl_3): δ 0.83 (d, 3H, $J = 6.5$ Hz), 0.84 (t, 3H, $J = 7.3$ Hz), 0.91 (d, 3H, $J = 6.7$ Hz), 1.18–1.35 (m, 21H), 1.93 (m, 1H), 2.09 (dd, 1H, $J = 8.2$, 14.7 Hz), 2.29 (dd, 1H, $J = 6.0$, 14.7 Hz), 3.65 (s, 3H). ^{13}C NMR (CDCl_3): δ 11.40 (C-15), 19.20 (Me-13), 19.72 (Me-3), 26.90, 27.10 (C-5, C-11), 29.48, 29.62, 29.64, 29.70, 29.73 (C-6 to C-10), 30.01 (C-14), 30.34 (C-3), 34.38 (C-13), 36.63 (C-12), 36.72 (C-4), 41.66 (C-2), 51.30 (OMe), 173.79 (C-1). Anal. calcd. for $\text{C}_{18}\text{H}_{36}\text{O}_2$ (%): C, 76.00; H, 12.76; found C, 75.73; H, 12.74.

Methyl 3,12-dimethylpentadecanoate (6c, Fig. 1, Scheme 1). Compound **6c** was synthesized analogous to **6a**, starting with LDA (21.4 mmol), trimethyl phosphonoacetate (2.60 ml, 18 mmol), and **4c** (3.20 g, 14.1 mmol). The resulting mixture of isomers was hydrogenated with 10% Pd/C catalyst (0.80 g). Yield of **6c** was 2.46 g (8.65 mmol, 61%

yield based on **4c**). Overall yield was 48%. ^1H NMR (CDCl_3): δ 0.83 (d, 3H, $J = 6.6$ Hz), 0.87 (t, 3H $J = 7.2$ Hz), 0.92 (d, 3H, $J = 6.7$ Hz), 1.18–1.41 (m, 21H), 1.93 (m, 1H), 2.10 (dd, 1H, $J = 8.2, 14.7$ Hz), 2.30 (dd, 1H, $J = 6.0, 14.7$ Hz), 3.66 (s, 3H). ^{13}C NMR (CDCl_3): δ 11.40, 19.65, 19.73, 20.13, 26.90, 27.06, 29.63, 29.67, 29.74, 30.03, 30.34, 32.46, 36.72, 37.07, 39.41, 41.67, 51.31, 173.80. Anal. calcd. for $\text{C}_{18}\text{H}_{36}\text{O}_2$ (%): C, 76.00; H, 12.76; found C, 75.84; H, 12.58.

3,13-Dimethylpentadecanoic acid (**7**). Ester **6b** (1.1 g) was stirred overnight in a mixture of ethylene glycol (10 ml), 95% ethanol (10 ml), and KOH (0.5 g). The mixture then was acidified with 10% HCl and extracted three times with ether. The extract was washed with H_2O and brine, dried (MgSO_4), and evaporated to afford crude acid **7** (1.0 g, 95% crude yield).

(3S)- and (3R)-2-Methyl-3-pentanol (**14** and **15**, Fig. 1, Scheme 2). 4-Methyl-1-penten-3-ol (**9**) was synthesized from propenal and isopropylmagnesium bromide (Georgoulis et al., 1984; Harayama et al., 1997). Kinetic resolution of 3.95 g of pure **9** (Martin et al., 1981) with D-(-)- and L-(+)-tartrate reagents [under argon and vigorous stirring at -25°C , racemic **9** (19.7 mmol) in CH_2Cl_2 (5 ml) and *t*-BuOOH (36.0 mmol; 3 M solution in toluene) were added to 150 ml of a solution of $\text{Ti}(\text{O}-i\text{Pr})_4$ (20 mmol) and D-(-)- or L-(+)-diisopropyl tartrate (21 mmol) in CH_2Cl_2 ; reaction mixtures were kept at -25°C for 7 d, followed by usual work-up] led to, respectively, 55% and 53% enantioselective conversion to the epoxides **10** and **12** (Jung and Jung, 1989) and alcohols **11** and **13**. The latter were separated from the epoxides by column chromatography (20% ether in pentane as eluent), and then hydrogenated with 5% Pd/C to (3*S*)- and (3*R*)-2-methyl-3-pentanol **14** and **15** (Grundon et al., 1971) in 0.5 and 0.4 g (25% and 20%) yields, respectively. The enantiomeric purity was determined by converting 10 mg of each alcohol to its corresponding lactate by overnight esterification in ether, using excess of (*S*)-lactic acid chloride in the presence of pyridine at room temperature (Slessor et al., 1985). The diastereomeric excess—monitored by GC (DB-5, 115°C isothermal)—was 98.7% for the ester derived from the *R*-alcohol, and 97.2% for the one derived from the *S*-alcohol.

1-Ethyl-2-methylpropyl 3,13-dimethylpentadecanoate (**8**) (Fig. 1, Scheme 1). A solution of acid **7** (0.5 g) in CH_2Cl_2 (5 ml) was stirred 0.5 hr with trifluoroacetic anhydride (0.45 g; Bolton et al., 1971). Then racemic 2-methyl-3-pentanol (0.2 ml) was added. After 1 hr, the mixture was diluted with ether/hexane (75 ml), washed with water and brine, dried (MgSO_4), and concentrated to yield ester **8** (0.35 g). After purification on silica gel (60 g; 5% ether in hexane as eluent), 100 mg of **8** as a mixture of four diastereomers was obtained. ^1H NMR (CDCl_3): δ ^1H : 4.68 (m, 1H), 2.31 (dd, 1H, $J = 6.1, 14.5$ Hz), 2.11 (dd, 1H, $J = 8.1, 14.5$ Hz), 1.93 (m, 1H), 1.83 (qdd, 1H, $J = 6.7, 6.7, 13.5$ Hz), 1.55 (m, 2H), 1.18–1.35 (m, 21H), 0.94 (d, 3H, $J = 6.7$ Hz), 0.83–0.90 (m, 15H). ^{13}C NMR (CDCl_3 ; carbons numbered as in Fig. 1): δ 9.87/9.88 (C-5'), 11.42 (C-15), 17.63/17.64 (C-1'), 18.59 (Me-2'), 19.22 (Me-13), 19.75/19.77 (Me-3), 23.97 (C-4'), 26.92, 27.11 (C-5, C-11), 29.49, 29.64, 29.71, 29.79 (C-6 to C-10), 30.02 (C-14), 30.39 (C-3), 30.84/30.86 (C-2'), 34.39 (C-13), 36.63 (C-12), 36.72/36.74 (C-4), 42.26 (C-2), 79.40 (C-3'), 173.33 (C-1). Spectral editing by the DEPT pulse sequence confirmed the ^{13}C peak assignments and revealed the presence of diastereomers (two signals for C-5', C-1', Me-3, C-2' and C-4, respectively). Anal. calcd. for $\text{C}_{23}\text{H}_{46}\text{O}_2$ (%): C, 77.90; H, 13.08; found C, 78.05; H, 12.91. Esterifications of acid **7** with optically active alcohols **14** and **15**, respectively, were conducted under the same conditions with comparable yields, and resulted in NMR data consistent with those of racemic ester **8**.

Methyl 3-, 4-, and 14-methylpentadecanoate standards were synthesized as described above by coupling, respectively, 2-tetradecanone, 2-methyltridecanal, and 12-methyltride-

canal with trimethyl phosphonoacetate, and by hydrogenating (H_2 and 10% Pd/C) the resulting unsaturated esters.

Field Experiments

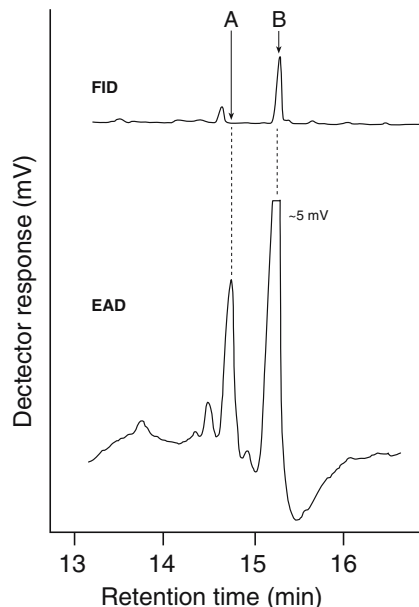
Field experiments were conducted in forests of Yishui County (Shandong Province, P. R. China; 118.64 east longitude and 35.78 north latitude) with 18- to 25-m tall *Paulownia tomentosa* trees. Experiments employed a randomized complete block design with 7–10 replicates each. Delta-like traps were made from 2-l milk cartons (Gray et al., 1984), coated with Tanglefoot (The Tanglefoot Company, Grand Rapids, Michigan, PA, USA), and suspended from trees 15 m above ground at ~30-m spacings. Traps were baited with a gray sleeve stopper (West Pharmaceutical Services, Lionville, PA, USA) impregnated with candidate pheromone components in HPLC-grade hexane. Every day the numbers of moths captured were recorded and treatment positions were re-randomized.

Experiment 1 (6–25 June 1998) consisted of four treatments: (1) racemic 1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate (**8**) (280 μg); (2) (1*S*)-1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate (140 μg); (3) (1*R*)-1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate (140 μg); and (4) an unbaited control. Experiment 2 (31 May to 8 June 2000) tested racemic 1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate at 0, 1, 10, 100, and 1,000 μg doses. Trap catch data were subjected to nonparametric analyses of variance (Friedman's test) followed by comparison of means by the Scheffé test (Zar, 1984; SAS/STAT, 1988). In all analyses, $\alpha = 0.05$.

Results and Discussion

GC-EAD analyses of pheromone gland extracts from female *C. variegata* revealed two components (**A** and **B** in Fig. 2) that elicited strong responses from male *C. variegata*

Fig. 2 Representative recording ($N = 5$) of flame ionization detector (FID) and electroantennographic detector (EAD: male *C. variegata* antenna) responses to one female equivalent of *C. variegata* pheromone gland extract. Chromatography: DB-210 column; 100°C/1 min, 15°C/min to 180°C, then 2°C/min to 220°C; **A** = unknown; **B** = (1*S*)-1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate



antennae. Component **B** appeared to be the major candidate pheromone component because it elicited the strongest responses and occurred in quantities greater than those of **A**. In this manuscript we have focused on the structural elucidation of **B**.

Retention indices (RI, relative to alkane standards; Van den Dool and Kratz, 1963) of **B**, and intercolumn RI differentials of **B** and of known ester pheromones (Table 1), suggested that **B** had an ester functionality. Mass spectral data of **B** in electron impact (Fig. 3) and chemical ionization mode (Tan et al., 1987) supported an ester functionality and disclosed that **B** had an unusually high molecular weight (354) (Tan et al., 1987; molecular ion not visible in Fig. 3) for a (candidate) lepidopteran sex pheromone component. Based on the strong fragment ion m/z 84 (the alkyl portion of the ester), the acylium ion m/z 253, and the m/z 271 ion [the fragment formed by transfer of a hydrogen atom from the alkyl portion to the carbonyl oxygen (McLafferty and Tureček, 1993)], we hypothesized that the ester had 17 carbon atoms in the acyl portion and six carbon atoms in the alkyl portion. We thus esterified (Nieses and Steglich, 1978) heptadecanoic acid with primary, secondary, and tertiary 6-carbon alcohols, including 1-hexanol, 2-hexanol, 3-hexanol, 2-methylpentan-2-ol, 3-methylpentan-2-ol, 4-methylpentan-2-ol, 2-methylpentan-3-ol, 3-methylpentan-3-ol, 2,3-dimethylbutan-2-ol, and 3,3-dimethylbutan-2-ol (all from Sigma-Aldrich). The resulting esters, however, had RIs on all three GC columns that were 70 to 200 RI units higher than those of **B**. Considering that the RIs of methyl-branched molecules are ca. 40–70 units lower than those of straight-chain molecules (Jennings and Shibamoto, 1980), we hypothesized that the acyl portion of **B** must have at least two methyl branches. Considering further that methyl esters, unlike other alkyl esters, are most likely to reveal GC-MS fragment ions indicative of methyl-branch positions in the acyl portion (McLafferty and Tureček, 1993), we decided to isolate **B** by HPLC and to transesterify it to a methyl ester.

Table 1 Retention indices (RI) and intercolumn RI differentials of components **A** and **B** in pheromone gland extracts of female *C. variegata* and of synthetic standards

Compounds	RI on GC column			Intercolumn RI differentials		
	DB-5	DB-23	DB-210	DB-23 vs. DB-5	DB-210 vs. DB-5	DB-210 vs. DB-23
A in Fig. 2 ^a	2256	2495	2522	239	266	27
B in Fig. 2 ^b	2279	2523	2548	244	269	25
1-Ethyl-2-methylpropyl 3,13-dimethylpentadecanoate ^b	2279	2523	2548	244	269	25
10,14-Dimethylpentadecyl isobutyrate ^c	2147	2421	2416	274	269	5
2-Pentyl dodecanoate ^d	1804	2064	2050	260	246	-14
2-Methylbutyl decanoate ^c	1650	1919	1905	269	255	-14
2-Hexyl heptadecanoate ^f	2395	2683	2665	288	270	-28
3-Hexyl 3,14-dimethylpentadecanoate ^f	2279	2516	2528	237	244	12

^a **A** in Fig. 2 = structure unknown.

^b **B** in Fig. 2 = 1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate (**8** in Fig. 1).

^c Sex pheromone component of tea tussock moth, *Euproctis pseudoconspersa* (Wakamura et al., 1994).

^d Sex pheromone component of the bagworm *O. kirbyi* (Rhains et al., 1994).

^e Saturated version of sex pheromone component 2-methylbutyl (*E*)-7,9-decadienoate of nettle caterpillar *Darna trima* (Sasaerila et al., 2000).

^f Available in Gries laboratory from unpublished project.

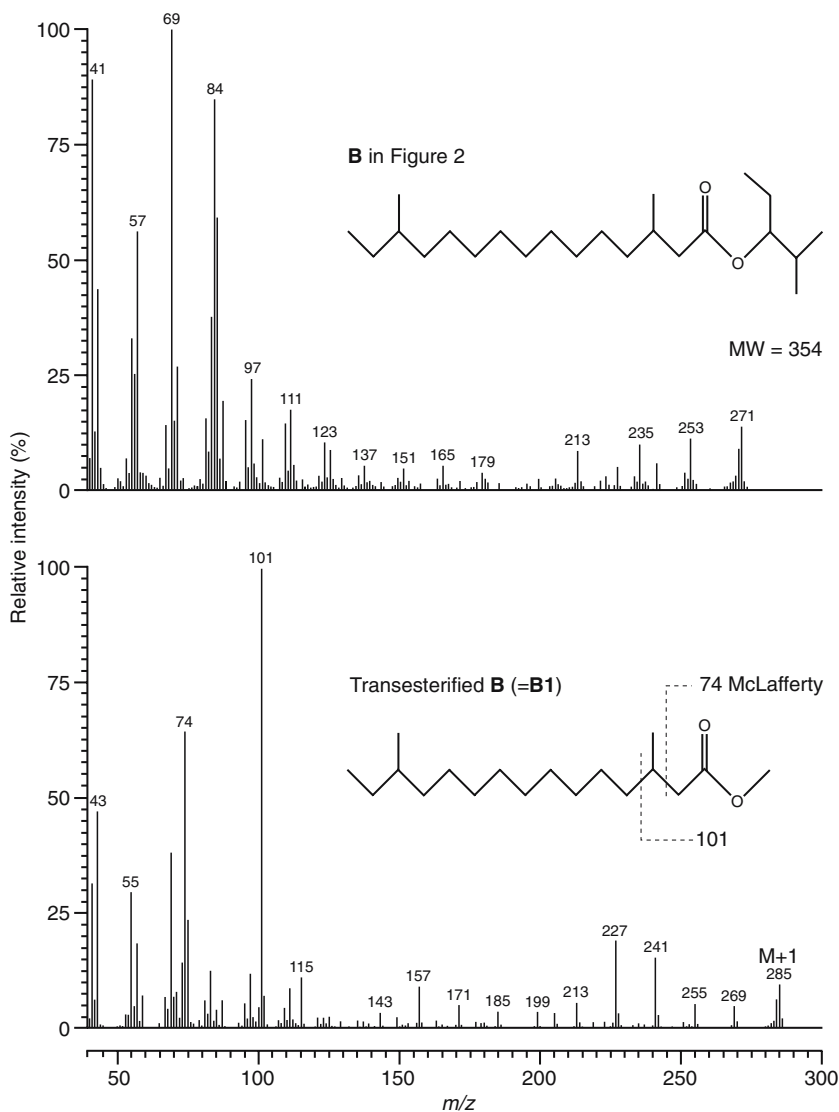


Fig. 3 Ion trap mass spectra (top) of (1*S*)-1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate (**B** in Fig. 2) from pheromone gland extracts of female *C. variegata* and (bottom) of methyl 3,13-dimethylpentadecanoate (**B1**) prepared by transesterification of HPLC-isolated **B**. Fragment ions in **B1** indicate a methyl branch at C3 in the acyl portion of the molecule

GC-MS analysis of the transesterified sample revealed **B** (75%) and a new compound (**B1**) with fragment ions m/z 74, indicative of a methyl ester, and m/z 101, indicative of a methyl branch at C3 (Fig. 3). To infer the second methyl-branch position in **B**, we synthesized and studied the mass spectra of pentadecanoic acid methyl esters with methyl branches near (e.g., C3, C4, C5) or widely separated (e.g., ω -1, ω -2, ω -3) from the carboxylate carbon. Whereas the mass spectrum of methyl 4-methylpentadecanoate indicated a methyl branch at C4 (data not shown), the mass spectrum of methyl 13-

methylpentadecanoate did not reveal the methyl branch at C13 (data not shown). Considering that the mass spectra of **B** and **B1** (Fig. 3) also did not reveal the second methyl branch position, we hypothesized that it might be at ω -1, ω -2, or ω -3.

We thus synthesized 3,12-, 3,13-, and 3,14-dimethylpentadecanoic acid and esterified each of the three acids (scheme 1 in Fig. 1) with each of ten 6-carbon alcohols (see above). Of all the resulting esters, only 1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate had mass spectral and GC retention characteristics (diastereomers not resolved on DB-5, DB-23, or DB-210 columns) entirely consistent with **B** in pheromone gland extracts. Moreover, synthetic 1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate and **B** elicited similar antennal responses from male antennae in GC-EAD analyses (data not shown). Finally, identical GC and GC-MS characteristics of methyl 3,13-dimethylpentadecanoate and of transesterified **B1** provided supporting evidence for the correct identification of **B**.

In field experiment 1, traps baited with (1*S*)-1-ethyl-2-methylpropyl 3,13-dimethylpentadecanoate attracted male moths, whereas the (1*R*)-stereoisomer was neither attractive by itself, nor did it reduce attractiveness of the (1*S*)-isomer in the racemic mixture (Fig. 4). In field experiment 2, only traps baited with the highest dose of racemic pheromone captured male moths (a total of 17).

Consistent with other psychid moths (Leonhardt et al., 1983; Rhainds et al., 1994; Subchev et al., 2000), female *C. variegata* produce an ester sex pheromone. This ester, however, has a molecular weight (MW = 354) 84–214 daltons heavier than that of pheromone components in congeners. Indeed, to our knowledge only three sex pheromone components identified to date in the Lepidoptera have higher molecular weights (Arn et al., 1992; El-Sayed 2005). (*Z,Z,Z*)-11,14,17-Icosatrienyl 4-methylvalerate (MW 390) and (*Z,Z,Z*)-11,14,17-icosatrienyl isobutyrate (MW 362) synergistically attract male *Euproctis pulverea* (Lymantriidae) (Wakamura et al., 2001), and (*Z,Z,Z,Z*)-7,13,16,19-docosatetraen-1-ol isobutyrate (MW 404) attracts male *E. chrysorrhoea* (Lymantriidae) (Leonhardt et al., 1991). Interestingly, these three pheromone components are also esters.

To fully characterize the sex pheromone blend of *C. variegata*, the molecular structure of candidate pheromone component **A** (Fig. 2) needs to be identified. Similar intercolumn RI differentials of **A** and **B** (Table 1), coupled with lower RIs of **A** than **B**, suggested that **A** might be another ester with lower molecular weight, possibly 340. Moreover, the relative and absolute configurations at the additional chiral centers C3 and C13 of the acyl portion of **B** need to be determined. Conceivably, any of the eight possible stereoisomers of **B** could be pheromone components, or pheromone inhibitors. Should the synthetic material field-

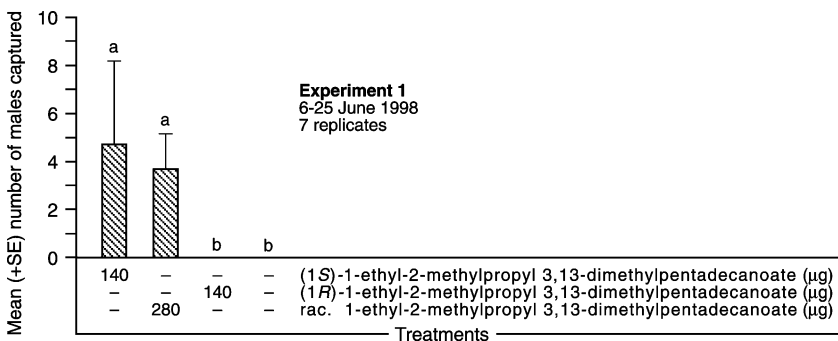


Fig. 4 Mean captures per night of male *C. variegata* in field experiment 1 in sticky traps baited with racemic or optically active candidate pheromone components, Yu Shan Forest Farm, Yu Shui County, Shandong Province, P. R. China. Bars with different letter superscripts are significantly different, $\alpha = 0.05$

tested in experiments 1 and 2 have contained one or more inhibitors, it might explain the relatively moderate trap captures. Alternatively, component **A** in Fig. 1 may be an important synergist that is required in the pheromone blend for strong attraction of male *C. variegata*.

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Characterization of a Female-Produced Courtship Pheromone in the Parasitoid *Nasonia vitripennis*

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Abstract Males of the parasitoid *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) show a characteristic courtship behavior. We demonstrate that male arrestment and key behavioral elements of the courtship sequence are mediated by a female-derived contact sex pheromone. Males were arrested on paper disks treated with female extracts but not on those treated with male extracts. Male responsiveness was influenced by the surface to which female extracts were applied. Extracts applied to an extracted beetle elytron arrested males more strongly than those applied to filter paper of comparable size. However, more complex behavioral elements, such as head nodding and copulation attempts, were shown only when extracts were applied to extracted male cadavers, suggesting that tactile or visual cues synergize the male response. The chemicals involved are stable, of low volatility, and nonpolar. Dead females arrested males and elicited courtship behavior for at least 8 d. Males showed no sign of attraction to live females at a distance of 3 cm in an olfactometer. Fractionation of female extracts demonstrated that the activity was exclusively located in the nonpolar fraction. Analysis of the active fraction by gas chromatography–mass spectrometry revealed that cuticular hydrocarbons with chain lengths between 25 and 37 carbon units were present. Comparison of hydrocarbon profiles from males and females showed qualitative and quantitative differences. These results suggest that sex-specific cuticular hydrocarbons are the key signals mediating the male courtship behavior in *N. vitripennis*.

Keywords Parasitoid · *Nasonia vitripennis* · Pteromalidae · Sex pheromone · Courtship behavior · Cuticular hydrocarbons

Introduction

Sex pheromones are assumed to play a major role in the sexual communication of parasitic wasps (Godfray, 1994; Quicke, 1997). Depending on the function of the

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pheromones, the range of activity varies: highly volatile compounds released by females are used by males for long-range orientation during mate finding (Eller et al., 1984; Swedenborg and Jones, 1992a,b; Swedenborg et al., 1994; McNeil and Brodeur, 1995; Jewett and Carpenter, 1999), whereas chemicals of relatively low volatility mediate male courtship behavior at close range (Simser and Coppel, 1980; Mohamed and Coppel, 1987; Shu and Jones, 1993; Syvertsen et al., 1995; Sullivan, 2002). Sex pheromones have been demonstrated in several families of the parasitic Hymenoptera, e. g., in Braconidae, Chalcididae, and Ichneumonidae (reviewed by Kainoh, 1999). Within the Pteromalidae, little is known. The sex pheromones hitherto demonstrated in this taxon are exclusively those eliciting courtship behavior at close range (van den Assem and Povel, 1973; Yoshida, 1978; Ruther et al., 2000; Sullivan, 2002). Studies on *Roptrocercus xylophagorum* (Sullivan, 2002) and *Lariophagus distinguendus* (Steiner et al., 2005) indicated that the active compounds are cuticular hydrocarbons. Interestingly, the female sex pheromone of *L. distinguendus* is already present in pupae of both sexes. However, males actively decompose the chemicals involved immediately after emergence (Steiner et al., 2005).

Nasonia vitripennis (Walker) (Hymenoptera: Pteromalidae) is a gregarious parasitoid attacking pupae of several fly species, including blowflies, flesh flies, and houseflies. The parasitoid has been intensely investigated in studies addressing genetic (Reed, 1993), ecological (King and D'Souza, 2004), behavioral (Baeder and King, 2004), developmental (Rivers et al., 1999), and evolutionary (van den Assem and Jachmann, 1982) aspects of its biology. As with many other parasitoids, the mating system of *N. vitripennis* is characterized by protandry, i.e., males emerge first and females follow shortly after. When male density is low, single dominant individuals aggressively defend the escape hole in the host puparium against competitors to ensure that they mate with the emerging females. Inferior males may wander away and find other puparia in the vicinity. At high density, however, competition degenerates into scramble competition, and the territorial system breaks down (van den Assem et al., 1980a).

Males of *N. vitripennis* perform a characteristic courtship behavior that has been studied in detail (Barras, 1960; van den Assem and Vernel, 1979; van den Assem and Werren, 1994). When encountering a female, the male immediately mounts. Subsequently, the male shows regular movements of the wings. These movements are slight lifts rather than the high-frequency wing-fanning as described from other pteromalids (e.g., Ruther et al., 2000). Wing movements may be accompanied by a characteristic head nodding consisting of rapid raising and lowering of the head in combination with chewing movements of the mouthparts. It has been hypothesized that head nodding serves to transfer a male aphrodisiac to the female (van den Assem et al., 1980b). As a result of the male courtship behavior, the female signals sexual receptivity by lowering her antennae and synchronously raising the abdomen to expose her genitalia. Subsequently, copulation may occur. It has been suggested that male courtship behavior in *N. vitripennis* is induced by a female-derived sex pheromone (van den Assem et al., 1980b), but the chemicals involved are unknown.

The present study was initiated to investigate more thoroughly the role of female sex pheromones in the sexual communication of *N. vitripennis*. We characterized the chemicals involved by studying their longevity, range of activity, and polarity. We also analyzed fractions eliciting behavioral activity. Finally, we investigated the possible influence of additional physical cues on male responsiveness and studied whether the pheromone is already present in the pupal stage.

Methods and Materials

Insect Rearing

Laboratory cultures were kept at 25°C and 60% relative humidity with a daily light/dark cycle of 16:8 hr. Thirty to 40 freshly emerged *N. vitripennis* were placed in petri dishes with 50 freeze-killed puparia of the green bottle fly *Lucilia caesar* and kept there until their death. After a development time of 14–15 d, adult parasitoids from the next generation emerged and were collected every second day.

General Methods for Experiments

To obtain virgin individuals for bioassays, parasitoid pupae were removed from host puparia 1–2 d before eclosion and sexed in the pupal stage according to the different-sized wing pads (van den Assem and Jachmann, 1999). Pupae were kept individually in 1.5-ml microcentrifuge tubes until emergence.

Freshly emerged parasitoids were held for 1–2 d in single-sex groups of 10–15 individuals in petri dishes lined with moistened filter paper and provided with one split raisin. One hr before experiments, parasitoids were transferred individually into microcentrifuge tubes.

All experiments except for experiment 4 were performed in a bioassay chamber (10 mm diam × 3 mm high) described elsewhere (Ruther et al., 2000). Behaviors were observed with a stereo microscope with illumination from a microscope light and recorded using The Observer 3.0 computer software (Noldus Information Technology, Wageningen, the Netherlands). Parasitoids were used only once in bioassays.

Experiment 1: Activity of Pheromone Extracts

Ten freshly emerged females were killed by freezing (1 hr, -20°C) and extracted for 30 min with 60 µl dichloromethane at room temperature. The resulting extracts were concentrated to one-third individual equivalent per microliter under nitrogen. Two female equivalents of the extract were applied on a filter paper disk (diam 5 mm, Melitta, Germany), and the solvent was allowed to evaporate for 15 min. The paper disk was offered to a male in the observation chamber, and arrestment time on the disk as well as key elements of the courtship sequence (head nodding, copulation attempts) were recorded for 5 min. Paper disks treated with pure solvent or male extract were used as controls ($N = 15$ for each treatment). Extract samples were changed after every five parasitoids.

Experiment 2: Influence of the Release Matrix on Male Courtship Behavior

This experiment was carried out to study whether male responsiveness was influenced by the matrix to which female extracts were applied. Extracts were prepared as described in experiment 1 and applied (two female equivalents) to (1) a piece (3 × 2 mm) of filter paper, (2) a piece (3 × 2 mm) of a solvent extracted elytra of the garden chafer *Phyllopertha horticola* (model insect cuticle), or (3) a solvent-extracted dead *N. vitripennis* male. Treated objects and solvent-treated control objects were presented to a male and arrestment time, head-nodding behavior, and copulation attempts were recorded for 5 min ($N = 25$ for each treatment).

Experiment 3: Stability of the Biologically Active Chemicals

This experiment investigated the time that dead females remained active to verify literature data suggesting that activity ceases within 2 d. For this purpose, freshly emerged females were killed by freezing and stored throughout the test period at a constant temperature of 25°C and a relative humidity of 60%. The cadaver was presented daily to a male during the first 6 d and finally 8 d after killing. Arrestment time on the cadavers, head-nodding behavior, and copulation attempts were recorded for 5 min. Solvent-extracted females were used as controls ($N = 10$ for each treatment).

Experiment 4: Test for Attraction of Males to Females

This experiment was performed in a static four-chamber olfactometer, which has been used successfully to study the long-range orientation of pteromalids (Steidle and Schöller, 1997; Ruther and Steidle, 2000). No airflow was generated. Ten freshly emerged virgin *Nasonia* females were placed in a petri dish (5.5 cm diam \times 1.2 cm high) covered with gauze (mesh 0.5 mm) and transferred into one chamber of the olfactometer. The remaining three chambers contained empty petri dishes as controls. The olfactometer was covered with a walking arena made from gauze (distance to the volatile source: 3 cm) and finally with a glass plate to prevent test animals from escaping. The olfactometer was placed on the bottom of a white bucket (29 cm diam, 36 cm high) and illuminated from above (2050 lux). A single male was introduced in the center of the walking arena, and the time the parasitoid spent in the area above the four chambers was recorded for 10 min. Males that rested more than 5 min of the total observation time were assumed to be unmotivated and excluded from statistical analysis. Virgin females were replaced after every five males ($N = 22$).

Experiment 5: Production of the Sex Pheromone During Pupal Development

To investigate whether *N. vitripennis* begins producing the sex pheromone during pupal development as shown for other pteromalids (Yoshida, 1978; Steiner et al., 2005), male and female pupae were dissected from host puparia and classified according to pigmentation. Behavioral responses of adult males to single male and female pupae from three stages (yellowish pupae, pupae with red mouthparts, and totally melanized pupae) were observed for 5 min in a bioassay chamber. Extracted male and female pupae were used as controls. Pupae used as odor sources were tested only once ($N = 20$ for each pupal stage of both sexes).

Experiment 6: Activity of Pheromone Fractions

Dichloromethane extracts of *N. vitripennis* females were fractionated by liquid chromatography, and the resulting fractions were bioassayed. Batches of 10 freshly emerged females were extracted with 60 μ l dichloromethane as described in experiment 1. The extracts were concentrated to 15 μ l under nitrogen, applied to 25 mg silica gel cartridges for solid phase extraction (IST, Mid-Glamorgan, UK), and eluted sequentially with 120 μ l each of hexane, 10% dichloromethane in hexane, dichloromethane, and methanol. Four female equivalents of the fractions were applied onto extracted male cadavers, and the behavioral responses of males (arrestment time, head nodding, and copulation attempts) to the treated dead males were recorded for 5 min ($N = 27$ for each fraction). Extracted male cadavers treated with pure solvent were used as controls ($N = 24$ for each fraction).

Chemical Analysis

Hexane fractions from females and males ($N = 7$ for each sex) were prepared as described in experiment 6 and analyzed by coupled gas chromatography–mass spectrometry (GC–MS) on a Fisons GC 8060 with a Fisons MD 800 quadrupole MS (Thermo Finnigan, Egelsbach, Germany). Analytical conditions were as follows: injector temperature 240°C, column 30 m \times 0.32 mm ID DB-5 ms, film thickness 0.25 μ m (J & W Scientific, Folsom, CA, USA), carrier gas: helium, and inlet pressure 10 kPa. The temperature program started at 150°C and increased 2°C/min to 280°C. One microliter of each fraction, representing 0.5 wasp equivalent, was injected together with 25 ng of tetracosane as an internal standard. Linear retention indices of methyl-branched and unsaturated hydrocarbons were calculated by coinjection of straight-chain hydrocarbons. Methyl-branched hydrocarbons were identified by diagnostic ions resulting from the favored fragmentation at the branching points (Lockey, 1988; Nelson, 1993) and by comparing linear retention index values with literature data (Carlson et al., 1998). Position of the double bonds of unsaturated hydrocarbons was determined by iodine-catalyzed methylthiolation using dimethyl disulfide (Francis and Velant, 1981; Howard, 1993). Peak areas for each compound were calculated and related to the total peak area for each run.

Statistical Analysis

Statistical analyses were performed with Statistica 4.5 scientific software (StatSoft, Hamburg, Germany). Mean arrestment times of males were analyzed by Kruskal–Wallis H test followed by multiple U tests for individual comparisons. The time parasitoids spent in the areas above the four chambers of the olfactometer (experiment 4) were analyzed by a Friedman analysis of variance. Number of males responding by head nodding and copulation attempts to different odor sources were compared by using a $2 \times 2 \chi^2$ test. Relative abundances of hydrocarbons in male and female hexane fractions were compared by a Mann–Whitney U test. Whenever multiple comparisons were carried out during statistical analysis, a sequential Bonferroni correction was applied (Sachs, 1992).

Results

Experiment 1: Activity of Pheromone Extracts

Males spent more time on filter paper disks treated with two equivalents of a female extract than on disks treated with male extract or pure solvent (Fig. 1). Apart from this arrestment, however, no other behaviors related to courtship (e.g., head nodding or copulation attempts) were observed. Paper disks treated with male extracts did not cause arrestment in responding males when compared with solvent controls.

Experiment 2: Influence of the Release Matrix on Male Courtship Behavior

Males were arrested on all objects treated with female extracts when compared with controls (Fig. 2). However, arrestment on treated beetle elytra was stronger than on filter paper of the same size. Male cadavers treated with female extracts arrested males more strongly than filter paper, whereas the difference between beetle elytra and male cadaver

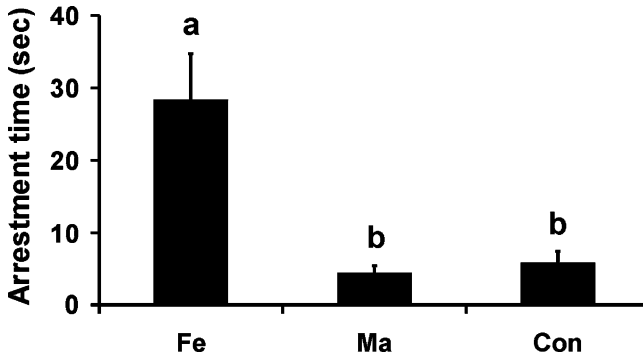


Fig. 1 Mean arrestment time (\pm SE) of *N. vitripennis* males on differently treated paper disks (Fe: female extract, Ma: male extract, Con: solvent control) during a 5-min observation period. Bars with different lowercase letters are significantly different at $P < 0.05$ (Kruskal–Wallis H test followed by multiple U tests; $N = 15$)

was not significant ($U = 222$, $P = 0.079$). When considering more complex behavioral elements such as head nodding and copulation attempts, only treated cadavers released male responses (head nodding: 28% response, $\chi^2 = 8.14$, $P = 0.004$; copulation attempts: 48% response, $\chi^2 = 12.58$, $P < 0.001$).

Experiment 3: Stability of the Biologically Active Chemicals

Females killed by freezing shortly after emergence and stored at 25°C and 60% relative humidity elicited responses from males for several days. During the first 8 d, males spent significantly more time on female cadavers when compared with controls (Fig. 3A). Head-nodding behavior and copulation attempts were elicited up to the sixth day of the test period (Fig. 3B–C). This shows that the bioactive chemicals are relatively stable.

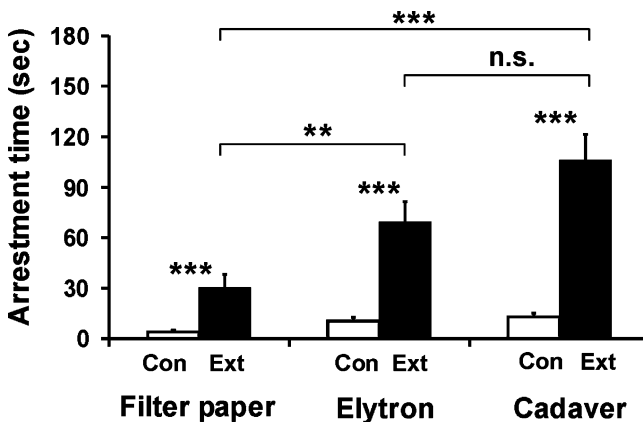


Fig. 2 Mean arrestment time (\pm SE) of *N. vitripennis* males on differently treated paper disks, extracted beetle elytra (model insect cuticle), and extracted male cadavers of *N. vitripennis* (Con: solvent control, Ext: treated with two equivalents of a female extract) during a 5-min observation period. Asterisks indicate significant differences (*** $P < 0.001$, ** $P < 0.01$, n.s.: not significant, Kruskal–Wallis H test followed by multiple U tests, $N = 25$)

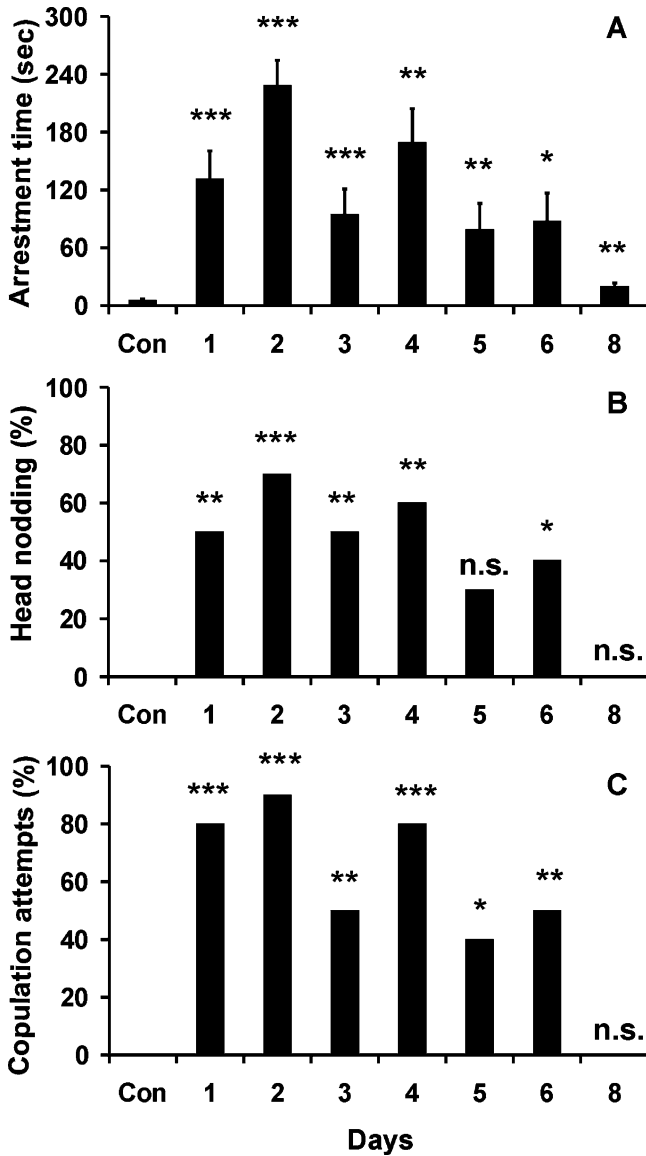


Fig. 3 Response of *N. vitripennis* males to female cadavers presented 1–6 and 8 d after killing. (A) Mean arrestment time (\pm SE). (B) Percentages of males showing head nodding. (C) Percentages of males showing copulation attempts. Asterisks indicate significant preferences for female cadavers when compared with control (***) $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s.: not significant). Mean arrestment times were compared by multiple Mann–Whitney U tests; head-nodding and copulation behavior was analyzed by $2 \times 2 \chi^2$ tests ($N = 10$)

Experiment 4: Test for Attraction of Males to Females

In the four-chamber olfactometer, males did not spend more time in the test field above the 10 virgin females than in the control fields ($\chi^2 = 4.087$, $P = 0.252$). This result indicates that females of this species do not produce a volatile sex pheromone that attracts males from a distance.

Experiment 5. Production of the Contact Sex Pheromone During Pupal Development

Male and female pupae of the three different stages elicited few behavioral responses from males. Males spent more time on totally melanized female pupae when compared with extracted female control pupae (Fig. 4). However, the arrestment time on pupae was smaller when compared with fully developed dead females (Fig. 3A). The arrestment on totally melanized male pupae did not differ significantly from the time spent on extracted male control pupae. Regardless of the sex, yellowish pupae and pupae with red mouthparts did not cause arrestment in responding males. Although head nodding was shown once and copulation attempts in two cases toward totally melanized pupae of either sex, these differences were not significant when compared with the respective control pupae, which elicited no responses at all (head nodding: 5%, $\chi^2 = 1.030$, $P = 0.311$; copulation attempts: 10%, $\chi^2 = 2.110$, $P = 0.147$).

Experiment 6: Activity of Pheromone Fractions

Only the hexane fraction of female extracts elicited arrestment and courtship behavior (head nodding and copulation attempts) in males when applied to extracted male cadavers. The other fractions did not elicit behavioral responses (Fig. 5A–C). This shows that the arrestant pheromone is comprised of nonpolar compounds.

Chemical Analysis

Sixty-seven straight-chain, methyl-branched, or monounsaturated hydrocarbons were identified in the hexane fractions of *N. vitripennis* (Table 1). The chain length varied

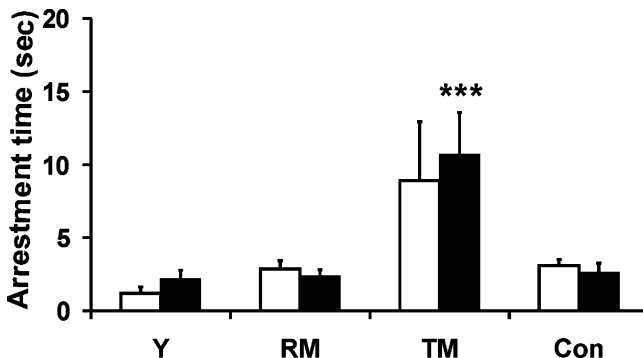


Fig. 4 Mean arrestment time (\pm SE) of adult *N. vitripennis* males on female (black bars) and male pupae (white bars) from different stages (Y: yellowish pupae, RM: pupae with red mouthparts, TM: totally melanized pupae, Con: extracted melanized pupae as control). Asterisks indicate significantly increased arrestment compared with control at $P < 0.001$ (Mann–Whitney *U* test, $N = 20$)

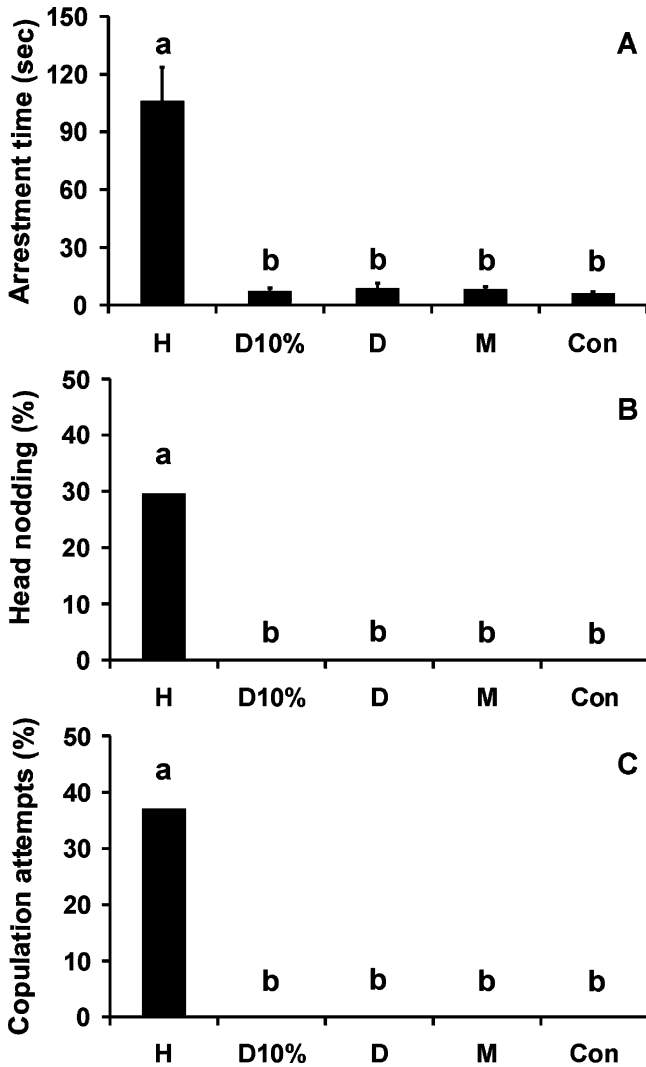


Fig. 5 Response of *N. vitripennis* males to extracted male cadavers treated with different fractions of female extracts (H: hexane, D10%: 10% dichloromethane in hexane, D: dichloromethane, M: methanol, Con: solvent control). (A) Mean arrestment time (\pm SE). (B) Percentages of males showing head nodding. (C) Percentages of males showing copulation attempts. Different lowercase letters indicate significant preferences at $P < 0.05$ (mean arrestment times were compared by Kruskal–Wallis H test followed by multiple U tests; head-nodding and copulation behavior was analyzed by multiple χ^2 tests; $N = 27$)

between 25 and 37 carbon units. Most compounds occurred both in male and female parasitoids, but in most cases the relative abundances of the individual hydrocarbons were substantially different between sexes. In both sexes, the majority (>80%) of hydrocarbons consisted of methyl-branched alkanes with one to four methyl groups. All compounds eluting before triacontane (peak 14) occurred in higher relative amounts in the hexane fractions from females than in those from males. The most striking sex-related differences among later-eluting hydrocarbons were found in homologous series of monomethylalkanes

Table 1 Relative composition of hexane fractions from freshly emerged *N. vitripennis* females and males

No.	LRI	Compound	Diagnostic ions	Female ^a	Male ^a	<i>P</i> level ^b
1	2500	C25	352	0.20 ± 0.04^c	0.06 ± 0.03 ^c	0.013
2	2551	5-MeC25	351 (M-15), 84/85, 308/309	0.17 ± 0.03^c	0.01 ± 0.01 ^c	0.001
3	2700	C27	380	0.48 ± 0.07	0.17 ± 0.09 ^c	0.025
4	2751	5-MeC27	379 (M-15), 84/85, 336/337	0.18 ± 0.03^c	0.01 ± 0.01 ^c	0.002
5	2773	3-MeC27	365 (M-29)	0.15 ± 0.02^c	0.02 ± 0.01 ^c	0.002
6	2800	C28	394	0.07 ± 0.01	0.02 ± 0.01	0.048
7	2900	C29	408	3.6 ± 0.4	1.1 ± 0.2	0.002
8	2931	13-MeC29	407 (M-15), 196/197, 252/253	0.3 ± 0.03	0.02 ± 0.01 ^c	0.001
	2933	+11-MeC29	407 (M-15), 168/169, 280/281			
9	2936	9-MeC29	407 (M-15), 140/141, 308/309	0.30 ± 0.04	0.02 ± 0.01 ^c	0.002
10	2941	7-MeC29	407 (M-15), 112/113, 336/337	2.2 ± 0.2	0.60 ± .12	0.002
11	2951	5-MeC29	407 (M-15), 84/85, 364/365	0.40 ± 0.03	0.15 ± 0.02	0.002
12	2973	3-MeC29	393 (M-29)	0.21 ± 0.02	0.04 ± 0.02	0.004
13	2981	5,17-DiMeC29	421 (M-15), 84/85, 379, 196/197, 266/267	0.17 ± 0.02^c	0.06 ± 0.02 ^c	0.009
14	3000	C30	422	0.58 ± 0.05	0.27 ± 0.04	0.003
15	3041	7-MeC30	421 (M-15), 112/113, 350/351	0.26 ± 0.01	0.38 ± 0.04	0.018
16	3075	C31:1(9)	528, 173, 355 (DMDS)	0.22 ± 0.01 ^c	1.1 ± 0.18^c	0.002
17	3084	C31:1(7)	528, 145, 383 (DMDS)	0.00	0.16 ± 0.03^c	0.007
18	3100	C31	436	11.5 ± 1.1	10.5 ± 0.8	0.22
19	3128	15-MeC31	435 (M-15), 224/225, 252/253	6.2 ± 0.3	0.58 ± 0.22	0.002
	3129	+13-MeC31	435 (M-15), 196/197, 280/081			
	3132	+11-MeC31	435 (M-15), 168/169, 308/309			
	3135	+9-MeC31	435 (M-15), 140/141, 336/337			
20	3141	7-MeC31	435 (M-15), 112/113, 364/365	7.8 ± 0.2	21.7 ± 1.0	0.002
21	3150	5-MeC31	435 (M-15), 84/85, 392/393	2.5 ± 0.1	6.3 ± 0.4	0.002
22	3157	11,15-DiMeC31	449 (M-15), 168/169, 322/323, 238/239, 252/253	0.90 ± 0.07	0.00	0.002
		+13,17-DiMeC31	449 (M-15), 196/197, 294/295, 266/267, 224/225			
23	3163	9,21-DiMeC31	449 (M-15), 140/141, 350/351, 322/323, 168/169	0.81 ± 0.06^c	0.00	0.002
24	3168	7,11-DiMeC31	449 (M-15), 112/113, 378/379, 182/183, 308/309	1.2 ± 0.09^c	0.72 ± 0.16 ^c	0.013
25	3174	3-MeC31	421 (M-29)	3.2 ± 0.3	2.7 ± 0.15	0.11
26	3181	5, <i>x</i> -DiMeC31	449 (M-15), 84/85, 406/407	0.97 ± 0.11 ^c	3.2 ± 0.2^c	0.002
		+7, <i>x</i> -DiMeC31	449 (M-15), 112/113, 378/379			
27	3191	unknown HC		0.34 ± 0.04	0.60 ± 0.05	0.003
28	3201	3,15-DiMeC31	449 (M-15), 435 (M-29), 238/239, 252/253	0.94 ± 0.04	0.38 ± 0.04 ^c	0.002
29	3208	3,7-DiMeC31	449 (M-15), 435 (M-29), 126/127, 364/365, 435	0.31 ± 0.02	0.72 ± 0.04	0.002
30	3229	3,11,19-TriMeC31	449 (M-29), 182/183, 322/323, 252/253	1.00 ± 0.03^c	0.52 ± 0.02 ^c	0.0012
		+3,11,15-TriMeC31	449 (M-29), 182/183, 322/323, 308/309, 196/197			
	3234	+3,7,11-TriMeC31	463 (M-15), 449 (M-29), 126/127; 378/379; 196/197; 308/309			

Table 1 (continued)

No.	LRI	Compound	Diagnostic ions	Female ^a	Male ^a	<i>P</i> level ^b
31	3245	6-MeC32	449 (M-15), 98/99, 392/393	0.12 ± 0.01 ^c	0.41 ± 0.04^c	0.002
32	3258	3,7,11,15-TetraMeC31	477 (M-15), 463 (M-29), 126/127, 393, 196/197, 323, 266/267, 252/253	1.3 ± 0.08	0.08 ± 0.01 ^c	0.0012
33	3277	C33:1(9)	556,173, 383 (DMDS)	0.09 ± 0.02 ^c	0.62 ± 0.04^c	0.002
34	3285	C33:1(7)	556,145, 411 (DMDS)	0.25 ± 0.02 ^c	0.54 ± 0.04^c	0.002
35	3300	C33	464	0.61 ± 0.07 ^c	0.88 ± 0.08^c	0.025
36	3327	15-MeC33	463 (M-15), 224/225, 280/281	8.5 ± 0.3	3.6 ± 0.2	0.002
	3328	+13-MeC33	463 (M-15), 196/197, 308/309			
	3331	+11-MeC33	463 (M-15), 168/169, 337/338			
37	3340	7-MeC33	463 (M-15), 112/113, 392/393	0.99 ± 0.07	3.9 ± 0.1	0.002
38	3350	5-MeC33	463 (M-15), 84/85, 420/421	1.1 ± 0.1 ^c	1.3 ± 0.1 ^c	0.34
39	3356	11,15-DiMeC33	477 (M-15), 168/169, 350/351, 239/240, 280/281	9.7 ± 0.2	1.7 ± 0.2	0.002
		+13,17-DiMeC33	477 (M-15), 196/197, 322/323, 266/267, 252/253			
		+15,19-DiMeC33	477 (M-15), 224/225, 294/295			
	3360	+11,21-DiMeC33	477 (M-15), 168/169, 3 50/351, 196/197, 322/323			
40	3368	7,19-DiMeC33	477 (M-15), 112/113, 406/407, 224/225, 294/295	3.7 ± 0.1	8.3 ± 0.5	0.002
	3372	+7,23-DiMeC33	477 (M-15), 112/113, 406/407,168/169, 351/352			
41	3380	5 _x -DiMeC33	477 (M-15), 84/85, 434/435	2.8 ± 0.1 ^c	5.8 ± 0.3^c	0.002
42	3388	11,15,23-TriMeC33	491 (M-15), 168/169, 364/365	1.4 ± 0.1	1.0 ± 0.1	0.025
43	3393	unknown HC		1.1 ± 0.1	0.33 ± 0.12	0.004
44	3405	3,17-DiMeC33	477 (M-15), 463 (M-29), 308/309, 238/239	0.00	1.10 ± 0.1^c	0.002
		+3,15-DiMeC33	477 (M-15), 463 (M-29), 280/281, 266/267			
45	3460	3,7,11,15-TetraMeC33	505 (M-15), 491 (M-29), 126/127, 421, 196/197, 351, 266/267, 280/281	1.6 ± 0.1	0.41 ± 0.05 ^c	0.002
46	3530	17-MeC35	491 (M-15), 252/253, 280/281	3.4 ± 0.1	1.8 ± 0.1	0.002
	3531	+15-MeC35	491 (M-15), 224/225, 308/309			
	3532	+13-MeC35	491 (M-15), 196/197, 336/337			
	3533	+11-MeC35	491 (M-15), 168/169, 364/365			
47	3542	7-MeC35	491 (M-15), 112/113, 420/421	0.08 ± 0.02	0.47 ± 0.03	0.002
48	3554	15,19-DiMeC35	505 (M-15), 224/225, 322/323, 294/295, 252/253	8.5 ± 0.2	2.0 ± 0.1	0.002
	3556	+13,17-DiMeC35	505 (M-15), 196/197, 350/351, 266/267, 280/281			
		+11,15-DiMeC35	505 (M-15), 168/169, 378/379, 238/239, 308/309			
49	3569	7,15-DiMeC35	505 (M-15), 112/113, 435/436, 280/281, 238/239	1.5 ± 0.1	6.9 ± 0.3	0.002
		+7,19-DiMeC35	505 (M-15), 112/113, 435/436, 252/253, 294/295			
		+7,23-DiMeC35	505 (M-15), 112/113, 435/436, 196/197, 350/351			

Table 1 (continued)

No.	LRI	Compound	Diagnostic ions	Female ^a	Male ^a	<i>P</i> level ^b
50	3579	5,17-DiMeC35	505 (M-15), 84/85, 462/463, 280/281, 266/267	3.5 ± 0.2	4.9 ± 0.3	0.006
	3585	+11,15,23-TriMeC35	519 (M-15), 168/169, 392/393, 238/239, 322/323, 196/197/364/365			
51	3722	+17-MeC37	519 (M-15), 252/253, 322/323	0.34 ± 0.03^c	0.26 ± 0.02	0.048
		+15-MeC37	519 (M-15), 224/225, 350/351			
		+13-MeC37	519 (M-15), 196/197, 378/379			
52	3558	15,19-DiMeC37	533 (M-15), 224/225 322/323, 294/295, 252/253	2.1 ± 0.1^c	0.51 ± 0.03 ^c	0.002
		+13,17-DiMeC37	533 (M-15), 196/197, 350/351, 266/267, 280/281			
		+11,15-DiMeC37	533 (M-15), 168/169, 406/407, 238/239, 308/309			
53	3770	7, <i>x</i> -DiMeC37	533 (M-15), 112/113, 462/463	0.21 ± 0.02	1.25 ± 0.08	0.002

LRI: linear retention index; DMDS: diagnostic ions after derivatization with dimethyl disulfide.

^a Means ± S.E.

^b Relative amounts were compared by Mann–Whitney *U* test. Significantly higher abundances are shown in boldface (*N* = 7).

^c Not previously reported in *N. vitripennis* by Carlson et al. (1999).

and dimethylalkanes. In hexane fractions from females, there were higher relative abundances of peaks resulting from the coelution of 9-, 11-, 13-, and 15-methylalkanes (peaks 19, 36, 46) and of those belonging to 9,*x*-, 11,*x*-, 13,*x*-, and 15,*x*-dimethylalkanes (peaks 22, 23, 39, 48, 52). Compounds with higher relative abundances in male fractions were some of the 5-methylalkanes and 7-methylalkanes (peaks 20, 21, 37, 47) as well as 3,*x*-, 5,*x*-, and 7,*x*-dimethylalkanes (peaks 26, 40, 41, 44, 49). Alkanes with more than two methyl branches (peaks 30, 32, 42, 45) were more abundant in female extracts, whereas alkenes (peaks 16, 17, 33, 34) were predominantly found in extracts from males.

Discussion

The study demonstrates that arrestment and key elements of the male courtship sequence in *N. vitripennis* are mediated by a female-derived contact sex pheromone. Bioassays of fractions of cuticular extracts revealed that the active compounds are nonpolar. The pheromone components from cuticular extracts were only active at close range, and were shown to be relatively stable because dead females elicited courtship in males for at least 6 d and caused arrestment for at least 8 d. The loss of activity after this period might be explained with residues left by male responders on the surface of the cadavers during previous trials. The only compounds detectable in the biologically active hexane fraction were cuticular hydrocarbons. The relative composition of cuticular hydrocarbons from males and females showed numerous qualitative and quantitative differences. These findings suggest that sex-specific differences in cuticular hydrocarbon profiles form the chemical basis of signals mediating male courtship behavior in *N. vitripennis*. Van den Assem et al. (1980b) assumed the presence of a sex pheromone in *N. vitripennis* after showing that

male courtship behavior toward dead females can be terminated by solvent extraction of the cadavers. However, they concluded that the chemicals involved were relatively unstable because dead females induced mounting responses in conspecific males for less than 2 d.

The primary function of insect cuticular hydrocarbons is to protect insects from desiccation (Lockey, 1988). However, numerous studies have demonstrated that these chemicals are also involved in orientation and recognition processes of insects, such as species, kin, and sex discrimination, as well as chemical mimicry by social parasites or parasitoids to gain access to their hosts (reviewed by Blomquist et al., 1993; Dettner and Liepert, 1994; Singer, 1998; Howard and Blomquist, 2005). Carlson et al. (1999) analyzed the cuticular hydrocarbon profiles of *N. vitripennis* and *Muscadifurax raptorellus* exuviae to develop a tool for discrimination of parasitoid species after these had left the host puparium. For comparison, the authors presented also some preliminary data on adult parasitoids which, however, did not allow for a systematic analysis of gender-specific differences. Our study reports also some additional components which have not been reported in the mentioned paper. This might indicate strain-specific variations or an influence of the host on the hydrocarbon profiles of the parasitoid, because Carlson et al. (1999) used house fly pupae to rear the parasitoids.

Only a few authors have studied the composition of cuticular hydrocarbons of parasitic wasps with regard to their possible role as contact pheromones. Syvertsen et al. (1995) found that some female-derived alkadienes arrested males of the braconid *Cardiochiles nigriceps*. As in the present study, pheromone activity was located in the hydrocarbon fraction of cuticular extracts of two other pteromalids, *R. xylophagorum* (Sullivan, 2002) and *L. distinguendus* (Steiner et al., 2005). In the latter species, as well as in the closely related *Anisopteromalus calandrae* (Yoshida, 1978) and in the braconid *Cotesia glomerata* (Tagawa, 1977), the pheromone was already present in the early pupal stage. It has been suggested that this might enable mating of the females before they leave the site of emergence to search for new host patches (Steiner et al., 2005). Because male pupae and freshly emerged *L. distinguendus* males also contained the courtship pheromone, Steiner et al. (2005) assumed that developing males use the courtship pheromone to fool sexual competitors. The present study shows a different situation for *N. vitripennis*: contact pheromones appear in the cuticular lipids much later, and only totally melanized female pupae are slightly attractive to males. Pheromone activity is fully developed in freshly emerged females but not in males. This explains why males of *N. vitripennis* are arrested on fly puparia containing females that are about to leave the host (King et al., 1969).

Interestingly, the response of *N. vitripennis* males to female extracts was influenced by the release substrate. Pheromone extracts applied to insect cuticles (extracted beetle elytra or male wasp cadaver) arrested males more strongly than those applied to pieces of filter paper. This might be because of differing adsorptive properties of the two matrices influencing the available amounts of the active compounds. Alternatively, physical cues associated with the insect cuticle might affect male responsiveness. More complex behavioral elements such as head-nodding or copulation attempts were shown only toward male cadavers treated with female extracts. In contrast, neither untreated male cadavers nor extracted female cadavers with reapplied male hydrocarbons elicited any responses in males (data not shown). This supports the hypothesis that visual and/or tactile cues synergize the male response to the female courtship pheromone. In other parasitic wasps, physical cues do not seem to play that crucial role because paper disks treated with pheromone extracts not only caused arrestment but also elicited elements of the courtship behavioral sequences (Takahashi and Sugai, 1982; Shu and Jones, 1993; Sullivan, 2002; Steiner et al., 2005).

Nasonia vitripennis females did not attract males from a distance. The present study revealed no apparent change in behavior of males in the four-chamber olfactometer when exposed to virgin females at a distance of only 3 cm. This suggests that this species does not use a volatile sex pheromone, unlike some other parasitic hymenoptera (e.g., Lewis et al., 1971; Field and Keller, 1993; McNeil and Brodeur, 1995). This reflects the natural history and the mating strategy of *N. vitripennis*: the parasitoid is gregarious, and protandrous males mate at the site of emergence with later emerging females. Thus, female-derived sex attractants are not necessary in this species. Males of the gregarious pteromalid *L. distinguendus* have been shown to use volatile cues other than sex pheromones for reaching or being arrested in the habitat of potential mates, orienting toward the host-associated volatiles that females use for host finding. These chemicals are emitted from the larval feces of the host *Sitophilus granarius* (Ruther and Steidle, 2000). Future studies will address the question of whether *N. vitripennis* males also use host-associated cues for long-range orientation during mate finding, and whether this strategy is a general feature among pteromalid wasps. Another challenge of future research is to identify the specific cuticular hydrocarbons that act as contact sex pheromones. However, the complexity of insect hydrocarbon profiles together with the nonavailability of synthetic reference compounds will complicate this undertaking.

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Molecular Cloning and *in Situ* Expression Patterns of Two New Pheromone-Binding Proteins from the Corn Stemborer *Sesamia nonagrioides*

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Abstract We describe the identification and characterization of two new cDNAs encoding pheromone-binding proteins (PBPs) from the male antennae of *Sesamia nonagrioides*, a species where no PBPs have been identified to date. Because PBPs are thought to participate in the first step of odor detection in a specific manner, we focused our investigation on this olfactory protein family using reverse transcription–polymerase chain reaction strategies. The deduced amino acid sequences of SnonPBP1 and SnonPBP2 revealed mature proteins of 142 and 143 amino acids, respectively, with six cysteine residues in conserved positions relative to other known PBPs. The alignment of the two mature *S. nonagrioides* PBPs with other noctuid PBPs showed high sequence identity (70–80%) with other full-length sequences from GenBank. Sequence identity between SnonPBP1 and SnonPBP2 was only 46%, suggesting that the two proteins belong to different classes of PBPs already described from the Noctuidae. Furthermore, analyses of expression patterns of SnonPBP1 and SnonPBP2 were performed by *in situ* hybridization on antennae of both sexes, and these studies revealed the expression of the two PBPs at the bases of olfactory sensilla (basiconica or trichodea) from both sexes. The possible binding properties of these two new PBPs are discussed according to their homologies with other known PBPs and *S. nonagrioides* pheromone components.

Keywords *Sesamia nonagrioides* · Noctuidae · (*Z*)-11-hexadecenyl acetate · Pheromone-binding proteins · Molecular cloning · *In situ* hybridization · RT-PCR · Stemborer

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Introduction

In insect antennae, odorant-binding proteins (OBPs) are soluble proteins that are thought to participate in the first step of olfactory detection by specific binding with volatile molecules. Well known in insects, OBPs are proposed to be specialized in solubilization, binding, and transport of odorants across the aqueous lymph of the olfactory sensilla (Vogt and Riddiford, 1981), allowing hydrophobic molecules to reach the membrane olfactory receptors. In addition, these proteins may also participate in dendritic odorant receptor activation or odorant signal deactivation (Kaissling, 2004). They are small hydrophilic proteins synthesized by accessory cells that surround the olfactory neuron and are secreted into the sensillar lymph where they are found at high concentrations. OBPs have been identified in a variety of insect species and appear to be present throughout the Neoptera (Vogt et al., 1999; Vogt, 2003). They are divided in two groups according to their potential ligands: the pheromone-binding proteins (PBP) are thought to bind pheromone components, whereas the general odorant-binding proteins (GOBPs) are believed to bind more “general” odorants such as those from plants. Although the designations PBP/GOBP are used to distinguish two lineages of OBPs with high sequence similarity within the Lepidoptera, this designation is also used across all insects to distinguish functional classes of OBPs based on their probable ligands.

Direct cloning and genomic analysis with insects have revealed the occurrence of multiple OBPs within the same species. For example, up to 50 OBP-related genes were identified in the fruit fly *Drosophila melanogaster* and a similar number in the mosquito *Anopheles gambiae* (reviewed in Vogt, 2003). Within OBPs, multiple PBPs are also expressed in a single species. Indeed, three PBPs are known in the silk moths, *Antheraea polyphemus* and *A. pernyi* (Saturniidae) (Raming et al., 1990; Krieger et al., 1991; Vogt et al., 1991; Maida et al., 2000) and the hawkmoth, *Manduca sexta* (Sphingidae) (Györgyi et al., 1988; Robertson et al., 1999), whereas two PBPs are known in different armyworm moths, e.g., *Mamestra brassicae* (Maïbèche-Coisné et al., 1998) and *Heliothis virescens* (Noctuidae) (Krieger et al., 1993; Abraham et al., 2003; direct GenBank submission no. AY301988). The presence of different subtypes of PBPs in the same species, together with the occurrence of different components in the sex pheromone, suggest a possible role of PBPs in specific binding/transport of pheromone components to the receptor neuron. PBPs could then contribute to the specificity of the olfactory system through an initial selective detection of odorant molecules (Leal, 2003). However, only a few studies have provided data suggesting some binding specificity between PBPs and pheromone components. For example, two different *M. brassicae* PBPs showed different binding affinities to the main pheromone component of this moth, (Z)-11-hexadecenyl acetate [(Z)-11-16:Ac] with PBP1, but not PBP2, binding the compound (Maïbèche-Coisné et al., 1997), although it may not be the only compound that PBP1 could bind (Campanacci et al., 2001). Similar results were obtained in *Lymantria dispar* (Vogt et al., 1989; Plettner et al., 2000), *A. pernyi* (Prestwich et al., 1995), and *A. polyphemus* (Du and Prestwich, 1995). In addition, *Bombyx mori* PBP showed binding to the pheromone component bombykol, but not to structurally unrelated chemicals (Wojtasek and Leal, 1999). In a competitive situation, each of the three *A. polyphemus* PBPs preferentially binds one of the pheromone components (Maida et al., 2000, 2003), and responses of the receptor neurons to pheromone appeared to depend on both the pheromone component and the type of PBPs (Pophof, 2002, 2004). Therefore, the PBPs appeared to contribute to the excitation of the receptor cells (Pophof, 2004).

The stemborer *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae), is an important pest of maize in the Mediterranean Basin. Integrated pest management strategies

are being developed against this species, including olfactory-mediated behavioral modification centered on the sex pheromone. This pheromone consists of a blend of (*Z*)-11-16:Ac (main component), (*Z*)-11-16:OH, (*Z*)-11-16:Ald, and 12:Ac (Mazomenos, 1989). Because no OBP have been described to date in *S. nonagrioides*, we investigated its PBP repertoire by polymerase chain reaction (PCR) strategies and compared the PBPs with previously reported sequences from other noctuid moths. This homology-based approach allowed us to describe two new PBPs from antennae of male *S. nonagrioides*, which are expressed in both male and female olfactory tissues.

Methods and Materials

Insect Rearing

A culture of *S. nonagrioides* was established from pupae collected in central Italy and reared with a meridic diet (Giacometti, 1995). Larvae, sexed pupae, and adults were kept inside screened plastic containers in an environmental chamber ($27 \pm 1^\circ\text{C}$, 60–80% relative humidity, 16-hr light/8-hr dark photoperiod).

RNA Extraction and cDNA Synthesis

Total RNA was extracted from 200 antennae from sexually mature *S. nonagrioides* males by using an Rneasy[®] MiniKit (Qiagen, Hilden, Germany). Single-stranded 3'RACE-ready-cDNA and 5'RACE-ready-cDNA were synthesized from 1 μg of RNA with the SMART[™] RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) with 200 U of SuperscriptII[™] (Gibco BRL, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Degenerate PCR for Isolation of PBP1 and PBP2 Gene Fragments

Degenerate primers corresponding to amino acid consensus regions of several PBPs from different species of Lepidoptera were used for PCR amplifications of *S. nonagrioides* cDNA templates: PBP1-forward (5'-GAYCAGTGYYAAGAAYGAGCTTAAT-3') and PBP1-reverse (5'-GTGAGGGATCCRTGYTTYWTNGCRAA-3'); PBP2-forward (5'-ATGAAYT TYGCYAAACCWYTA-3') and PBP2-reverse (5'-ACTCCTGAGCCTTCCCGTGATGCA-3'). These primers have already been used successfully for the molecular cloning of PBP1 and PBP2 from *M. brassicae*, a species that also uses (*Z*)-11-16:Ac as its main pheromone component (Maibèche-Coisné et al., 1998). PCR was carried out with 1 U of Taq polymerase (Promega, Madison, WI, USA) and consisted of 40 cycles of 30 sec at 94°C , 30 sec at 40 and 50°C for PBP1 and PBP2, respectively, and 30 sec at 72°C .

3'RACE-PCR

3'RACE amplifications were conducted on 2 μl of the *S. nonagrioides* 3'RACE-ready cDNA, with sense gene-specific primers deduced from the sequences obtained following degenerate PCR amplifications of PBP1 and PBP2 (PBP1Sn-forward: 5'-CTGGCGCGAG GAGTACGAGCTGGT-3'; PBP2Sn-forward: 5'-TGTGCCATACTCTGTCTCTCATCT-3') and with an antisense Universal Primer Mix (UPM, Clontech). The 50- μl amplification mix was prepared according to the SMART[™] RACE cDNA protocol using the Advantage2[™]

polymerase mix (Clontech). Touchdown PCRs were performed in the following conditions: 1 min at 94°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.

5'RACE-PCR

5'RACE amplifications were conducted on 2 µl of the *S. nonagrioides* 5'RACE-ready cDNA using Universal Primer Mix (Clontech) as a sense primer and antisense gene-specific primers (PBP1Sn-reverse: 5'-GCACATCACCATGCATCCCAGGTCGCG-3'; PBP2Sn-reverse: 5'-CTTAAGGTCTGGATCGAGCAGTTCCAG-3'). Touchdown PCRs were performed as described for the 3'RACE-PCR.

Cloning and Sequencing

After gel extraction (Genelute, Sigma, St. Louis, MO, USA), the amplified cDNAs were ligated into the plasmid pCRTM-II using the TOPO cloning kit from Invitrogen. Recombinant plasmids were isolated using the Plasmid Mini kit (Qiagen), and both strands were subjected to automated sequencing by Genome Express (Grenoble, France). 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).

In Situ Hybridizations

Digoxigenin-labeled RNA sense and antisense probes (200 bp) were obtained for each PBP. The pCRTMII-cDNA plasmids resulting from degenerate PCR amplifications of the internal gene fragments of PBP1 and PBP2 were PCR amplified by using M13 forward and reverse primers. The PCR products were used to generate digoxigenin-labeled RNA probes by *in vitro* transcription using T7 and SP6 RNA polymerase (Promega) following the manufacturer's protocol and in the presence of 1.5 U of Rnasin (Promega).

For hybridization, antennae were removed from the heads of adults of both sexes, cut into pieces, and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Fixed tissues were dehydrated in 100% methanol. The hybridization protocol was performed as described (Jacquin-Joly et al., 2000), with a hybridization temperature of 65°C, and digoxigenin was detected using alkaline-phosphatase-conjugated antidigoxigenin Ig (1:4000; Roche, Basel, Switzerland) and revealed colorimetrically with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate, toluidine salt (Roche). After sufficient staining, specimens were washed in PBS and fixed in 4% PFA, dehydrated, and embedded in Epon (Agar Scientific, Stansted, Essex, UK). Six-micrometer sections were counterstained with acridine orange. Sections were photographed, and pictures were digitized and processed with Adobe Photoshop 5.0.

Phylogenetic Analysis with Other Noctuid PBPs

Several noctuid PBP sequences were retrieved from GenBank for phylogenetic analysis. Amino acid sequences of PBPs without the leader sequence were aligned by using Multalin program (Corpet, 1988). Maximum parsimony was used to build a strict consensus tree in PAUP 4.0b10 software (Sinauer Associates, Sunderland, MA, USA). Branch support was

assessed by bootstrap analysis based on 1000 replications, and the *H. virescens* GOBP1 and 2 (GenBank accession nos. X96862 and X96863, respectively) were used as outgroups.

Results

Molecular Cloning and cDNA Sequencing of *S. nonagrioides* PBPs

By using degenerate PCR and RACE-PCR strategies, two full-length cDNAs, encoding potential PBPs, were cloned in *S. nonagrioides*. They both contained an open-reading

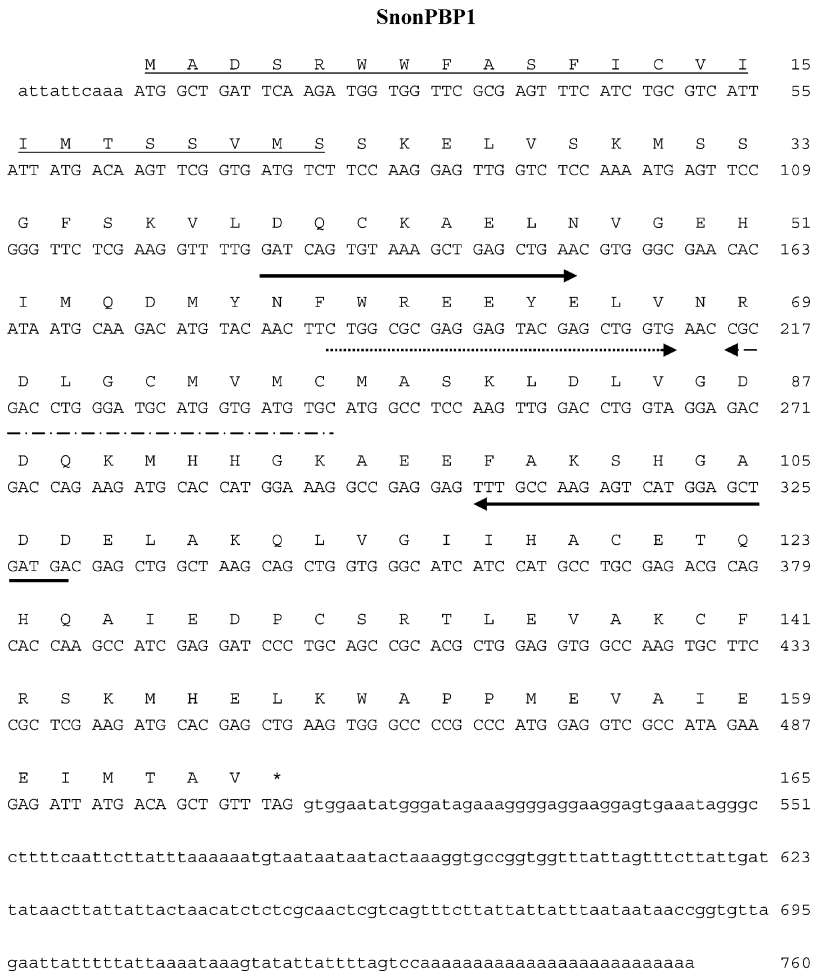


Fig. 1 Nucleotide and deduced amino acid sequences of SnonPBP1 and SnonPBP2 cDNAs from antennae of male *Sesamia nonagrioides* (GenBank accession nos. AY485219 and AY485220). The stop codons are indicated by an asterisk. The positions of the degenerate primers used in PCR for gene fragment amplifications are underlined with a bold line (PBP1-forward and -reverse; PBP2-forward and -reverse), whereas gene-specific primers for 3'RACE (PBP1Sn-forward and PBP2Sn-forward) and 5'RACE (PBP1Sn-reverse and PBP2Sn-reverse) are marked with dotted and dashed lines, respectively. Putative signal peptides at the N-termini are underlined

Snon PBP2

<u>M A L H R S P I M S A R L A L V L</u>	17
aca ATG GCG CTG CAT CGA TCG CCC ATC ATG TCG GCA CGC TTG GCG CTG GTA CTG	54
<u>I A S L F I V V K C</u> S Q E V M K N L	35
ATC GCC AGT CTG TTC ATC GTC GTG AAA TGT TCT CAA GAA GTC ATG AAG AAT CTG	108
T H H F S K P L E D C K K E M D L P	53
ACC CAT CAT TTC TCT AAG CCT TTG GAA GAC TGT AAG AAG GAG ATG GAC CTC CCG	162
D S V I T D F Y N F W K E G Y E F T	71
GAC TCA GTG ATC ACA GAT TTC TAC AAT TTC TGG AAA GAA GGC TAC GAG TTC ACG	216
S R H T G C A I L C L S S K L E L L	89
AGC AGA CAT ACA GGC TGT GCC ATA CTC TGC CTC TCA TCT AAG CTG GAA CTG CTC	270
D P D L K L H H G K A Q E F A Q K H	107
GAT CCA GAC CTT AAG TTG CAT CAT GGA AAG GCG CAG GAG TTC GCG CAG AAA CAT	324
G A D E A M A K Q L V G L I H G C M	125
GGC GCT GAC GAG GCC ATG GCG AAG CAG CTG GTA GGC CTG ATC CAC GGC TGT ATG	378
E T I R E P A D D P C V R A Q N V V	143
GAG ACA ATC CGC GAA CCG GCC GAC GAC CCC TGC GTG AGG GCT CAG AAC GTA GTC	432
M C F K A K I H E L X W A P S L D L	161
ATG TGC TTC AAG GCC AAG ATA CAT GAG CTG ANC TGG GCG CCT AGC TTG GAC CTC	486
I V G E V L A E V *	171
ATC GTG GGA GAA GTC TTG GCT GAA GTC TAG catgatgcaccttggttccogtgatataccttt	547
atcttctcttcogtcatagaaggccatcattgcatgtgatagtgatgttgttttgaatgcaaaacatagt	619
ttcatctttttcattgttttctgtgagtgttttcagctagagacattacgtaaatcaaaagtctttttatc	691
aaatatcattctctgttaagaaccaataaccagtgctcagacaacattaatgtttatgtgcggttgaatgt	763
aatgcaatgcttatgacctgcaggaataaaatgcaaaataagtttatctacattacattatgtttatacatt	835
acagtcattgtgttatacaagtctgattgtttctatctctactttaacgacaaggcttgttcaatggact	907
acagatatttctacagttagttattttgattaatatttaataattccttgtaagagctctcctgtctccagct	979
tctcatcaaaggagtggttacattgtaagttgcaagttctggatgtcatattaataaagaatacatcttta	1051
caaaaaaaaaaaaaaaaaaaaaaaaaaaaaa	1080

Fig. 1 (continued)

frame encoding two putative proteins of 165 and 170 amino acids, named SnonPBP1 and SnonPBP2, respectively, according to their high identity with proteins from these classes in BLAST searches. The sequences for SnonPBP1 and SnonPBP2 have been deposited in GenBank under the accession numbers AY485219 and AY485220, respectively. The deduced amino acid sequences for both proteins possess a putative signal sequence of 23 (SnonPBP1) and 27 (SnonPBP2) amino acids (Fig. 1). Mature predicted SnonPBP1 protein consisted of 142 amino acids with a molecular mass of 16,117 Da and a pI of 5.49, whereas SnonPBP2 consisted of 143 amino acids with a molecular mass of 16,039 Da and a pI of 5.00. 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 PBPs: positions C¹⁹, C⁵⁰, C⁵⁴, C⁹⁷, C¹⁰⁸, and C¹¹⁷ (Fig. 2).

The alignment of the two mature *S. nonagrioides* PBPs with other noctuid PBPs showed high sequence identity. *S. nonagrioides* PBP1 showed 83% identity with *M. brassicae* PBP1 (MbraPBP1), 72% with *Spodoptera exigua* PBP1 (SexiPBP1), and 88% with the partial sequence of *H. virescens* PBP2 (HvirPBP2; Fig. 3). Similarities were even higher,

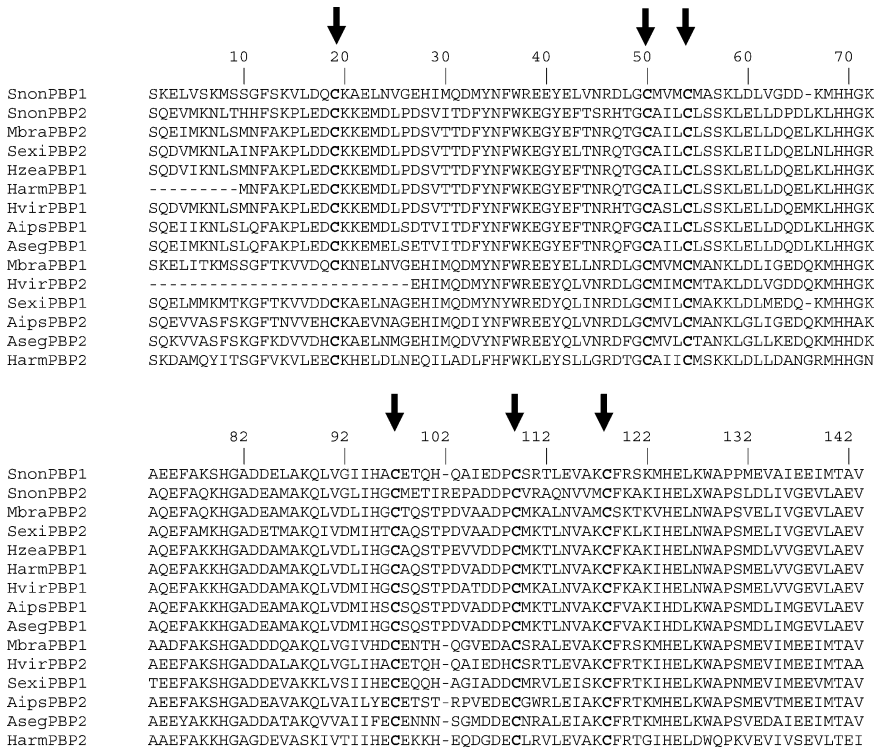


Fig. 2 Comparison of the predicted amino acid sequences of SnonPBP1 and SnonPBP2 from antennae of male *S. nonagrioides* with pheromone-binding proteins from other noctuids. The six conserved cysteines are in bold and marked by arrows. GenBank accession numbers—SnonPBP1: AY485219, SnonPBP2: AY485220 (*S. nonagrioides*); MbraPBP1: AF051143, MbraPBP2: AF051142 (*Mamestra brassicae*); SexiPBP1: AY743351, SexiPBP2: AY743352 (*Spodoptera exigua*); HzeaPBP1: AF090191 (*Helicoverpa zea*); HarmPBP1: AJ278992, HarmPBP2: AF527054 (*Heliothis armigera*); HvirPBP1: X96861, HvirPBP2: AY301988 (*Heliothis virescens*); AipsPBP1: AY301985, AipsPBP2: AY301986 (*Agrotis ipsilon*); and AsepPBP1: AF134253, AsepPBP2: AY301987 (*Agrotis segetum*)

Snon PBP1																			
83	Mbra PBP1																		
88	80	Hvir PBP2																	
70	72	75	Aips PBP2																
67	67	68	77	Aseg PBP2															
72	71	76	70	65	Sexi PBP1														
46	44	49	42	42	46	Snon PBP2													
45	44	49	45	43	45	80	Mbra PBP2												
48	46	51	46	46	46	78	87	Hvir PBP1											
48	46	52	46	45	45	79	88	92	Hzea PBP1										
49	47	53	48	45	47	77	83	83	86	Aips PBP1									
49	47	54	48	46	49	77	83	83	84	97	Aseg PBP1								
50	47	53	46	46	48	79	92	94	98	87	86	Harm PBP1							
45	42	48	42	41	45	73	85	84	86	80	80	89	Sexi PBP2						
51	50	53	44	46	53	45	46	47	46	43	44	49	47	Harm PBP2					

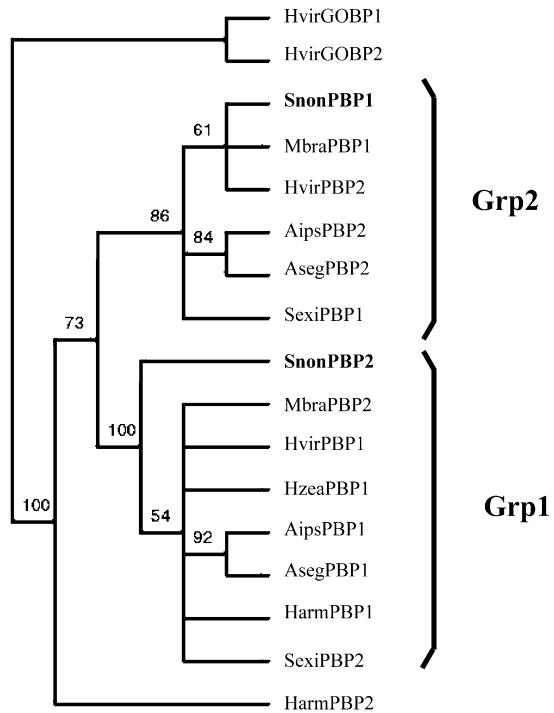
Fig. 3 Percentage identity of pheromone-binding proteins (PBPs) of the Noctuidae based on conserved amino acid residues. Sequences were retrieved from GenBank (accession numbers listed in Fig. 2), and signal peptides were deleted for this comparative analysis. Identities greater than 55% are in bold. Two different groups of PBPs are evident according to level of identity: Grp1 (names not bold) and Grp2 (names in bold), as defined by Picimbon and Gadenne (2002)

e.g., 95% with MbraPBP1. SnonPBP2 presented the highest identities with MbraPBP2 (80%), HzeaPBP1 (79%), and HarmPBP1 (79%). The degree of identity between SnonPBP1 and SnonPBP2 (46%) was much lower than in the interspecific comparisons within a protein (Fig. 3). Sequence similarities of noctuid PBPs were summarized in a neighbor-joining tree (Fig. 4). This analysis revealed two clusters of PBPs from various species, designated as Grp1 and Grp2, according to the two PBP groups already described in noctuids (Picimbon and Gadenne, 2002). Grp2 contained SnonPBP1, whereas Grp1 contained SnonPBP2 (Fig. 4).

PBP Expression Patterns in *S. nonagrioides*

Expression patterns of the two *S. nonagrioides* PBPs were established by *in situ* hybridization to RNA in both male and female antennae (Figs. 5 and 6). *S. nonagrioides* antennae contain about 40 flagellomeres. In males, they are comb-like (bipectinate) and 7.20 mm long and 0.70 mm wide at mid-length (Fig. 5A), whereas in females, they are filiform and 6.60 mm long and 0.15 mm wide at mid-length (Fig. 6A). In both sexes, the

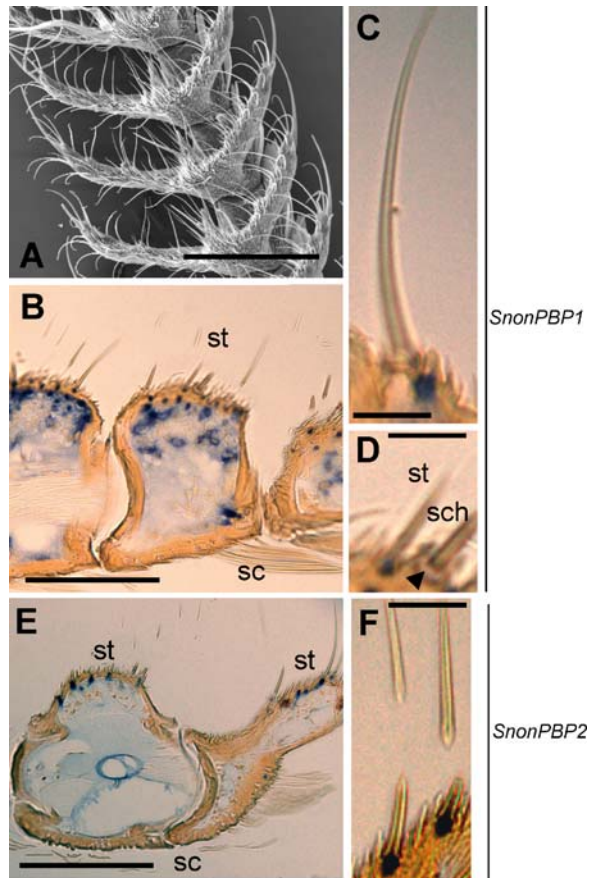
Fig. 4 Phylogenetic analysis of *S. nonagrioides* SnonPBP amino acid sequences with various noctuid PBPs. GenBank accession numbers are listed in Fig. 2. Bootstrap support values (in percent) based on 1000 replicates are indicated. Analysis indicates two groups (Grp1 and Grp2), as defined by Picimbon and Gadenne (2002)



antennae are covered dorsally with overlapping scales. The olfactory hairs, the sensilla trichodea and the sensilla basiconica, are all set on the ventral side of the flagellomere. In males, the olfactory sensilla are distributed across both portions of the lateral processes and in the medial process (Fig. 5A). In particular, the branches of the male antennae carry a great number of long sensilla trichodea, which have been characterized functionally by single cell recording (Quero et al., 2004). Although the number and the distribution of sensilla are quite different in females and males, both sexes share the same types of sensilla (Solinas and Trona, 2002).

Hybridization of male antennae with either SnonPBP1 or SnonPBP2 sense probes showed no signal (data not shown). Hybridization with either SnonPBP1 (Fig. 5B–D) or SnonPBP2 (Fig. 5E and F) antisense probes showed signals restricted to the sensillar side of the antennae (Fig. 5B), with labeling present in both lateral and medial processes (Fig. 5E). No labeling was visible on the dorsal-scaled side of the antennae. On the sensillar side, labeling was observed at the base of olfactory sensilla (Fig. 5C, D, and F), with no labeling at the bases of the sensilla chaetica (Fig. 5D), which are thought to function in gustation in other Lepidoptera (Jørgensen et al., 2006). In some sections (Figs. 5B, C, E, 6B and C), labeling could be clearly associated with numerous long sensilla trichodea. However, it was not possible to exclude expression in sensilla basiconica that differs from sensilla trichodea by their small size because a whole sensillum was rarely visible on a section. In longitudinal sections of female antennae, *in situ* hybridization revealed that both PBP1 and PBP2 are also abundantly expressed in this sex, with labeling observed at the base of the olfactory sensilla with both probes (Fig. 6B and C).

Fig. 5 Expression patterns of SnonPBP1 and SnonPBP2 revealed by *in situ* hybridization to mRNA in longitudinal and transversal sections of *S. nonagrioides* male antennae. (A) SEM of the ventral side of a male antenna. (B) Longitudinal section showing SnonPBP1 labeling on the sensilla side of the antenna. (C) SnonPBP1 labeling at the base of a sensillum trichodeum. (D) Magnification of (B) showing labeling at the base of a sensillum trichodeum with no labeling associated with a sensillum chaetium (arrow). (E) Transversal section showing SnonPBP2 labeling on the sensilla side of the antenna. (F) SnonPBP2 expression at the base of sensilla trichodea. sc: scales; st: sensilla trichodea; sch: sensilla chaetia. Bars: 200 μ m (A); 100 μ m (B, E); 25 μ m (C, D, F)



Discussion

Heterogeneity of PBPs Expressed in *S. nonagrioides* Antennae

In *S. nonagrioides*, no OBPs had been described prior to our study. In this species, the responses of the olfactory receptor neurons have been recently investigated, defining functional types of long sensilla trichodea distributed on the lateral branches of male antenna (Quero et al., 2004). Deciphering the PBP repertoire in this species provides the opportunity to relate PBP expression with the functional types of sensilla. Here, we have cloned two cDNAs encoding proteins that can be classified in the lepidopteran PBP family. Indeed, both of 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; and (4) their expression is associated with the olfactory sensilla, as revealed by *in situ* hybridization. The two deduced proteins are grouped in the PBP1 and PBP2 families, according to sequence homologies with noctuid PBPs and phylogenetic analyses, and thus have been named SnonPBP1 and SnonPBP2. With only 46% identity, these two PBPs clearly represent divergent proteins.

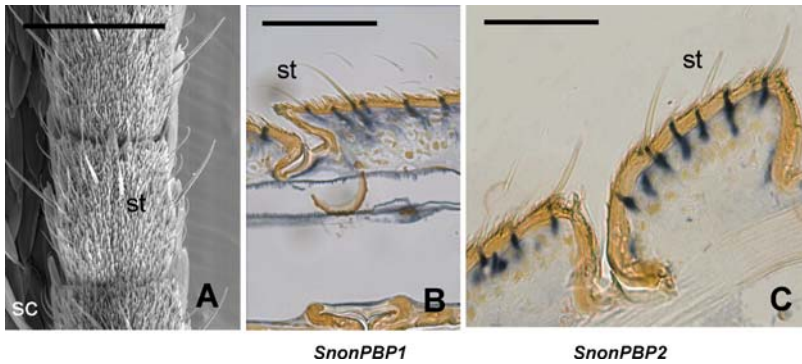


Fig. 6 Expression patterns of SnonPBP1 and SnonPBP2 revealed by *in situ* hybridization to mRNA in longitudinal sections of *S. nonagrioides* female antennae. (A) SEM of the ventral side of a female antenna. (B) Longitudinal section of female antenna with SnonPBP1 expression pattern. (C) Longitudinal section of female antenna with SnonPBP2 expression pattern. st: sensilla trichodea; sc: scales. Bars: 100 μm (A); 50 μm (B, C)

With the discovery of at least two PBPs in its antennae, *S. nonagrioides* is another example of a moth species expressing multiple PBP-like proteins (Györgyi et al., 1988; Raming et al., 1990; Krieger et al., 1991; Vogt et al., 1991; Maïbèche-Coisné et al., 1998; Robertson et al., 1999; Maida et al., 2000). In particular, noctuid species tend to have PBPs belonging to both the PBP1 and the PBP2 families. Because lepidopteran pheromone blends usually consist of several components, the occurrence of divergent PBPs within a species accounts for a possible role of PBP in specific binding. In particular, *M. brassicae* PBP1 has been shown to bind (Z)-11-16:Ac (Maïbèche-Coisné et al., 1997). As *S. nonagrioides* and *M. brassicae* share this main pheromone component, SnonPBP1 could be the ortholog of MbraPBP1, considering that these proteins share 83% identity. This hypothesis is supported by the establishment of the PBP1 expression pattern in correlation with the functional types of the olfactory sensilla. Indeed, PBP1 appeared to be expressed in association with numerous sensilla distributed on the lateral branches of the male antennae (Fig. 5B). These branches have been shown to carry different functional sensilla types (Quero et al., 2004), and among them, 90% containing at least one olfactory neuron responding to (Z)-11-16:Ac.

Because the sex pheromone of *S. nonagrioides* consists of several different components, SnonPBP2 may bind one or more of these other components. In particular, *S. nonagrioides* female pheromone also contains (Z)-11-16:Ald (Mazomenos, 1989), another common pheromone component between *M. brassicae* and *S. nonagrioides* (Renou and Lucas, 1994), which is detected by *S. nonagrioides* olfactory neurons housed in 48% of the sensilla trichodea located on the branches of male antennae (Quero et al., 2004). High conservation between SnonPBP2 and MbraPBP2 (80% identity), together with PBP2s from other noctuids that also use (Z)-11-16:Ald in their pheromone blend (*H. virescens*, *H. zea*, and *H. armigera*; see the Pherolist: <http://www-pherolist.slu.se/pherolist.php>), further supports the hypothesis that these PBP2s could be involved in (Z)-11-16:Ald binding, although no functional data are available on either SnonPBP1 or SnonPBP2. The correlation between PBP sequence identities and pheromone components has already been investigated, but there is no evidence for convergence of PBP sequences in species that use similar pheromone components (Willett, 2000a). However, when alleles of PBPs were sampled from members of the tortrix moth genus *Choristoneura* (Tortricidae) that use

pheromones with different functional groups, evidence for episodes of selection acting on PBPs was demonstrated (Willett, 2000b). However, it is not clear whether pheromone changes are causing this selection.

Through our neighbor-joining tree analysis (Fig. 4), the two newly identified SnonPBPs were easy to classify in the two previously defined groups of PBPs (Picimbon and Gadenne, 2002; Abraham et al., 2005). Indeed, SnonPBPs segregate into two subclasses. Subclass 1 (Grp1) contains PBPs reported only in noctuid species (Picimbon, 2003) and includes SnonPBP2, HzeaPBP1, HvirPBP1, AsegPBP1, and AipsPBP1. Subclass 2 (Grp2) includes SnonPBP1 together with MbraPBP1, HvirPBP2, AipsPBP2, AsegPBP2, and SexiPBP1, as well as non-noctuid PBPs (Picimbon, 2003). The classification of the two SnonPBPs in the two subgroups confirms the occurrence of two orthologous groups of PBPs in noctuids.

The Two New *S. nonagrioides* PBPs are Expressed in Male and Female Antennae

From our *in situ* hybridization results, we showed that SnonPBP1 and SnonPBP2 were abundantly expressed in both male and female antennae. Moreover, the expression is associated with the olfactory sensilla in both sexes, with no labeling associated with gustatory sensilla (Fig. 5), consistent with a role in olfaction.

Considering their role in pheromone detection, PBPs were first thought to be expressed exclusively in male antennae (Vogt and Riddiford, 1981), in contrast to GOBPs, which were present in both sexes. We now know from several examples that PBPs are expressed in antennae of both sexes (Györgyi et al., 1988; Maïbèche-Coisné et al., 1997; Callahan et al., 2000; Vogt et al., 2002), but with differences according to the insect family. In the Saturniidae, Bombycidae, and Sphingidae, PBPs are present in much higher levels in males than in females (e.g., Györgyi et al., 1988; Steinbrecht et al., 1992, 1995; Vogt et al., 2002), whereas in the Noctuidae, relatively high expression has been observed in female antennae (Maïbèche-Coisné et al., 1997; Callahan et al., 2000; Zhang et al., 2001). Our results on PBP expression in *S. nonagrioides* female antennae are consistent with these previous observations from noctuid moths. However, the function of PBPs in females remains unclear. Although many studies have failed to detect female response to sex pheromone (reviewed in Callahan et al., 2000), electrophysiological recordings from female antennae showed that *Spodoptera littoralis* as well as *M. sexta* females could respond to at least one of their pheromone components (Ljungberg et al., 1993; Kalinova et al., 2001). The behavioral significance of female PBPs and/or female detection of their own pheromone remains unknown.

The molecular characterization of SnonPBP1 and SnonPBP2 are significant not only because olfactory-mediated behavioral modification of the pest is used in integrated pest management strategies, but also because *Telenomus busseolae* Gahan (Hymenoptera: Scelionidae), an egg parasitoid, is being used effectively in crop protection against *S. nonagrioides*. Possible explanations for such efficacy is that *T. busseolae* uses the *S. nonagrioides* sex pheromone component (Z)-11-16:Ac as a host location kairomone and has developed morphofunctional adaptations, behavioral strategies (Conti and Bin, 2000), as well as molecular adaptations of odorant detection. Indeed, like other parasitoids, *T. busseolae* utilizes a semiochemical espionage strategy (Vinson, 1984, 1998). In behavioral laboratory tests, *T. busseolae* are attracted by the *S. nonagrioides* main pheromone component, (Z)-11-16:Ac (Colazza et al., 1997), as well as to contact chemicals from *S. nonagrioides* (Colazza and Rosi, 2001). The long-term goal of this work is to compare the olfactory elements involved in (Z)-11-16:Ac recognition in both the host and its parasitoid.

A molecular analysis of these elements should provide useful information on the coevolution of this host–parasitoid complex.

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Effect of Wind Speed on the Pheromone-Mediated Behavior of Sexual Morphs of the Potato Aphid, *Macrosiphum euphorbiae* (Thomas) Under Laboratory and Field Conditions

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Abstract The effect of wind on the potato aphid, *Macrosiphum euphorbiae*, male responsiveness to the sex pheromone, and on the calling behavior of virgin oviparae, was studied under constant wind speeds in the laboratory and in the field. A significant proportion of females called at wind speeds up to 4 m/sec, whereas male flight behavior in clean air was inhibited at velocities > 2 m/sec. However, males continued to walk over the range of wind speeds at which females called. Under constant wind velocities in a wind tunnel, males downwind of calling females oriented on the upwind edge of the release stand, and fewer individuals took flight at low constant wind speeds than in a clean air plume. In all cases, whether calling females were present or not, the males that took flight moved downwind. However, when a bridge was available, a significant proportion of males walked upwind to the pheromone source. The same orientation and walking behaviors were observed when males were placed downwind of calling females under variable wind conditions in the field. However, contrary to the laboratory results, 30% of the males tested flew upwind and landed on the source. A more detailed examination found that males orienting toward a source would walk if wind speeds were high but initiated flight in an “upwind” direction if there was a lull in wind velocity. These findings suggest that for the potato aphid, and probably for many other insect species with weak flight capacity, walking behavior is a significant component of pheromone-mediated mate location. This would permit males to continue foraging for calling females in the vicinity when wind velocities inhibit flight.

Keywords Potato aphid · *Macrosiphum euphorbiae* · Sex pheromone · Wind tunnel · Calling behavior · Mate location behavior

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Introduction

Many abiotic and biotic factors influence chemically mediated reproductive behaviors in insects (McNeil, 1991). Wind is one of the important abiotic factors and may affect reproductive success in two distinctly different ways. The first relates to the temporal and spatial movement of the infochemicals, because wind velocity and the level of turbulence will influence parameters such as the distance the pheromone moves from the source, the plume structure, and the concentration of the message at any given point within the plume (see Elkinton and Cardé, 1984; Murlis et al., 1992; Bell et al., 1995 and references therein). Second, variations in wind speed may directly influence the behaviors of both sexes. Wind speed can influence the proportion of individuals that emit pheromone (Kaae and Shorey, 1972) or change the behavior associated with pheromone release (Conner et al., 1985). In the case of the responding sex, wind speed may influence the proportion of individuals reaching the source, possibly related to the plume characteristics mentioned above. In addition, there will be an upper limit to wind velocity, above which upwind flight is not possible, and this threshold will be determined by the flight capacity of each species.

The sex pheromones of several aphid species have been identified (Dawson et al., 1987, 1989; Campbell et al., 1990; Lilley et al., 1994/1995; Gabry et al., 1997; Boo et al., 2000; Goldansaz et al., 2004) and a few studies of pheromone-mediated behaviors have been carried out under controlled laboratory conditions (Eisenbach and Mittler, 1980, 1987; Guldemond and Dixon, 1994; Thieme and Dixon, 1996). However, there has been little work examining the pheromone-mediated behavior of aphid sexual forms under field conditions in late summer and early fall when these morphs are present in nature. Virgin, apterous female oviparae raise their back legs off the substrate when releasing sex pheromone from glands on the tibia. Fluctuating climatic conditions, including wind velocity, had a marked effect on the expression of this calling behavior in the potato aphid, *Macrosiphum euphorbiae*, in the field (Goldansaz and McNeil, 2003). Aphids are weak flyers, and the speed and direction of flight is usually governed by the wind (Dixon and Howard, 1986). Furthermore, the inhibitory effect of wind speeds >2 m/sec on the flight behavior of parthenogenetic female alate aphids has been documented (Kennedy and Thomas, 1974; Kennedy, 1990; Storer et al., 1999). However, despite the fact that wind velocity could markedly influence the reproductive biology of aphids, no detailed studies have examined the effect of this parameter on alate males. We, therefore, carried out experiments under both laboratory and field conditions to examine the effect of wind speed on the flight behavior of alate male potato aphids and determined how this related to the calling behavior of wingless oviparae under similar conditions.

Methods and Materials

Insects

All aphids used came from a multiclinal laboratory culture, established, and restocked annually with parthenogenetic individuals collected from potato fields around Quebec City, Canada. Colonies were maintained on potato seedlings *Solanum tuberosum* c.v. Norland, at $20 \pm 1^\circ\text{C}$, $60 \pm 10\%$ relative humidity (RH), under a light/dark 16:8 hr photoperiod, conditions that ensure continuous asexual production. In all experiments, aphids were held on four- to six-leaf stage potato seedlings, and new plants were provided every 3 d. To obtain sexual morphs, aphids were reared at $20 \pm 1^\circ\text{C}$, $60 \pm 10\%$ RH, under a light/dark

10:14 hr photoperiod. The gynoparous and androparous females (which produce the sexuals) were produced in the first generation and were held in groups on host plants. Their offspring were collected daily to ensure production of known-age oviparae and males, which were held on potato seedlings under these conditions until used in experiments.

Wind Tunnel Bioassays

Experiments were conducted in a laminar airflow wind tunnel (290 cm long, 120 cm wide, and 100 cm high) with a capacity to generate wind speeds from 0 to 120 cm/sec. The tunnel was located in an environmental chamber maintained at $18 \pm 0.5^\circ\text{C}$ with RH 40–50%, with lighting provided by eight 40-W fluorescent bulbs, placed uniformly above the tunnel ceiling. Light intensity was 150 lux at the points where the potato plants with calling females or male release cages were placed. All assays, using 5- to 6-d-old virgin individuals, were carried out in the 3rd to 6th hr of the photophase because this was the period of maximum calling activity under controlled laboratory conditions (Goldansaz and McNeil, 2003). Individuals were used only once, and all bioassays testing males were terminated after 3 min if the test animal did not respond.

The effect of wind on the calling behavior of oviparae was tested by placing a calling virgin female in the wind tunnel, and classified as either “calling” or “noncalling” after 3 min at wind speeds of 0, 20, 40, 60, 80, 100, and 120 cm/sec. Fifteen oviparae were tested at each wind speed and the entire experiment was replicated $\times 3$, using a randomized block design.

Wind speed is known to affect the probability of take off in parthenogenetic alates (see Kennedy, 1990) so the flight behavior of males was tested over the same range of wind speeds in clean air. Each male was placed downwind in a plastic cage (5 cm long and 3 cm diam) on a metal release platform. The time to take off and flight direction were recorded for 15 males at each wind speed, and the entire experiment was replicated $\times 3$, using a randomized block design.

We then examined the responses of males to the presence of five calling virgin females on a single potato seedling, 40 cm upwind, at wind speeds of 40, 80, and 120 cm/sec, velocities at which $> 80\%$, $< 20\%$, and 0% of males initiated flight in the clean air assays described above. Despite the presence of a pheromone source, any males taking flight still flew downwind, as they did in clean air. Therefore, we repeated the experiment but this time providing a bridge, a single white string connecting the male release platform and the plant with the calling females, which allowed males to reach the source by walking upwind (Goldansaz et al., 2004). We recorded whether males orientated to the pheromone source (moving to the upwind edge of the release cage/platform and exhibiting rapid antennal movement), as well as the time taken to either walk to the source or initiate flight. The experiment was replicated $\times 3$ with 15 males per replicate at each wind velocity, using a randomized block design.

A split plot design was used to test the effect of distance of males from the pheromone source (at 40, 70, and 100 cm) at the same three wind speeds, 40, 80, and 120 cm/sec. There were three replicates, each testing 12 males at all distance/wind speed combinations.

Field Experiments

The distinct female calling posture observed under constant laboratory regimes may be less evident under variable field conditions (Goldansaz and McNeil, 2003), so we examined the effect of wind on female calling and male-searching behaviors under field conditions on the

campus of Laval University, Ste-Foy, Quebec, on September 15 and 27, 2001. These were clear days, and during observation periods temperatures varied between 14–17°C and 12–14°C, respectively. On both days, the calling behavior of 30 oviparae from the laboratory culture, each held on an individual potato seedling placed on a large table around 10:00 hr, was recorded every 30 min from 11:00 to 15:00 hr. Over the same time period, the behavior of males in clean air was also tested hourly on both days. One hundred males, in individual release cages, were placed on the table each morning and two cohorts of 10 individuals were tested each hour. We removed the covers from the release cages, and the behaviors of the 20 males were recorded over a 3-min period, noting if they remained immobile, walked (around the cage and/or the release platform site), or took flight. During each observation period, the wind speed were monitored for 1 min, and because of the variability during the experiment the results were grouped in categories 0– X m/sec, where X was the maximum wind speed observed during a given recording interval.

The behaviors of individual males in the presence of five calling females on a potato seedling were examined under field conditions between 14:00 and 16:00 hr on the October 11 ($N = 29$) and 24 ($N = 17$), 2001. Both days were clear, and during the bioassays air temperatures varied between 17–15°C and 16–13°C, respectively. As in the controlled wind tunnel experiments, females were placed 40 cm upwind of the male, and a string bridge connected the platforms holding the potato plant and the release cage. The cover of the release cage was removed and the behavior of individual males was recorded for 3 min.

Statistics

The proportion of males taking flight in the presence or absence of calling females as a function of wind speed was compared with an analysis of contrasts, using the GENMOD procedure (SAS, 1999). Categorical responses (orient/not orient; reach the source/not reach the source) of males exposed to calling females were compared with a logit model using the GENMOD procedure of SAS (SAS, 1999), whereas the times to reach the source as a function of wind speed were compared with an ANOVA, using the GLM procedure of SAS. The proportions of males taking flight, orienting to, and/or reaching the source at different wind speeds and distances were compared with a logit model.

Results

Laboratory Bioassays

There was no change in the calling behavior of oviparae under the range of constant wind velocities tested in the wind tunnel (Fig. 1). In contrast, the propensity of males to take flight in clean air varied with wind speed ($df = 5$, $\chi^2 = 126.89$, $P < 0.001$: because winds of 120 cm/sec totally inhibited flight, we excluded this treatment from the analysis). The proportion taking flight increased with increasing wind speeds up to 40 cm/sec and then declined at higher wind speeds, with practically 100% inhibition at > 80 cm/sec (Fig. 1).

When a pheromone source was present, $> 80\%$ of males oriented toward the source at all wind speeds tested. However, at a wind velocity of 40 cm/sec, fewer males took flight compared with those tested in clean air ($df = 1$, $\chi^2 = 14.63$, $P < 0.001$; Fig. 2). At wind velocities of 80 ($df = 1$, $\chi^2 = 0.11$, $P = 0.74$) and 120 cm/sec ($df = 1$, $\chi^2 = 1.40$, $P = 0.24$), relatively few males took flight and the proportions were not affected by the presence or absence of pheromone (Fig. 2). In all cases, orienting males moved to the upwind edge of

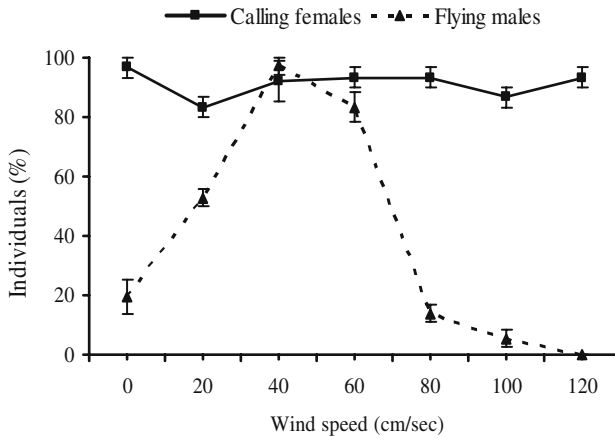


Fig. 1 Effect of wind speed on the proportion of *M. euphorbiae* oviparae calling and males taking flight ($\bar{x} \pm$ SEM; three replicates of 15 individuals at each wind speed) in a wind tunnel at $18 \pm 0.5^\circ\text{C}$, 40–50% RH

the release cage/platform but, if taking flight, they all flew downwind and not toward the source. However, when there was a bridge available, some males reached the calling females by walking. Wind speed had a significant effect on the proportion of males orienting toward ($df = 2$, $\chi^2 = 11.11$, $P = 0.004$; Fig. 3a), and walking to the source ($df = 2$, $\chi^2 = 16.78$, $P < 0.001$; Fig. 3b), both of which were higher at the higher wind velocities when male flight was inhibited (see Fig. 2). The time taken to reach the source was also influenced by wind speed ($df = 2$, $F = 8.63$, $P = 0.035$), taking longer at 120 cm/sec than at the lower wind velocities (Fig. 3c). Varying the distance to the source did not significantly modify the effect of wind speed on the proportion of males orienting to the source, initiating downwind flight, or reaching the source (Tables 1 and 2). As expected, at any given wind speed, there was an obvious effect of distance on the time to reach the source (Table 1). There was also a significant wind speed \times distance interaction (Table 2), due to the fact that for any given distance between the release site and the calling females, males at

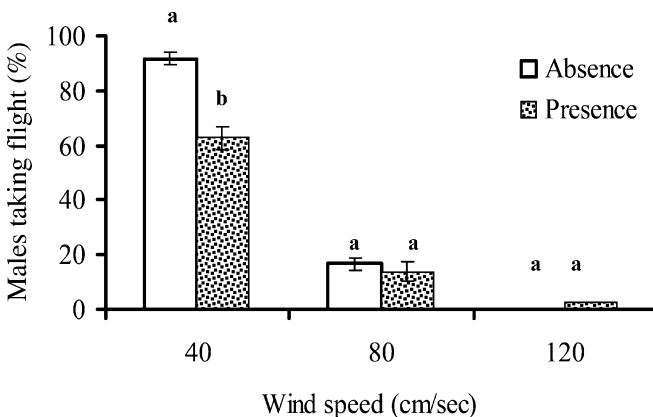


Fig. 2 Effect of wind speed on the proportion of *M. euphorbiae* males ($\bar{x} \pm$ SEM; three replicates of 15 individuals) taking flight in the presence or absence of calling sexual females in a wind tunnel at $18 \pm 0.5^\circ\text{C}$, 40–50% RH

Fig. 3 Effect of wind speed on the proportion of *M. euphorbiae* males ($\bar{x} \pm \text{SEM}$) orienting (a) and walking toward the source (b), and the time to reach the source in a wind tunnel at $18 \pm 0.5^\circ\text{C}$, 40–50% RH (c). The source was five calling oviparae 40 cm upwind of the release site

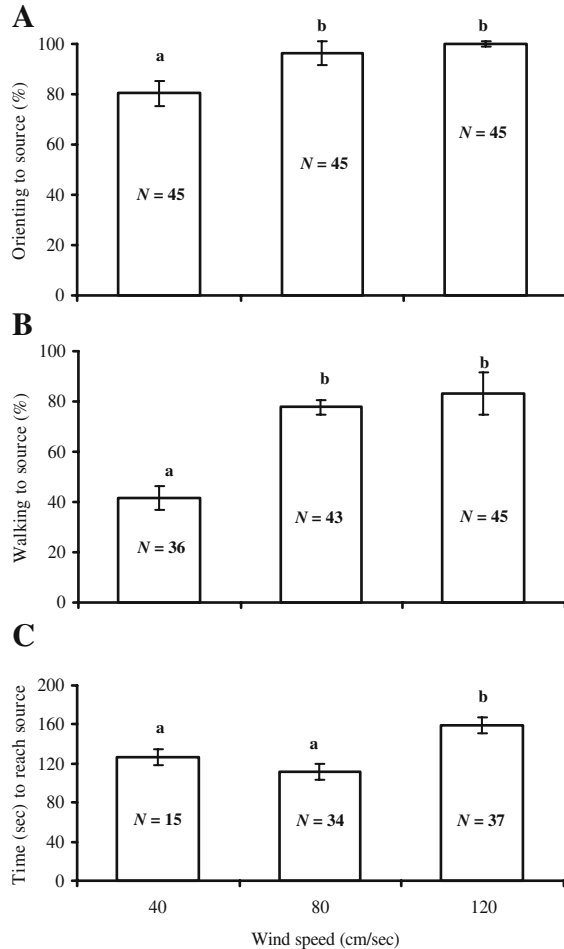


Table 1 Proportion of *M. euphorbiae* males responding to sexual females as a function of distance to the source at different wind speeds in a wind tunnel at $18 \pm 0.5^\circ\text{C}$, 40–50% RH

Wind speed (cm/sec)	Distance (cm)	Orientation ^a	Fly down wind ^a	Walk to source ^a	Time to reach source (sec)
40	40	80.5 ± 2.8 a	52.8 ± 2.8 a	41.7 ± 4.9 a	126.5 ± 6.6 a
	70	83.3 ± 8.5 a	61.1 ± 5.7 a	38.9 ± 5.7 a	204.4 ± 7.9 b
	100	80.5 ± 2.8 a	66.7 ± 4.9 a	30.1 ± 5.7 a	266.5 ± 9.2 c
80	40	94.4 ± 2.8 a	11.1 ± 2.8 a	77.8 ± 2.8 a	112.2 ± 1.6 a
	70	94.4 ± 2.8 a	13.9 ± 2.8 a	80.6 ± 2.8 a	148.1 ± 6.0 b
	100	88.9 ± 5.7 a	11.1 ± 5.7 a	72.2 ± 2.8 a	240.4 ± 10.2 c
120	40	100 ± 0.0 a	0 ± 0.0 a	83.3 ± 8.5 a	159.6 ± 11.2 a
	70	96.1 ± 2.8 a	2.8 ± 2.8 a	69.4 ± 2.8 a	331.0 ± 5.0 b
	100	88.9 ± 7.5 a	0 ± 0.0 a	72.2 ± 2.8 a	396.1 ± 6.0 c

^a For each wind speed, proportions with the same letter are not significantly different ($P < 0.05$).

Table 2 Two-way ANOVA results summarizing the effect of distances from source within a given wind speed on *M. euphorbiae* male behavior in a wind tunnel at 18 ± 0.5°C, 40–50% RH

Sources	Orientation			Fly down wind			Reach the source			Time to source (s)		
	df	F	P	df	F	P	df	F	P	df	F	P
Replicate	2,4	0.04	0.96	2,4	0.27	0.78	2,4	2.46	0.20	2,4	1.85	0.27
Wind speed (<i>W</i>)	2,4	5.56	0.07	2,4	121.32	<0.001	2,4	60.75	<0.001	2,4	111.45	<0.001
Distance from source (<i>D</i>)	2,12	1.77	0.21	2,12	1.03	0.39	2,12	3.25	0.07	2,12	176.93	<0.001
<i>W</i> × <i>D</i>	4,12	1.25	0.348	4,12	0.63	0.65	4,12	1.11	0.40	4,12	11.88	<0.001

a wind speed of 80 cm/sec took less time to reach the source than those tested at either 40 and 120 cm/sec (Table 1).

Under field conditions, females sustained calling behavior at variable wind speeds up to 4 m/sec, but above this level calling was rarely observed (Fig. 4a,b). A proportion of males exhibited downwind flight behavior if wind speeds did not exceed 2 m/sec, but above this velocity flight was inhibited (Fig. 4a,b). However, some males exhibited active walking behavior at wind speeds up to 4 m/sec, the upper limit at which females called (Fig. 4a,b). When males were tested in the presence of calling females, the behaviors observed in the field differed somewhat from those under laboratory conditions. As before, responding males oriented by moving to the upwind edge of the release cage/platform, and 52% of the 46 males tested still walked to the source. However, about 30% flew upwind to the calling females, with few flying downwind (Fig. 5). Detailed studies of male behaviors during the assay carried out on October 11 (Fig. 6) showed that males walked upwind when there were wind currents, but if the wind dropped to < 25 cm/sec (indicated as 0 in Fig. 6 reading

Fig. 4 Effect of wind speed on the proportion of *M. euphorbiae* oviparae calling and males taking flight ($\bar{x} \pm \text{SEM}$) under variable wind conditions in the field on September 15 (a) and 27 (b), 2001. Thirty females and 20 males were observed at each observation period

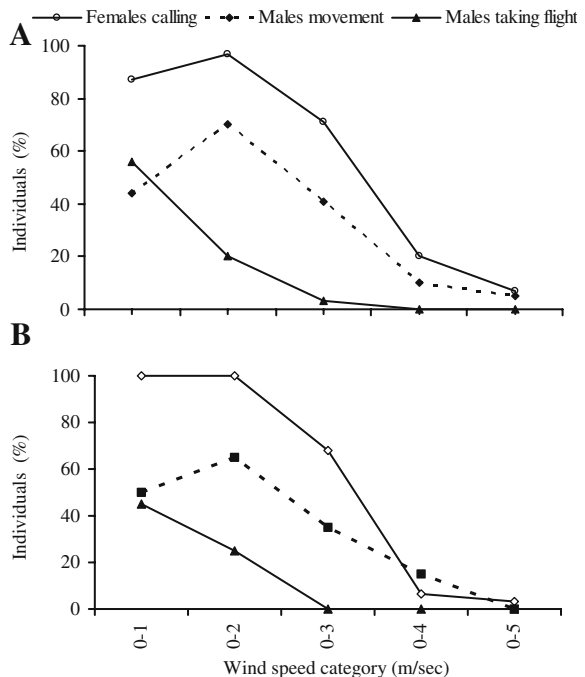
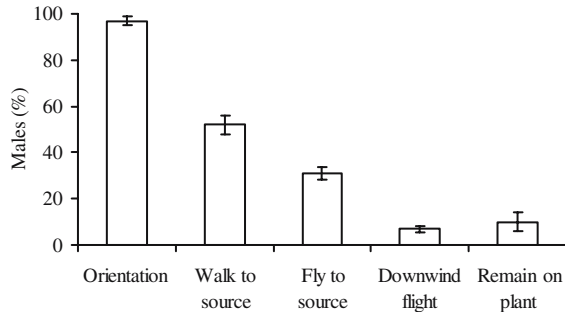


Fig. 5 Responses of *M. euphorbiae* males ($N = 46$; $\bar{x} \pm \text{SEM}$) to calling females located 40 cm upwind under variable wind conditions in the field assays carried out on October 11 and 24, 2001. Males could either fly or walk to the source



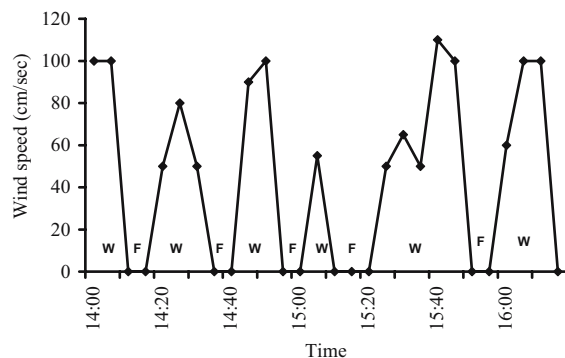
because of the sensitivity of the anemometer) during their approach toward the females they profited from the lull to take off and fly to the source. We verified this behavior experimentally in the laboratory by cutting off the wind when walking males were half way across the bridge: within seconds, the males ($N = 12$) took flight and moved in the “upwind” direction toward the calling females.

Discussion

Our results show that the wind speeds prevailing during late summer and fall could have a significant effect on the manner in which *M. euphorbiae* males forage for receptive females. Whether sex pheromone was present or not, male flight was inhibited at constant wind speeds > 1 m/sec in the laboratory, and under variable field conditions, if the maximum velocity exceed 2 m/sec. This is not surprising because many small insects stop flying at relatively low wind speeds (Juillet, 1964; Kring, 1972; Nealis, 1986; Kennedy, 1990; Fink and Völkl, 1995; Messing et al., 1997; Weisser et al., 1997; Marchand and McNeil, 2000). The values observed here for *M. euphorbiae* males are similar to those reported for flight by virginoparous alates of *Aphis fabae* in clean air (Kennedy, 1990), and *Cavariella aegopodii* to plant odors (Chapman et al., 1981).

Although the initiation (Dixon and Mercer, 1983) and termination (Thomas et al., 1977) of flight are active decisions, once in flight aphids are rather clumsy fliers, and movement is generally governed by prevailing wind direction (Dixon and Howard, 1986). Thus, under windy conditions, oriented flight toward a pheromone source may be ineffective and certainly energetically costly (see Keller, 1990). Prevailing environmental conditions

Fig. 6 Temporal pattern of wind velocity during the assay carried out on October 11, 2001, to test the response of *M. euphorbiae* males to calling females located 40 cm upwind under variable wind conditions in the field. The periods identified as W and F represent the conditions under which males either walked or flew to the source



fashion how animals search (Bell, 1991), so the evolution of walking as a major component of mate location in species which are not strong fliers would certainly be beneficial because the inability to fly during strong winds decrease a small insect's reproductive success (Weisser et al., 1997). Our laboratory and field experiments supported this hypothesis in the case of potato aphid males. First, both female calling and male ambulatory behaviors continued at winds approaching 4 m/sec, well above the threshold that inhibited male flight behavior. Second, at lower wind speeds, the proportion of males initiating downwind flight decreased for males orienting toward a pheromone source, which would not be expected if flight were the principal means of locating calling females. However, as seen in the field studies, flight is an integral component of aphid mate searching under appropriate conditions, providing a more rapid means of displacement toward receptive females and facilitating movement from branch to branch when branches are not in direct contact. Kennedy (1990) reported an acceleration in the incidence of “taking off” behavior in black bean aphid parthenogenetic alates following a period when flight was inhibited by winds of 2 m/sec. A similar behavioral postinhibitory rebound by males could play an important role in mate location and merits further investigation. The combined use of ambulatory and flight movement in response to varying environmental conditions has been demonstrated in other insects, such as the Colorado potato beetle (Boiteau, 2001) and parasitic wasps (e.g., Weisser et al., 1997), as well as with male moth responses to pheromone sources (e.g., Willis and Baker, 1987).

We used secondary (summer) rather than primary (fall) host for maintenance of our colonies, so another aspect of the mate location behavior of *M. euphorbiae* males that merits further investigation is the possible role of host plant volatiles. Clean potato plants, or those infested with parthenogenetic aphids, did not induce orientation and walking to the source, but these behaviors were elicited when males were exposed to oviparae on potatoes or just to synthetic pheromone lures (Goldansaz et al., 2004). It is probable that primary plant cues could increase these two responses, but just as the presence of the sex pheromone reduces the incidence of flight (Fig. 2), so the presence of these volatiles might reduce, rather than increase, the probability of take off by males.

It is likely that *M. euphorbiae* males use flight as the primary means of locating habitats where there are receptive females, possibly integrating olfactory cues such as primary host plant volatiles and the female sex pheromone, as suggested for other aphid species (Campbell et al., 1990, Hardie et al., 1994) and phytophagous insects (Landolt and Phillips, 1997). Gynoparae of the black bean aphid, *A. fabae*, appear to have an obligatory “migrant phase” in their maiden flight, flying for a significantly longer time before responding to visual cues that alate virginoparae (Nottingham and Hardie, 1989; Hardie, 1994). Whether male aphids, which also appear in the fall, show a similar tendency with respect to their response to host plant cues remains to be determined. However, prior sustained flight is not a prerequisite for male response to a pheromone source, because the potato aphid males used in our bioassays had no previous flight experience but nevertheless oriented to and approached calling females. However, because many males will be produced by females who are already on primary host plants, extended flight by males in search of receptive ovipare may not be necessary.

Species-specific differences may exist in the respective roles of flight and walking behavior in mate location in aphids. Under seminatural conditions, male hop aphids exhibited upwind flight to a pheromone source (Hardie et al., pers. comm.), but in the present study potato aphid males only flew toward the source during wind lulls. Therefore, comparative research on the behaviors associated with mate location in aphids will be required, within a meaningful ecological framework, to clarify species-specific differences

and to establish whether any generalized trends exist for the Aphididae. This should include detailed studies on the interactions between wind speed and distance from the source affecting male movement, for these relationships certainly are not straightforward (Tables 1 and 2). In addition, there is a need to examine how the complex canopy structure of primary host plants influences plume structure and, thus, the behaviors of aphid sexuals. Such experiments would provide information on the temporal and spatial aspects of the chemical cues actually reaching males and how male aphids respond to such variability, and possibly provide broader insight into the evolution of mate location strategies of insects with poor flight capabilities.

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Sustained Production of the Labile Pheromone Component, (Z,Z)-6,9-Heneicosadien-11-one, from a Stable Precursor for Monitoring the Whitemarked Tussock Moth

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Abstract The principal sex pheromone component of the whitemarked tussock moth (WMTM), *Orgyia leucostigma*, was recently identified as (Z,Z)-6,9-heneicosadien-11-one (Z6Z9-11-one-21Hy). However, it is thermally unstable and quickly degrades under field conditions so that baited traps are effective for only one night. We have developed a solution to this problem that combines two techniques: (1) the use of a stable pheromone precursor, (Z,Z)-6,9-heneicosadien-11-one ethylene ketal, which is hydrolyzed to the dienone by an acidic aqueous solution (2% *p*-toluenesulfonic acid in 35% aqueous sorbitol), and (2) use of a small, off-the-shelf, autonomous pump (the Med-e-Cell Infu-disk™) to deliver the precursor continuously to a suitable substrate where it is converted rapidly into the attractive dienone pheromone component. The pump and hydrolysis substrate fit inside sticky traps and because generation and release of pheromone is continuous, the instability of the pheromone is not an issue. In electroantennogram bioassays, dose-dependent responses were obtained with 1 to 1000 ng of hydrolyzed ketal on filter paper, but no response was obtained to 1000 ng of the ketal itself. In wind tunnel bioassays, males were attracted to lures emitting the dienone pheromone component generated from 0.1 to 100 ng of the hydrolyzed ketal. Field tests in 2004 and 2005 showed that sticky traps fitted with the pump delivering the ketal (0.1–1 µg/µL in heptane) at 10 µL/hr to a cotton pad soaked with the hydrolyzing solution were attractive to male WMTM. No moths were caught in controls or traps baited with (Z)-6-heneicosen-11-one. An average of 0.51 moths per trap night was caught over an 18-night period in 2005. The

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results represent a first step toward developing a sensitive and practical monitoring tool for the WMTM by using a ketal precursor of its unstable dienone pheromone component.

Keywords Labile pheromone · Propheromone · Infu-disk pump · *Orgyia leucostigma* · Tussock moth · Hydrolysis · Ketal · (Z,Z)-6,9-heneicosadien-11-one ethylene ketal · (Z,Z)-6,9-heneicosadien-11-one · (Z)-6-heneicosen-11-one · EAG · Wind tunnel

Introduction

The whitemarked tussock moth (WMTM), *Orgyia leucostigma* (J. E. Smith) (Lepidoptera: Lymantriidae), is a serious forest pest in eastern Canada. In 1998, over 5.9 million ha of balsam fir forest were severely defoliated or killed by an outbreak of WMTM in Nova Scotia (van Frankenhuyzen et al., 2002; Grant et al., 2003). Fruit crops were also threatened. In 2004, a new outbreak of WMTM was discovered in the balsam fir forests in the Cape Breton highlands of Nova Scotia (R. Guscott, Nova Scotia DNR, personal communication). This population increased substantially in 2005 and, along with two other balsam fir pests, caused visible defoliation. The infestation is now targeted for research and operational spray programs.

Whitemarked tussock moth populations in the maritime provinces of Canada have been monitored with traps baited with a commercial lure containing (Z)-6-heneicosen-11-one (Z6-11-one-21Hy). Although Z6-11-one-21Hy is produced by the WMTM female (Liu, 1999) and is attractive to males (Grant, 1977; Grant et al., 2003), few WMTM are caught, particularly at low population levels, owing to the lure's low potency. Thus, these monitoring traps were not effective in providing early detection of the aforementioned outbreaks in Nova Scotia.

A substantially more attractive sex pheromone component of WMTM was recently identified as (Z,Z)-6,9-heneicosadien-11-one (Z6Z9-11-one-21Hy; Liu 1999; Grant et al., 2003). It was effective at low doses (1 µg/lure) and low population levels, and it was more than 100-fold more attractive in laboratory bioassays than the minor pheromone component, Z6-11-one-21Hy, currently used for monitoring (Grant et al., 2003). Furthermore, the dienone pheromone component does not appear to attract other tussock moth species in North America, such as the rusty tussock moth, *Orgyia antiqua*, or *Dasychira* tussock moth species, which are attracted by Z6-11-one-21Hy (Daterman et al., 1976; Grant 1977) and which occur in the Maritimes. Hence, Z6Z9-11-one-21Hy would be a superior lure for detecting WMTM outbreaks.

Unfortunately, Z6Z9-11-one-21Hy is thermally unstable and degrades after one night in baited traps (Grant et al., 2003). This compound is also a key pheromone component for another economically important tussock moth species, the painted apple moth, *Teia anartoides*, in New Zealand (El-Sayed et al., 2005; Gries et al., 2005). Thus, a new type of lure is needed to overcome the limitations imposed by the compound's instability, so that baited traps will be effective throughout the flight season without requiring rebaiting. We have developed a solution to this problem that combines two techniques: (1) the use of a stable synthetic precursor or propheromone (Liu et al., 1984; Pickett et al., 1984) that can be readily converted to Z6Z9-11-one-21Hy and (2) the use of a small, off-the-shelf, autonomous pump to deliver the precursor continuously to a suitable substrate where it is converted into the dienone pheromone. Because the generation and release of Z6Z9-11-one-21Hy is continuous, its instability is not an issue. Here, we report the results of experiments that demonstrate the success of this approach.

Methods and Materials

Precursor Chemistry

After preliminary experimentation with two potential precursors of the WMTM dieneone pheromone component, we focused our attention on the ketal (*Z,Z*)-6,9-heneicosadien-11-one ethylene ketal (Fig. 1). Details of its synthesis and purification (>98%) will be described elsewhere (Liu, unpublished data). The ketal is readily converted to *Z,Z*9-11-one-21Hy by acidic hydrolysis (Fig. 1).

Wind Tunnel Bioassays

The effect of ketal concentration on pheromone production and WMTM male behavior was evaluated in a wind tunnel, 2 m long with a 1 × 1 m cross section, and a wind speed of about 0.35 m/sec (Grant et al., 2003). Bioassays were conducted between 10:30 and 13:30 hr, at 23–24°C and ambient relative humidity. Laboratory-reared males from the WMTM colony at the Great Lakes Forestry Centre were held under continuous light and assayed when 1–2-d-old. Males were transferred individually to small screen cages and conditioned in the wind tunnel for at least 1 hr before a test.

Ketal solutions were made up in 10-fold dilutions in hexane at concentrations from 10 to 0.01 ng/μL. Just before the start of a bioassay, 10 μL of a ketal solution were deposited onto a 3-mm-thick, 1 × 1 cm pressed cotton pad (Med-e-Cell, San Diego CA, USA) that had been treated 5–10 min beforehand with 600–700 μL of a 2% solution of *p*-toluenesulfonic acid in 35% aqueous sorbitol (Sigma-Aldrich, Oakville, ON, Canada). Sorbitol was included because it was used in field tests described below to retard evaporation of the solution on the substrate. The pad was then placed in a small nylon screen cage to facilitate handling. The cage was suspended from a wire support about 40 cm above the wind tunnel floor and about 10 cm from the upwind end of the tunnel. Bioassays were started within 10 min of treating the pad with the ketal solution and lasted about 1–1.5 hr.

Caged males were introduced one at a time into the pheromone plume. They were held there until they began wing-fanning behavior and then were released about 20 sec later. If a male failed to become active after 60 sec, a new male was tested. The following behavioral events were recorded: (1) the number of males that began wing fanning when placed in the pheromone plume, (2) the number of males that “locked on” to the plume when released, (3) the number of males that reached (touched) the lure after being released and the time required to do so, and (4) whether or not the male landed on the lure. Thirty males were tested with each dosage of hydrolyzed ketal. To accommodate the small number of males available per day and to simplify the handling of lures, males were tested against only one or two dosages per day until 30 males per dosage had been tested. Controls consisted of cotton pads treated only with ketal at dosages of 1, 10, 50, or 500 ng. Five males were tested against each dosage.

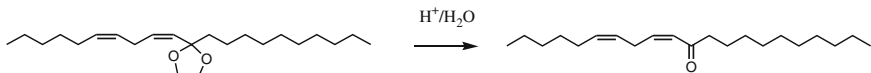


Fig. 1 Hydrolysis of (*Z,Z*)-6,9-heneicosadien-11-one ethylene ketal with an acidic aqueous solution (2% *p*-toluenesulfonic acid in 35% aqueous sorbitol) generates the labile WMTM pheromone component, (*Z,Z*)-6,9-heneicosadien-11-one

Electroantennogram Bioassay

Antennal responses from male WMTM to hydrolyzed ketal (i.e., to Z6Z9-11-one-21Hy) were obtained by electroantennogram (EAG) bioassays. Adults (1–2-d-old) were decapitated and the head and antennae fastened with thin strips of masking tape to a small platform. Glass electrodes filled with 0.9% NaCl were inserted into the distal and proximal segments of an antenna. Gold wires from each electrode were connected to a Syntech portable INR-2 amplifier (Syntech, Hilversum, NL), which was connected in turn to a personal computer containing a Syntech data acquisition interface board (type IDAC-02) and Syntech EAG software for recording and analyzing the EAG responses.

Test stimuli were applied to filter paper strips (1.8×0.8 cm) held in glass tubes (8×0.5 cm id) made from disposable Pasteur pipettes. Pipettes were connected to an airstream (500 mL/min) produced by a tank of high-purity compressed air. A 1.5 sec puff of air, controlled by a solenoid-actuated valve, passed through each glass tube containing a stimulus and over the antenna. The following stimuli were tested: blank filter paper; 15 μ L of the ketal hydrolyzing solution (2% *p*-toluenesulfonic acid in 35% aqueous sorbitol) on filter paper; 1000 ng of the ketal (dry) on filter paper; and filter paper strips dosed first with 1, 10, 100, or 1000 ng of ketal and then treated with 15 μ L of the hydrolyzing solution. The latter were tested successively within 30 sec of applying the hydrolyzing solution. They were reused once with a second antenna some 10–15 min later. A new set of stimuli was prepared for the next set of antennae. Each stimulus was puffed over an antenna at about 1-min intervals in the order given above.

Field Tests

We used a small, commercially available autonomous pump, the Infu-disk™ manufactured by Med-e-Cell (<http://medecell.com/>), to deliver the ketal precursor to a hydrolyzing substrate and generate the pheromone component, Z6Z9-11-one-21Hy. The pump operates by means of an electrochemical cell that is activated manually just before use. The pump forces liquid from a reservoir (5 and 10 mL capacities are available) at a preset rate. Experiments were conducted with the 5 mL Infu-disk (about 4.1 diam \times 1 cm) with a delivery rate of 10 μ L/hr (0.01 mL/hr), which provided an operational life of about 21 d. A few pumps with a higher delivery rate of 30 μ L/hr were also tested to provide a threefold higher level of pheromone for comparison although these pumps had a correspondingly shorter operational life.

A short plastic delivery tube, which emerges from the top of the Infu-disk, terminates with either a metal canula tip or a male luer tip to which a 20- or 22-gauge syringe needle can be attached (Fig. 2A,B). In either case, the end of the delivery tube was attached by means of staples to a 3-mm-thick, 2×7 cm cotton pad (Med-e-Cell) that served as the hydrolysis substrate (Fig. 2A). Care was taken to ensure that the opening of the canula or syringe needle was in contact with the pad but not obstructed by it. When readied for use, the pump reservoir was filled with the ketal solution by means of a syringe, the electrochemical cell was activated, and the pump was then taped into the thin plastic shell used for shipping the pump. The unit was attached to a Pherocon IC sticky trap by passing its wire hanger although the shell before the trap bottom was attached so that the pump and substrate could sit inside the trap (Fig. 2A). When traps were set out, the reaction substrate was soaked with the solution of 2% *p*-toluenesulfonic acid in 35% aqueous sorbitol applied with a syringe. In this initial field trial, we were interested in assessing how long the hydrolyzing substrates remained moist and active, so they were checked for dryness and, if deemed necessary, an aliquot of the solution was added to the pad to remoisten it.

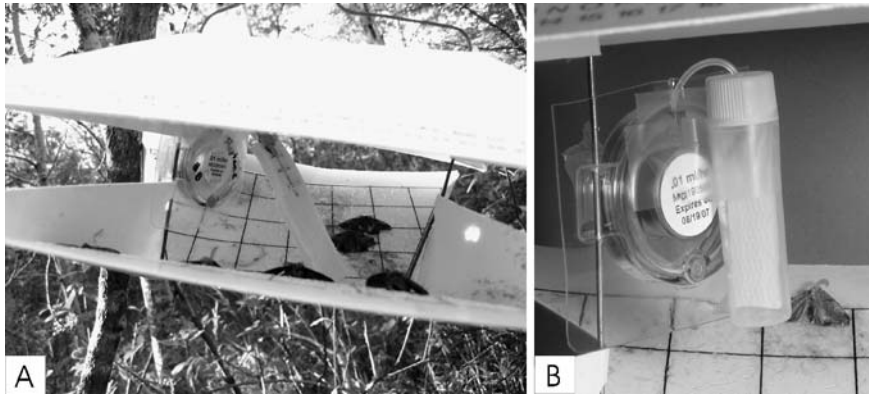


Fig. 2 (A) Pherocon IC sticky trap fitted with a ketal-containing, 5-mL Infu-disk pump connected via a syringe needle to a 1×7 cm cotton pad (hydrolysis substrate), which has been soaked with a solution of 2% *p*-toluenesulfonic acid in 35% aqueous sorbitol. (B) Ketal-containing Infu-disk pump fitted with 6-mL plastic vial containing the cotton pad and several milliliters of hydrolyzing solution. The pump and vial are taped to the thin plastic shell used to ship the pump. The delivery tube from the pump enters the vial through one of the five holes drilled in the cap and is attached to the cotton pad inside. The dienone pheromone component escapes through the perforated cap

The field test in 2004 was conducted late in the WMTM flight season between 24 August and 20 September in four mixed deciduous stands in Sault Ste. Marie, ON, known to harbor low, endemic populations of WMTM. The test was exploratory and designed to determine if our preliminary set of pump parameters and ketal concentrations would produce sufficient pheromone in the traps to attract male moths. Heptane and 2-propanol were tested as ketal solvents because they have a low vapor pressure, reducing the potential for back pressure in the pump, and they were compatible with its plastic components. Ketal concentrations of 0.1 and 1.0 $\mu\text{g}/\mu\text{L}$ (1 and 10 mg/mL) were selected to match the 10 $\mu\text{L}/\text{hr}$ delivery rate of the pump and provide an estimated 0.28 and 2.8 ng/sec , respectively, of Z6Z9-11-one-21Hy on the substrate. Because the test was exploratory, traps were deployed and taken down on different days. They were inspected daily, and moths were counted and removed. This procedure allowed us to confirm that pheromone was being produced continuously during the test. Comparisons were made with unbaited control traps ($N = 3$) and traps ($N = 5$) baited with 200 μg of (*Z*)-6-heneicosen-11-one (Bedoukian Research, Danbury CT, USA) in polyethylene vial closures (caps; no. 60975d-3 Kimball).

By using the results of the 2004 test as a guide, we selected 5 mL Infu-disks containing ketal at a concentration of 0.35 $\mu\text{g}/\mu\text{L}$ in heptane, and a delivery rate of 10 $\mu\text{L}/\text{hr}$ for the 2005 field test. This concentration of ketal was chosen as a reasonable compromise between the 0.1 and 1.0 $\mu\text{g}/\mu\text{L}$ concentrations tested in 2004. Although heptane is immiscible with water (on the hydrolysis substrate), heptane appeared as effective as 2-propanol in the 2004 tests, and hexane was acceptable as the ketal solvent in the flight tunnel bioassays.

In 2005, the cotton pad used as the hydrolysis substrate was enclosed in a 6-mL plastic vial to serve as a reservoir for the hydrolyzing solution to keep the substrate uniformly moist for the duration of the test. Five 5-mm holes were drilled in the vial cap to allow pheromone to diffuse out. The delivery tube of the Infu-disk was threaded through one of the holes in the cap and attached to the cotton pad (about 4.5×1 cm) with staples. The pad was then inserted into an empty vial, which was screwed onto the cap. The pump was activated, placed in its plastic shell, and the vial and pump were taped to the shell. The

entire unit was attached to the wire hanger of the Pherocon IC sticky trap (Fig. 2B). About 4 mL of the hydrolyzing solution were syringed into the vial through a hole in the cap when the trap was hung in the field.

Traps were deployed at seven sites in Sault Ste. Marie on 12 and 13 July 2005. Six sites each received a trap baited with the ketal-loaded Infu-disk matched with a second trap baited with 100 μg of Z6-11-one-21Hy for comparison. Traps were positioned about 25 m apart in each site. The seventh site received two ketal-baited traps and two traps baited with Z6-11-one-21Hy. With one exception, traps were taken down on 30 July. The exception involved one of the ketal-baited traps at the seventh site. This trap had failed to catch any moths between 13 and 26 July in contrast to the other ketal-baited trap at this site. Its pump was not functioning, so the pump was replaced on 26 July with a fresh ketal-loaded pump and allowed to operate until 19 August 2005.

Statistical Analysis

Means are shown with standard errors ($\pm\text{SE}$). The percentages of males successfully reaching different dosages of the lure in the flight tunnel bioassays were compared with the 1-ng dosage with the use of the Fisher exact test (Zar 1984). Mean EAG responses to the nonhydrolyzed ketal and blank filter paper were compared with the paired *t*-test (Zar 1984).

Results

Wind Tunnel Bioassay

Hydrolysis of the ketal at all dosages tested resulted in $>85\%$ of the males initiating wing-fanning behavior, and all but the 0.1 ng dosage induced a high percentage (77–90%) of males to lock on to the plume and fly upwind (Fig. 3). More males (83%, $P < 0.001$, Fisher

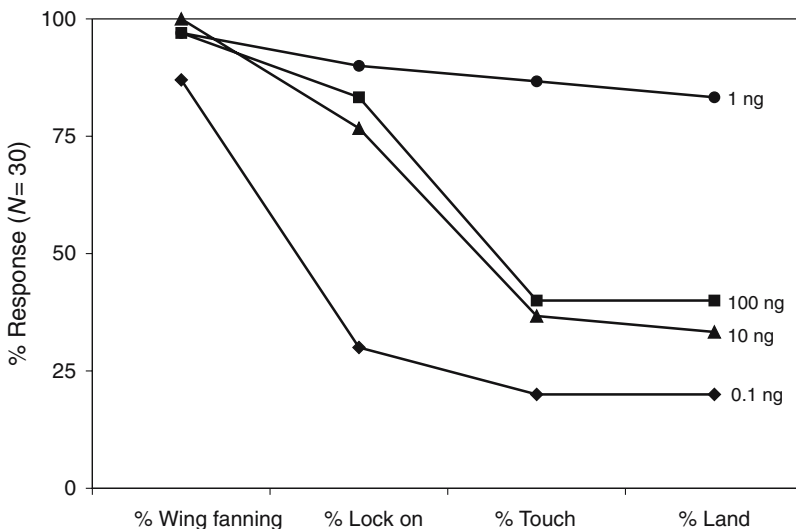


Fig. 3 Results of wind tunnel bioassays showing the effect of different dosages of the hydrolyzed ketal on the behavioral responses of male WMTMs

exact test for each comparison with the 1 ng dose) reached the 1 ng dosage lure and landed on it than with the other three dosages tested. The 0.1 ng dose apparently generated too little pheromone because only 30% of the males locked onto the pheromone plume and flew upwind. The two higher ketal doses (10 and 100 ng) apparently generated too high a level of pheromone. Males released into the higher-dose plumes usually locked on to the plume but then remained at or near the release point, casting from side to side before making slow, hesitant progress toward the lure. Otherwise, males in the high-dosage plume drifted slowly backwards from the release point, until they went out the back of the flight tunnel. No males were activated or took flight when exposed to substrate treated only with the ketal.

EAG Bioassay

There was no difference in the mean EAG response to 1000 ng of the dry ketal on filter paper (0.05 ± 0.01 mV, $N = 8$) and the mean EAG response to blank filter paper (0.06 ± 0.01 mV, $N = 8$; $P = 0.65$, paired t -test). In contrast, dose-dependent responses were elicited by the ketal on filter paper when treated with the hydrolyzing solution (Fig. 4). Because no significant EAGs were obtained in response to the ketal by itself or to the hydrolyzing solution (0 ng; Fig. 4), the response to the hydrolyzed ketal was due to the hydrolysis product, Z6Z9-11-one-21Hy.

Field Tests

All ketal-baited traps in the initial trial in 2004 caught WMTM (Table 1). Traps fitted with pumps loaded with a ketal concentration of $1 \mu\text{g}/\mu\text{L}$ delivered at $10 \mu\text{L}/\text{hr}$ caught more moths than similar traps with pumps loaded with $0.1 \mu\text{g}/\mu\text{L}$ ketal delivered at the same rate. Catches in traps fitted with pumps delivering ketal at $30 \mu\text{L}/\text{hr}$ (presumably producing a

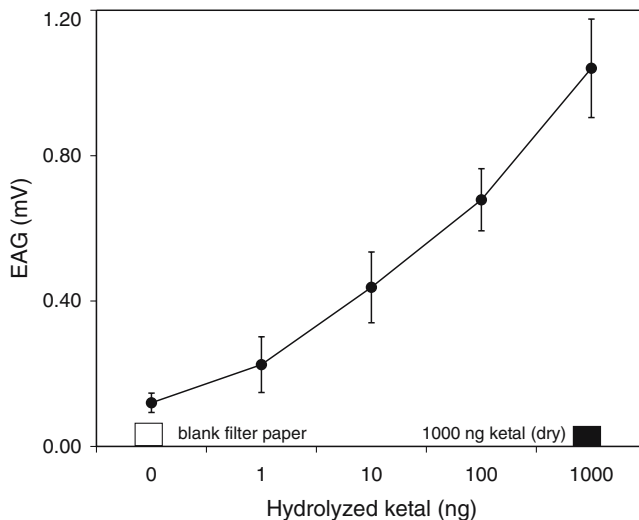


Fig. 4 Mean (\pm SE) EAG responses from male WMTM antennae ($N = 9$) to various doses of ketal on filter paper following hydrolysis with $15 \mu\text{L}$ of 2% *p*-toluenesulfonic acid in 35% aqueous sorbitol. The 0 dosage = $15 \mu\text{L}$ of the hydrolyzing solution only on filter paper. EAG responses ($N = 8$) to blank filter paper and 1000 ng ketal (dry) on filter paper are shown by columns at bottom left and right of the figure, respectively

Table 1 Catch of WMTM in Pherocon IC sticky traps fitted with ketal-loaded Infu-disk pumps operated under various conditions, Sault Ste. Marie, ON, 24 August–20 September 2004

	Site								
	A	A	A	A	B	B	C	C	D
Delivery rate ($\mu\text{L/hr}$)	10	10	10	10	10	10	30	30	30
Ketal concentration ($\mu\text{g}/\mu\text{L}$)	0.1	0.1	1	1	0.1	1	0.1	1	1
Ketal solvent ^a	2-P	2-P	2-P	2-P	2-P	H	H	H	2-P
Trap nights	23	23	23	23	20	19	6 ^b	9 ^c	8 ^c
Total WMTM	1	3	10	12	2	12	5	4	2
Mean ^d moths/night	0.04	0.13	0.43	0.52	0.10	0.63	0.83	0.44	0.25

^a Solvents were 2-propanol (2-P) and heptane (H).

^b Ketal reservoir was exhausted by the 6th day.

^c Ketal reservoir was exhausted by the 7th day. One trap caught one WMTM on the 8th day.

^d Standard errors were not shown because of large number of zero catches.

higher level of pheromone) were no better than traps with the lower delivery rate of $10 \mu\text{L/hr}$. Also, as expected, their operational life was considerably shorter (6–7 d; Table 1) and, thus, not as practical for monitoring purposes. Heptane and 2-propanol appeared to be equally suitable as solvents for the ketal. All but one trap had catches on more than one night, indicating that Z6Z9-11-one-21Hy was being generated throughout the experiment. The cotton pads in two traps that ran for ≥ 19 d required an additional aliquot of the hydrolyzing solution during the course of the test, whereas the pads in the remaining four traps were almost dry at the end of the test. No WMTM were caught in the unbaited control traps or traps baited with Z6-11-one-21Hy, although rusty tussock moths, *O. antiqua* (0.96 moths/trap night), were caught in the latter.

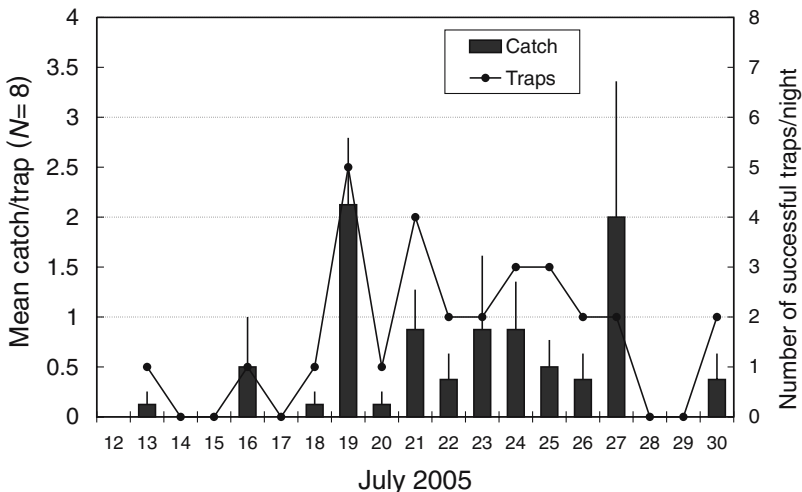


Fig. 5 Mean (\pm SE) daily catch of WMTMs in sticky traps fitted with Med-e-Cell Infu-disk pumps delivering $0.35 \mu\text{g}/\mu\text{L}$ ketal at the rate of $10 \mu\text{L/hr}$ to a cotton pad substrate treated with 2% *p*-toluenesulfonic acid in 35% aqueous sorbitol. The right-hand axis shows the number of traps per night that successfully caught moths

For the 2005 field test, we selected 5 mL Infu-disks containing ketal at a compromise concentration of 0.35 $\mu\text{g}/\mu\text{L}$ in heptane and a delivery rate of 10 $\mu\text{L}/\text{hr}$. All but one malfunctioning trap caught moths by 19 July and all but this one trap caught moths on three or more nights between 13 July and 30 July (Fig. 5). After the pump in the inactive trap was replaced on 26 July, this trap caught moths on 27 and 30 July when the test ended (except for this trap). A total of 74 WMTM were caught in eight traps over 18 nights, an average 0.51 moths/trap night. All the vials still contained the hydrolysis solution at the end of the trial and the cotton pad substrates inside were still completely wet. None of the traps baited with the Z6-11-one-21Hy caught WMTM, but rusty tussock moths were again caught in these traps.

The trap in which the ketal pump was replaced on 26 July subsequently caught moths on 18 out of the 24 nights between 26 July and 19 August, with an average catch of 4.8 moths/night. On the nights of 3, 4, and 5 August, this trap caught 17, 21, and 17 moths, respectively, suggesting that the peak WMTM flight occurred after the termination of the earlier part of the test on 30 July.

Discussion

Several studies have examined the potential for using pheromone precursors or propheromones to address various types of pheromone instability (Beever et al., 1977; Liu et al., 1984; Pickett et al., 1984; Streinz et al., 1993). Typically, these compounds were converted to the active pheromone by exposure to air or light. Our approach required hydrolysis of a ketal precursor and was dependent on finding a suitable device for delivering the precursor to the hydrolysis substrate, such as the Infu-disk pump. A different version of this device had been used in an experimental trial to emit the antiaggregation pheromone, 3-methyl-2-cyclohexen-1-one, to control attacks of the spruce beetle, *Dendroctonus rufipennis* (Holsten et al., 2003). The appeal of the pump device in that case was its constant delivery rate. In our case, the small size, autonomous operation, the 5 and 10 mL reservoir capacities, and the low delivery rates of the Infu-disk were qualities well suited for use in a monitoring trap. The challenge was to match the ketal concentration to the delivery rate of the Infu-disk pump to provide a level of Z6Z9-11-one-21Hy attractive to WMTM under field conditions.

The 2004 field trials showed that ketal concentrations between 0.1 and 1 $\mu\text{g}/\mu\text{L}$ delivered at a rate of 10 $\mu\text{L}/\text{hr}$ to the hydrolyzing substrate were effective. In that trial, the cotton pad substrates dried out as the trial proceeded. When some of these substrates were dried completely in the laboratory and then retreated with the hydrolyzing solution, they attracted males in the flight tunnel, indicating that not all the ketal reaching the substrate had been converted to the dienone pheromone. The wind tunnel and EAG bioassays indicated that the ketal by itself was not bioactive unless hydrolyzed. We improved the substrate system by enclosing the cotton pad in a perforated 6-mL vial that served as a reservoir for the aqueous solution, thereby keeping the substrate wet throughout the trial.

The seemingly low rate of catch (0.51 moths/trap night) in 2005 with the dienone-baited traps was probably a reflection of the low endemic population of WMTM (no larvae or cocoons were ever visible) and the deployment of the experiment before the usual flight peak in August. The one trap left operating in August had higher catches. Further increases in trap catch might be obtained by optimization of the emission of the dienone from traps through improvements in the pump design or its configuration with the hydrolysis substrate.

Nonetheless, males were caught consistently in the dienone traps on a nightly basis over the test period, and they outperformed traps baited with 100 µg of Z6-11-one-21HY, which caught no moths. In 1975, in the same test area, sticky traps baited with 100 µg of Z6-11-one-21HY in polyethylene caps caught an average of 0.84 moths/trap night, whereas female-baited traps caught 1.95 moths/trap night (Grant 1977). Larvae, cocoons, and egg masses of WMTM were visible on trees and buildings that year (unpublished observations), indicating a much higher WMTM population level than in 2005. This suggests that sticky traps emitting the dienone by hydrolysis of the ketal are a more sensitive method of monitoring WMTM than traps baited with Z6-11-one-21HY.

The response of males in the flight tunnel to hydrolyzed ketal had a narrow dose–response profile with respect to males reaching the lure. This narrow dose–response profile could reflect the absence of the minor pheromone components Z6-11-one-21HY or (*Z,E*)-6,8-heneicosadien-11-one (Liu 1999), although field tests did not show that these components combined with the dienone component increased attraction of males (Grant et al., 2003). The availability of a method for generating the dienone on a continuous basis, however, provides an opportunity to revisit this question with a more stable level of the dienone component. Also, the unstable dienone quickly degrades to several isomers (Gries et al., 1997, 2005; Grant et al., 2003; El-Sayed et al., 2005). Their presence in the pheromone plume could have adversely affected male attraction in the wind tunnel, particularly when the dose of the dienone was high. Finally, the hydrolysis reaction is rapid, because males began wing fanning a few seconds after the ketal was added to the hydrolyzing substrate. Thus, the reaction could produce an initial burst of dienone that for high-dosage lures may be too concentrated for the males. A longer delay before testing the dienone lures to allow for equilibration may improve the male's responses to the high-dosage lures.

Our experiments combining a ketal precursor of the unstable dienone pheromone component, Z6Z9-11-one-21Hy, with an autonomous pump are a first step toward developing a practical monitoring system for WMTM. This system may also prove useful for other species utilizing the same dienone pheromone component. The Infu-disk is also available with a 10-mL reservoir, which has a theoretical life of about 42 d with a 10 µL/hr delivery rate, and which may be more useful for monitoring traps. This slow release system also may offer a potential means of incorporating the unstable diene pheromone component into mating disruption programs for the WMTM (Grant 1978) and the painted apple moth (Suckling et al., 2002).

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Introduction

Oryctes monoceros Oliv. (Coleoptera, Scarabaeidae, Dynastinae) is one of the main pests in the coconut and oil palm plantations of tropical Africa (Lever, 1969; Mariau, 1981), particularly in Ivory Coast (Echimane et al., 1992). The Asian species, *Oryctes rhinoceros* L., which is found throughout the Asia-Pacific area, has a similar ecology and is equally harmful to cultivated palms (Waterhouse and Norris, 1987). Damage is generally caused by adults making feeding galleries in the apical section of young palms, but also of mature palms when populations are large. Larvae develop in decaying dead palm wood. Damage has increased over the last 20 yr as a result of large-scale replanting programs, which leave substantial numbers of felled stems on the ground, multiplying suitable sites for egg laying and larva development (Julia and Brunin, 1974). Periodic surveys carried out in plots at the Marc Delorme research station in Port Bouet (Ivory Coast) have revealed up to 40% damage in 0- to 5-yr-old oil palms (Mariau, 1981). In Africa, damage is worsened through attacks by *Rhynchophorus phoenicis* (Fabr.), which lays eggs in the galleries made by *O. monoceros* and often kills palms.

Removing or burning felled stems, or covering them with a legume, *Pueraria javanica* (Benth.), to prevent larval infestation has been recommended (Mariau and Calvez, 1973); however, these measures are rarely applied because of cost and the high planting density of palms (130–160/ha). Biological control has also been studied, but no pathogen (not even *Baculovirus oryctes*, which is quite active against *O. rhinoceros*) or parasitoid tested has provided long-lasting action against *O. monoceros* (Huger, 1966; Julia and Mariau, 1976a; Purrini, 1998; Seguni et al., 1999). The only control method currently used is manual extraction of adults from feeding galleries and insects (especially larvae) present in decaying old stems. This is highly labor intensive and is impossible in tall standing palms. Frequent insecticide applications are only possible in agroindustrial sectors, but they entail serious environmental risks (Echimane et al., 1992).

Other ways have been explored to control this pest, in particular mass trapping with attractants. Ethyl chrysanthemumate was rapidly abandoned because of insufficient catches (Julia and Mariau, 1976b). More recently, ethyl 4-methyloctanoate (**1**), reported as the male aggregation pheromone of *O. monoceros* (Gries et al., 1994) and *O. rhinoceros* (Hallett et al., 1995; Morin et al., 1996), proved to be a powerful attractant for *O. rhinoceros* (Purba et al., 2000). In a preliminary study, it also showed promise for *O. monoceros* (Allou et al., 2002).

We conducted trials in Ivory Coast to determine if the advances made for *O. rhinoceros* control (Morin et al., 2001a; Sudharto et al., 2001) could be applied to *O. monoceros*. We focused our assessment on: (1) the plant material [oil palm empty fruit bunches (EFB) and coconut wood (CW)] as co-attractants with **1**, (2) the efficiency of two types of traps, and (3) the study of 4-methyloctanoic acid (**2**), an untested molecule regularly emitted by *O. monoceros* males (Gries et al., 1994; Rochat et al., 2004).

Materials and Methods

Attractants

Palm material: About 15 L of plant material were used per trap. We tested the following: (1) 1-wk-old oil palm EFB obtained after processing in the oil mills and (2) CW disks

(6 cm thick and 30 cm diam) from 40- to 60-yr-old trees that we cut and left on the ground under natural conditions for 0.5, 1, 1.5, 2.5, and 3.5 mo before testing. The disks were sprayed with water from time to time during the dry season. For a given age category, three disks were cut into four pieces and placed in the trap. Plant material was not renewed once the trials had started.

Synthetic Chemicals

Ethyl 4-methyloctanoate: *O. monoceros* pheromone (**1**) and 4-methyloctanoic acid (**2**; 99% chemical purity by gas chromatography; racemic mixtures) were synthesized by EGNO-Chimie (Tancarville, France). The dispensers used were sachets made from high-density polyethylene (Alplast, Sainte-Marie-aux-Mines, France). Compound **1** was formulated in model D dispensers (25 × 50 mm, 200- μ m-thick film) containing about 1 mL of pheromone, which emitted 13–14 mg/d under field conditions in an earlier assessment (Allou et al., 2002). Compound **2** was formulated in dispensers made of 100- μ m-thick film. Four models were used: d1, 25 × 50 mm; d2, 12.5 × 50 mm; d3, 12.5 × 12.5 mm; and d4, 6.2 × 12.5 mm. To estimate average daily release rates, all dispensers were filled to last >1.5 mo and were weighed monthly and quarterly in trials 1–5, and 6, respectively.

Traps

Two types of trap were used. One is the pail trap that consisted of a covered 30-L plastic bucket with five holes (diam 5 cm) in the lid. The pheromone dispenser was suspended from the lid. Another type, the tube trap, based on the model described by Morin et al. (2001b), consisted of a vertical polyvinyl chloride (PVC) tube (height 2 m, diam 16 cm) with two apertures (20 × 10 cm) in its upper section 140 cm from the ground. The top of the tube was open while the base stood in a recipient with holes for rainwater drain-off. The dispenser was hung inside the tube, above the two apertures, and protected from direct sunlight with a piece of cardboard.

Trials

The study consisted of six trials conducted by using the same layout and procedure. They comprised eight randomized blocks 100 m apart, and within each block the traps, hung on poles 1.5 m from the ground, were spaced 50 m apart. They were inspected every 3 d and rerandomized every 6 d. Trial 5 compared both types of traps.

The majority of the trials were carried out at the Robert Michaux plantation, in Dabou, 65 km west of Abidjan. The traps were installed in a 19-ha plot, a coconut seed garden, located in the middle of a 4000-ha oil palm estate, planted in 1977 with the Malayan Yellow Dwarf variety. However, trial 3 was conducted in a 12-ha experimental coconut plantation in Assinie (30 km east of Abidjan on the coast) planted in 1999 with PB 121 hybrids combined with tree legumes, *Acacia mangium* and *Acacia auriculiformis*.

- Trial 1 (effect of addition of EFB to **1**) was carried out from 24 January to 21 February 2002, and compared the following three treatments: (1) **1** only, (2) **1** + EFB, and (3) EFB only.
- Trial 2 (comparative effects of EFB and CW with **1**) was conducted from 16 September to 16 October 2002, and comprised six treatments: (1) **1** only, (2) **1** + EFB, (3–6) **1** + 3.5-, 2.5-, 1.5-, and 0.5-mo-old CW, respectively.

- Trials 3 and 4 assessed the effect of 2 with 1 + EFB. Trial 3 was carried out from 2 August to 1 September 2002, with five treatments: (1) 1 + EFB, (2–5) 1 + EFB + 2 emitted from dispensers d1, d2, d3, and d4, respectively. Trial 4 was conducted from 23 January to 22 February 2003, with the same protocol but with an additional treatment, (6) EFB + 2 from dispenser d1.
- Trials 5 and 6 compared the effectiveness of both types of traps (pail and tube) with 1 + either EFB or CW. They comprised four treatments: (1) 1 + EFB in the pail trap, (2) 1 + EFB in the tube trap, (3) 1 + CW in the pail trap, and (4) 1 + CW in the tube trap. Trial 5 took place from 19 September to 22 December 2003, and plant material was not renewed. In trial 6, EFB and CW were renewed each month and every 6 mo, respectively. Pheromone dispensers were renewed every 3 mo in both trials. In trial 6, bait renewal was postponed by ± 10 d on some occasions because of unforeseeable events. Trial 6, started on 7 April 2004, was set up for pest control and served, secondarily, to assess the monthly renewal of EFB (as compared to trial 5), and, as it is ongoing, we present 15 mo of results (last data from 1 July 2005).

Effect of Trapping on Coconut Damage

The coconut seed garden at the Dabou plantation had been planted to produce hybrid seeds but was abandoned in the 1990s. In 2000, it was decided to restore it to its initial function, and new management practices, including monthly inspections, were introduced to monitor the phytosanitary condition of the palms. A report is prepared yearly, including the percentage of palms killed by combined *O. monoceros* and *R. phoenicis* (Coleoptera, Curculionidae) attacks. From January 2000 to December 2001, no special effort was made to control the beetle due to the large size of the palms and the absence of traps. However, damage was high, with surrounding oils palms a constant source of beetles, so it was decided to start pheromone trapping as a control option with trials 1–2 and 4–6 starting in January 2002. At that time, 24–48 traps (1.2–2.5 traps/ha) were installed in the block, on three occasions for 1 mo, in 2002 and early 2003 (trials 1, 2, and 4). Thirty-two traps (1.7 per ha) were installed for another 12 mo, then permanently from April 2004 (trials 5–6). The effect of olfactory trapping was assessed by studying annual trends in coconut palm mortality over a 5-yr period (2000–2004).

Statistical Analyses

In all the trials, the individual in each trap were pooled together over 6-d periods. Trials 1–4: The effect of the treatments was assessed by ANOVAs on values transformed by $(x + 0.5)^{1/2}$. Differences in average catches were assessed by Newman–Keuls multiple comparison tests at 5% (Statitcf Software, 1991). Trials 5–6: Data were analyzed by using a multifactor ANOVA with a GLIM procedure after transformation by $\ln(x + 1)$. The experimental factors were trap type (pail or tube), the kind of palm coactractant (EFB or CW), and the block. The trapping period was an additional factor included in the model, with corresponding interactions for trial 5. The average ages of the baits after installation (EFB, CW, and pheromone dispensers) were introduced as covariables, as catches clearly fluctuated periodically in trial 6 (Fig. 4). Differences in average catches were assessed by Tukey multiple comparison tests (Minitab v12.2, 1998).

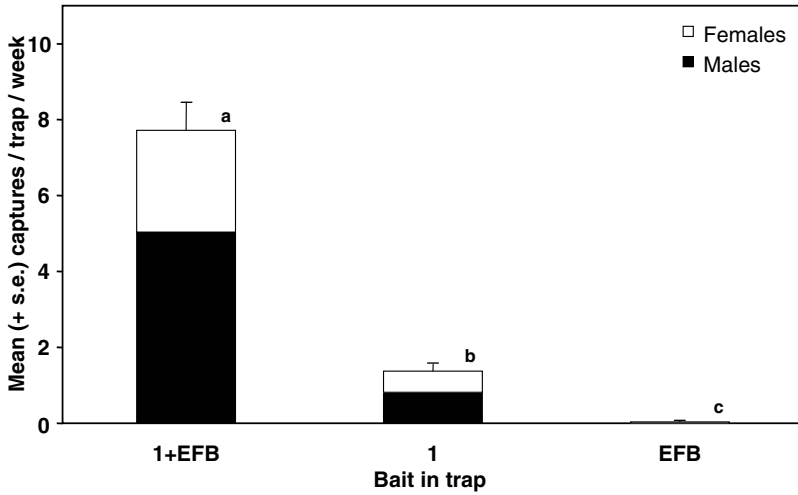


Fig. 1 Comparative catches of *O. monoceros* in 30-L pail traps baited with synthetic pheromone: ethyl 4-methyloctanoate (**1**) diffused at a rate of 15 mg/day, 1-wk-old oil palm EFB, or the two combined (trial 1). Treatments with different letters differed significantly (Newman–Keuls test at $\alpha = 0.05$ on $(x + 0.5)^{1/2}$ transformed data)

Results

Compound **1** was emitted at 12.7 ± 1.9 mg/d (mean \pm S.D.; $N = 384$) over all trials, with a minimum of 9.1 mg/d in trial 5 to a maximum of 15.2 mg/d in trial 1. Compound **2** was emitted at 3.8 ± 0.5 , 2.4 ± 0.6 , 1.3 ± 0.4 , and 0.6 ± 0.2 ($N = 16$) from dispensers d1, d2, d3, and d4, respectively.

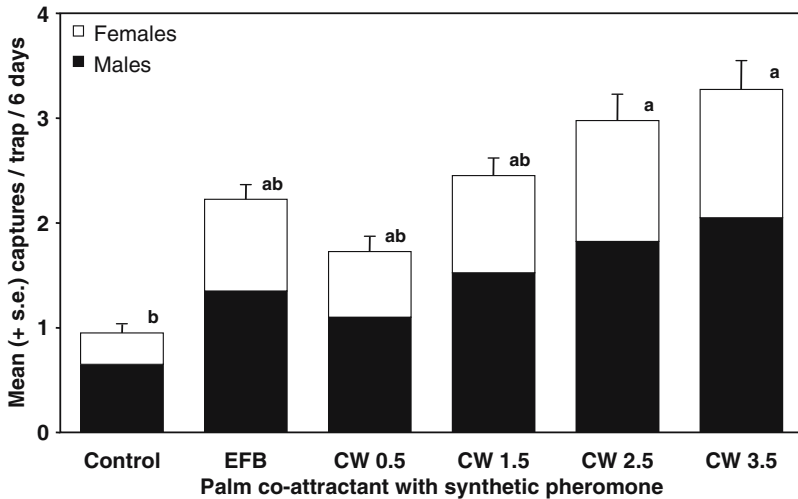


Fig. 2 Comparative catches of *O. monoceros* in 30-L pail traps using synthetic pheromone: ethyl 4-methyloctanoate (**1**) without plant material (control) or with various palm co-attractants: 1-wk-old oil palm EFB, CW after aging outdoors for 0.5, 1.5, 2.5, or 3.5 mo (trial 2). Treatments with different letters differed significantly [Newman–Keuls test at $\alpha = 0.05$ on $(x + 0.5)^{1/2}$ transformed data]

More males than females were caught in trials 1–4 (64% of 794) and 6 (57% of 4,989), while in trial 5 both sexes were equally represented (51% of 704).

Effect of Addition of Palm Material to Compound 1

A total of 292 beetles was captured in trial 1. The majority were caught in EFB + **1** traps, and while there were significantly fewer in **1** traps, **1** alone was more effective than EFB alone (Fig. 1; Newman–Keuls tests, $P < 0.05$).

A total of 340 beetles was caught in trial 2. Whereas there were no significant differences between the treatments that included a source of palm volatiles, traps baited with

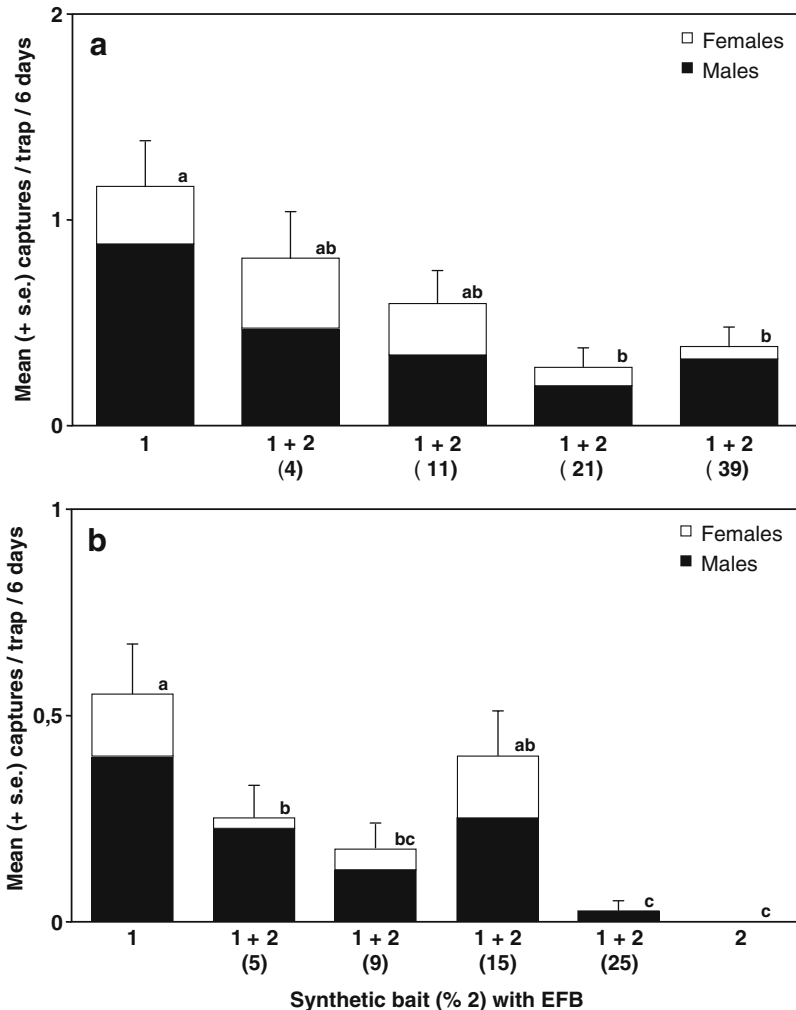


Fig. 3 Effect of 4-methyloctanoic acid (**2**), a volatile emitted by males, assessed at different ratios with synthetic pheromone: ethyl 4-methyloctanoate (**1**) in 30-L pail traps containing 1-wk-old oil palm EFB on *O. monoceros* catches in (a) trial 3, 2002, and (b) trial 4, 2003. Treatments with different letters differed significantly [Newman–Keuls test at $\alpha = 0.05$ on $(x + 0.5)^{1/2}$ transformed data]

the pheromone **1** and either 3.5- or 2.5-mo-old CW captured significantly more than **1** alone (Fig. 2).

Effect of Compound 2

In 2002, the release rate of **2** did not significantly affect the trap catch. However, at the two highest release rates, the number of beetles captured was significantly lower than in traps without **2** (Fig. 3a). In 2003, very few beetles were captured, due in part to heavy rainfall (some traps were flooded). However, as in the previous year, traps with EFB + **1** captured more beetles than EFB + **1** with **2** at the highest release rate although lower rates gave variable results (Fig. 3b). EFB + **2** captured no beetles at all.

Comparison of Trap Types with EFB or CW

Trap type and the kind of palm coacttractant added to **1** significantly affected catches (trap: $F_{1,41} = 21.8$; $F_{1,24} = 141$; palm: $F_{1,41} = 16.1$; $F_{1,24} = 15.0$ in trials 5 and 6, respectively; $P < 0.001$). Tube traps caught more beetles than pail traps, whatever the bait, whereas CW + **1** was more attractive than EFB + **1** regardless of trap type and renewal frequency (Table 1).

A significant “palm × trap” interaction was observed in trial 6 ($F_{1,24} = 4.44$; $P < 0.05$; Table 1) due to the unexpectedly high performance of EFB in tube traps, although globally CW was better than EFB, irrespective of trap type. The presence of a significant interaction in trial 6 but not in trial 5 could be the result of renewing EFB monthly in trial 6, combined with other unknown factors.

Seasonal Variation in Catches in Trial 6

About 5000 beetles were removed from the plot in 15 mo. The sex ratio of the beetles fluctuated from 26% to 70% female, with an average value of 43%. Overall, no significant decrease in catches (C as the mean catch per period) was recorded over time (T as the 6-d period numbered from 1 to 75; $C = -0.008 + 2.38T$; $F_{1,73} = 2.50$; $P = 0.12$). Catches ranged

Table 1 Comparative Captures (Mean ± S.E.) of *O. monoceros* in Traps with Pheromone Bait (**1**) as a Function of Trap Type (Pail or Vertical Tube) and Palm Coacttractant

Trial	No. periods (mo)	Trap	Palm coacttractant ¹		
			EFB	CW	Both
5	15 (3)	30-L pail	0.95 ± 0.10	1.40 ± 0.13	1.18 ± 0.09
		Vertical tube	1.48 ± 0.14	2.04 ± 0.16	1.77 ± 0.10 ²
		Both	1.22 ± 0.08	1.73 ± 0.11 ²	
6	75 (15)	30-L pail	1.28 ± 0.07 ^c	1.69 ± 0.08 ^b	1.48 ± 0.07
		Vertical tube	2.58 ± 0.11 ^a	2.76 ± 0.11 ^a	2.67 ± 0.07 ²
		Both	1.93 ± 0.07	2.22 ± 0.11 ²	

EFB: oil palm empty fruit bunches; CW: coconut stem wood.

¹Catches analyzed by GLIM ANOVAs on Ln($x + 1$) transformed data and means compared using Tukey multiple comparison tests.

²Significant differences between tube and pail traps, and EFB (empty fruit bunches) and CW (coconut wood), irrespective of the bait and trap, respectively ($\alpha = 0.01$). Average catches (per treatment and per 6-d period) with different letters were significantly different ($\alpha = 0.05$) in the case of significant palm × trap interactions (trial 6 only).

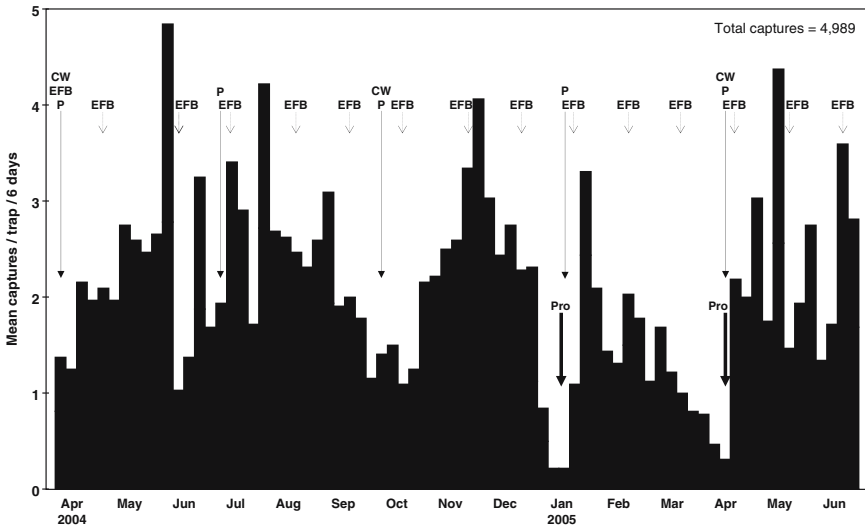


Fig. 4 Variation in *O. monoceros* catches at the Robert Michaux Plantation over 15 mo with 32 (16 pail plus 16 tube) traps baited with synthetic pheromone: ethyl 4-methyloctanoate (**1**) and 15 L of either oil palm EFB (renewed monthly) or coconut stem wood (CW renewed quarterly) from April 2004 to July 2005 (trial 6). Arrows indicate actual renewal of CW, EFB, and pheromone (P) and unavoidable run out of most of the pheromone dispensers (Pro)

from 0.2 to 4.8 beetles/trap/6 d, with an average of 2.1 (Fig. 4). These fluctuations were not correlated to the age of the palm material (EFB: $F_{1,24} = 0.40$ and CW: $F_{1,24} = 0.22$; $P > 0.5$) but significantly decreased with pheromone dispenser age ($F_{1,24} = 16.06$; $P < 0.001$). On two occasions, when bait renewal was unavoidably delayed, most of the dispensers ran out and resulted logically in lower catches (Fig. 4).

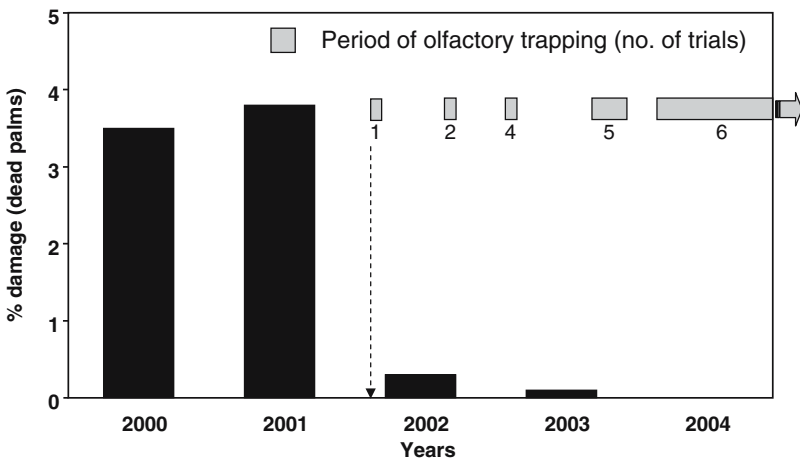


Fig. 5 Reduction in damage (dead palms) in the 19-ha coconut seed garden at the Robert Michaux plantation (Dabou) after 15 mo of olfactory trapping from January 2002 to December 2004 without any modification to the other plant protection measures. The dotted line indicates when trapping was initiated and the gray bars when trials 1, 2, and 4–6 were run

Effectiveness of Olfactory Trapping in the 19-ha Seed Garden at the Robert Michaux Plantation

Damage (dead coconut palms) caused by *O. monoceros* + *R. phoenicis* in the coconut seed garden at the Dabou plantation was 3.5% in 2000 and 3.8% in 2001 (Fig. 5). No insect control (neither insecticide applications nor particular cultural practice) was carried out in those 2 yr, except the elimination of dead coconut palms. Trapping clearly had an effect as palm mortality dropped to 0.5%, 0.2%, and 0.0% in 2002, 2003, and 2004, respectively (Fig. 5).

Discussion

Synergistic Effects of Decaying Palm Material with Pheromone

Our results show that decaying palm material, either EFB or CW, is a powerful coattractant with the aggregation pheromone **1** of *O. monoceros*, as previously reported for *O. rhinoceros* (Hallett et al., 1995; Alfiler, 1999; Sudharto et al., 2001). The effect of these host plant odors is another similarity of the two *Oryctes* species, which use the same aggregation pheromone. Although both odors are synergistic with **1**, CW was more efficient than EFB. CW can be prepared shortly before trapping starts, and is effective for 6 mo in both trap types. In contrast, EFB only works in a confined environment (straight pipe) and must be replaced each month.

Although the use of readily available plant material will enhance trap efficacy, the need to change it on a regular basis is labor intensive for vast trap networks. For instance, about 1 m³ of EFB is needed to service one trap for 1 yr. However, in using CW that only needs to be changed every 3 mo instead of monthly, one needs a chainsaw to prepare the pieces of CW effectively. Thus, the financial investment of this approach limits the availability to owners of small holdings.

The synergistic effect of plant odors on pheromone communication is common in insects, particularly in long-lived Coleoptera (Landolt and Phillips, 1997). This has been well documented for palm weevils Rhynchophorinae (e.g., *Rhynchophorus* and *Metamasius* spp.; Giblin-Davis et al., 1996; Rochat et al., 2000; Avand-Faghih 2004; Saïd et al., 2005) and certain scarabs, Dynastinae (e.g., *Scapanes australis* Bsdv. and *Oryctes elegans*: Rochat et al., 2002, 2004), where volatiles from freshly cut host plant tissues serve as cosignals with the pheromones for beetle aggregation on sites where both feeding, mating, and egg laying take place.

However, *O. rhinoceros* and *O. monoceros* feed individually rather than in aggregations on fresh tissues of living palms, although a palm may be attacked by several insects in succession (Morin, 1999). Thus, in these species, the synergistic effect of volatiles from decaying palm woody material (CW or EFB) with the pheromones is only important for aggregation at mating and egg-laying sites (Morin and Ollivier, 2001)

Inhibiting Effect of Compound 2

It is clear that when 4-methyloctanoic acid is combined with the aggregation pheromone (~30:70 acid/ester), there is a significant decline in the number of *O. monoceros* captured. This is in marked contrast with the findings for two other *Oryctes* species. In the case of *O. elegans*, 4-methyloctanoic acid serves as an aggregation attractant when combined with odors of freshly cut date palm stem (Rochat et al., 2004), whereas at 30 mg/d, either

evaluated alone or combined with the ethyl ester, it has no effect on *O. rhinoceros* (Hallett et al., 1995). Thus, *Oryctes* species appear to use 4-methyloctanoic acid and the ethyl ester derivative differently.

Interestingly, *O. monoceros* males emit variable amounts and ratios of both compounds (Gries et al., 1994; Morin et al., 1996). Although the origin and meaning of this variability are unknown, males may modulate the amount of acid emitted under certain circumstances and this may act as an antiaggregation signal to regulate densities at breeding sites (decaying woody material). Further research is required to clarify the situation, including the importance of chirality on the responses observed.

Comparison of Trap Types

The tube trap was more efficient than the pail trap for *O. monoceros*, as it was for *O. rhinoceros* (Morin et al., 2001b). The height and shape appear to be more adapted to the flight and approach behavior of the beetle, and insects entering the trap are prevented from escaping, without the need for insecticide or soapy water. The shape also helps keep the plant material moist for longer than in the more aerated pails, thereby lengthening the amount of time the pheromone cocontractants remains effective. Thus, as long as the bait remains active, the tube trap, which is sturdier than the pail trap in field conditions, could stay in the field for a long time without inspection or maintenance, making it suitable for use in coconut small holdings. The disadvantage remains to be its higher initial cost, and the availability of PVC tubes in remote areas.

Effect of Trapping on Damage to Coconut Palms

Despite continuous and stable catches over 3 yr, the damage caused by *Oryctes* (+ *Rhynchophorus*) decreased substantially in the 19-ha plot. The impact of olfactory trapping regimes have generally examined the reduction in a pest populations and this is, to our knowledge, the first comprehensive study showing reduced damage, as earlier reports analyzing 1–2 yr of trapping, reported mitigated effects (Ho 1996; Chung 1997). The insects caught since 2004 probably came from the oil palm plots surrounding the nursery, and the olfactory trapping clearly reduced attacks on palms whose considerable height (>8 m) prevented manual extraction of the insects from the galleries and insecticide applications. Our work has shown that olfactory trapping can be beneficial, and trials are under way to assess whether trapping intensity can be reduced while maintaining the benefit. This could be performed by either decreasing the trap density or by carrying out intermittent trapping at regular intervals. Studies are also in progress to evaluate the effect of trapping in immature crops.

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healthy plants, regardless of compatibility. These results are indicative of lowered herbivore defenses due to disease progression on the plants.

Keywords Pepper · Volatiles · Methyl salicylate · Insect feeding · Beet armyworm · Bacterial infection · Induced defense

Introduction

Plants have innate defense mechanisms that can be differentially activated in response to insect and pathogen attack. For example, plants may contain significant amounts of constitutive secondary metabolites including phenolics, terpenoids, and steroids, which are toxic to invading organisms. In addition to constitutive chemical defenses, there are also compounds that are actively produced or induced after penetration of the tissues has occurred. These inducible defenses are energetically expensive and are only produced upon specific recognition of an invading organism (Ryan and Jagendorf, 1995; Hahn, 1996; Schröder, 1998). Plants that are able to recognize an invading organism activate responses including a rapid localized cell death, also known as a hypersensitive response (HR), at the site of penetration and activation of biochemical defense responses. Biochemical responses include the following: production of reactive oxygen species, structural changes in the cell wall, accumulation of defense-related proteins, and phytoalexin biosynthesis. These chemical defenses can directly affect the development and survival of attacking organisms (Mür et al., 1997). Upon attack, plants may also produce secondary defense chemicals, volatile organic compounds (VOC's), which are released into their immediate airspace. These VOC's are produced by plants in response to attack by herbivores (McCall et al., 1994; Loughrin et al., 1995; Röse et al., 1996; Paré and Tumlinson, 1997) and pathogens (Cardoza et al., 2002; Huang et al., 2003). These VOC's are of great ecological relevance because they have been shown to attract parasitoids of insect pests (Turlings et al., 1991, 1993; Röse et al., 1998; Cardoza et al., 2003a) and to hinder pathogen development (Zeringue and McCormick, 1989, 1990; Zeringue et al., 1996; Cardoza et al., 2002).

While the responses of plants to individual attacks by insects and pathogens have been well studied, regulation of defense mechanisms that help plants cope with multiple stress factors remains to be elucidated. Similarly, it is not yet clear what defense pathways are involved in the production of VOC's by plants in response to combined insect and pathogen attack. Recent studies suggest that activation of plant defenses by pathogens interferes with plant defenses against herbivorous arthropods and *vice versa* (Karban et al., 1987; Bostock, 1999; Felton et al., 1999; Fidantsef et al., 1999; Stout et al., 1999). In light of these possible conflicts, studies on the interactions between insect and pathogen species, and the induced chemical changes that such interactions have on the host plant are needed.

An understanding of the dynamics involved in triggering plant innate defense mechanisms can lead to the development of crop protection based on genetic or chemical manipulation of such responses. The key to exploitation may lie in our ability to decipher the extent to which the biochemical defense cascades triggered within the plant by pathogen and herbivore damage interfere with one another, and to minimize these trade-offs. In this study, we evaluated VOC emissions elicited in bell pepper plants, *Capsicum annuum* L., by compatible and incompatible strains of the leaf spot pathogen, *Xanthomonas campestris* pv. *vesicatoria*, pepper race 1 (XCVP3; compatible) and tomato race 1 (XCVT1; incompatible). We also evaluated the effect of infection by these pathogens on the plants' ability to produce VOC's in response to feeding by beet armyworms (BAW), *Spodoptera exigua* Hübner. VOC

profiles of healthy plants and plants exposed to attack by the insect and the pathogen, individually and in combination, were analyzed and compared. The effect of infection with the two types of bacteria on the insects' feeding preference and larval development, from third instar to pupa, was also evaluated.

Methods and Materials

Plant and Insect Material

Pepper seeds "Early CalWonder" (Grimes, Concord, OH, USA) were sown in pairs in 1-gal pots (16 cm diam) containing Metromix 300 (Scotts-Sierra Horticultural Company, Marysville, OH, USA). Plants were grown in an insect-free greenhouse with natural light, under Florida summer conditions (14:10 light/dark cycle). The greenhouse temperature was kept between 25°C and 30°C. After emergence, seedlings were thinned to 1 individual per pot. Each plant received 100 ml liquid fertilizer [20:20:20 (N/P/K); Peters, W.R. Grace, Fogelsville, PA, USA] every 2 wk starting on the first week after emergence. Six-wk-old pepper plants with eight fully developed leaves were used in all experiments. BAW eggs were obtained from the rearing facilities at the USDA-IBPMRL (Tifton, GA, USA). Larvae were reared on a pinto bean-based artificial diet following the methodology described by King and Leppla (1984). Insects were maintained on a 14:10 L/D cycle and maintained at 28°C. Third instars were used in all experiments.

Bacterial Culture and Plant Inoculation

The initial cultures of *X. campestris vesicatoria*, pepper race 3 (XCVP3; compatible) and tomato race 1 (XCVT1; incompatible), were obtained from Jeffrey B. Jones (Department of Plant Pathology, University of Florida, Gainesville, FL, USA) and were grown on nutrient agar (NA) Petri plates. Subsequent cultures were started by using nutrient broth, and were stored in 15% glycerol at -70°C for later use. Viable cells for plant inoculation were obtained incubating 100 µl of the frozen culture in 50-ml conical centrifuge tubes containing 15 ml nutrient broth. Tubes were placed into a 200-rpm orbital shaker within a biological incubator kept at 28°C for 18 hr. Cells were harvested by spinning at 3000 × g for 10 min, and pellets were resuspended in tap water. Bacterial cell concentration was estimated by measuring absorbance with a spectrophotometer set at 600 nm. Concentration was adjusted to 10⁷ CFU/ml with water and 400 µl/l of Silwett L-77 were added to help cell penetration into the leaf. Plants were inoculated by dipping their aerial portions into the bacterial suspension for 20 sec. Control plants were mock-inoculated by dipping in the water Silwett L-77 mixture without bacterial cells.

Volatile Collections from Pathogen Infected and BAW-Damaged Peppers

Plant treatments consisted of (1) control (uninfected/undamaged) dipped in Silwett L-77 water, as described above; (2) BAW-damaged; (3) XCVP3-infected; (4) XCVT1-infected; (5) XCVP3-infected plus BAW damage; and (6) XCVT1-infected plus BAW damage. Plants were inoculated with bacteria as described above 12 hr before the start of the first sampling period. BAW-damaged plants were also exposed to feeding by six third instars within the volatile collection chambers 12 hr before the start of the first sampling period.

The aerial portion of each intact, nonexcised plant was contained within a cylindrical glass chamber with a guillotine-type Teflon base, which surrounded the plant stems (Röse et al., 1996). Purified air was pushed into the top of the chamber at a rate of 5 l min^{-1} . Samples of air from the whole-plant chambers were collected by pulling air at 1 l min^{-1} through 25 mg Super Q (80–100 mesh) (Alltech, Deerfield, IL, USA) adsorbent traps located around the bottom of each chamber (12). Volatiles were sampled for 4 d in three consecutive periods each day: (1) 6:00 A.M.–12:00 P.M., (2) 12:00–6:00 P.M., and (3) 6:00 P.M.–6:00 A.M. The experiment was set up in single replicates and repeated on different days for a total of six replicates. Data presented as total volatile production are for combined diurnal collection periods (1 + 2). Total volatile production represents the sum of the amounts of all the individual compounds produced by a given treatment.

Sample Extraction and Analysis

Compounds from individual traps in the volatile collection experiments were eluted with $170 \mu\text{l}$ dichloromethane (GC/GC-MS Solvent; B&J, AlliedSignal, Inc, MI, USA), and then 400 ng each of *n*-octane and nonyl acetate were added to each eluted sample as internal standards. Samples were analyzed by gas chromatography with flame ionization detection (HP5890 Gas Chromatograph, HP7673 auto sampler; Hewlett-Packard, Palo Alto, CA, USA) equipped with a $15 \text{ m (H)} \times 0.25 \text{ mm (ID)} \times 0.25 \mu\text{m}$ film thickness DB-1 capillary column (Quadrex, New Haven, CT). The splitless mode injector system was set at 220°C , the column oven was maintained at 40°C for 1 min after injection, and then programmed at $14^\circ\text{C min}^{-1}$ to 180°C . The carrier gas used was helium at an average flow velocity of 19 cm sec^{-1} .

For identification of volatile compounds, selected samples were analyzed via GC/MS (HP 6890/5893) in electron impact mode. Individual compounds were identified by comparing their retention times and mass spectra to those of commercially obtained authentic samples and/or by comparing their mass spectra to those available in a database from the Environmental Protection Agency/National Institute of Standards and Technology.

Effect of Bacterial Infection on BAW Larval Feeding and Performance

To determine if herbivore-induced defenses were compromised in bacterially infected plants, we conducted BAW feeding choice experiments at different times during disease development on the plants. Additionally, the performance of BAW, from third instar to pupa, on healthy and bacteria-infected peppers was evaluated.

To determine the feeding preference of BAW larvae for plants at different times during infection with both bacterial strains, insects were given a choice between leaves from healthy plants and leaves of plants 0, 2, 4, or 6 d after bacterial inoculation. Plants with eight fully developed leaves were used. The fifth oldest leaf of an infected plant was paired with its counterpart from a healthy plant by confining them, while still attached to the plants, side by side within Petri dish clip cages with six third instar BAW larvae (Alborn et al., 1996). Larvae were allowed to feed on the leaves for 24 hr. Leaves exposed to the feeding were photocopied, and the leaf images were scanned and imported into an imaging software program (ImagePC beta version 1; Scion Corporation, Frederick, MD, USA) to calculate leaf area eaten and leaf area remaining for each of the treatments. This experiment was repeated over time for a total of six replicates.

To evaluate larval performance on healthy and infected pepper, plants were individually placed within $46 \times 46 \times 46 \text{ cm}$ plexiglass cages into which 6 third instar BAW were introduced. Although insects were not weighed at the time of introduction, care was taken so that

all larvae used were newly molted third instars. Infected plants had been inoculated 3 d before the beginning of the experiment. Cages were kept in the greenhouse throughout the experiment. One plant provided more than enough food for all insects to develop until pupation. Insects were observed daily and were removed from the plants when they reached the wandering stage and no more feeding activity was observed. All insects from each of the treatments were collectively placed into a Petri plate labeled to indicate plant treatment and replicate number, and kept in the incubator until pupation. At the time of pupation, the number of surviving pupae and their weights were recorded. Eight replicates of this experiment were set up at one time in the greenhouse. This experiment was set up at two different times, by using either the compatible or the incompatible strain of the pathogen to inoculate plants.

Statistical Analyses

Data for total volatile emission were analyzed with ANOVA (SAS Institute, 1996). Significant ANOVAs were followed by Tukey's mean separation test. Data for BAW feeding preference were analyzed by paired *t* test (SAS Institute, 1996) for each day. Data for performance of BAW on healthy and bacteria-infected pepper were subjected to an analysis of variance (ANOVA; SAS Institute, 1996).

Results

Effect of BAW Feeding and Compatible and Incompatible Bacterial Inoculations on Volatile Production

Hypersensitive response symptoms were observed on the XCVT1-infected plants as early as 24 hr after inoculation, whereas chlorotic spots on the leaves in response to XCVP3 were seen only 3–4 d after inoculation. Volatile emission was highest during the second collection period (12:00–6:00 P.M.) and lowest during the third collection period (6:00 P.M.–6:00 A.M.) for the length of the experiment, regardless of plant treatment. Because of the high degree of variability, VOC emissions from the two diurnal collection periods were not different and were combined. The volatile blend emitted by plants under different biotic challenges consisted of mono- and sesquiterpenes, lipxygenase products, and methyl salicylate. However, there were differences in the amounts of each of these compound classes among the different treatments. For instance, total monoterpene release by plants damaged by BAW alone was the highest, followed by plants damaged by combinations of the herbivore and compatible and incompatible bacteria, respectively (Table 1). Monoterpene release by plants infected with the compatible bacterial strain were comparable to those of control plants; however, plants infected with the incompatible bacteria released higher amounts of these compounds on days 3 and 4 compared to controls (Table 1). Sesquiterpene release was significantly higher for plants under the XCVT1/BAW treatment during all 4 d sampled (Table 1). Plants exposed to XCVT1 and XCVT1/BAW had their highest sesquiterpene release on the first day of sampling, decreased on day 2, and remained constant during days 3 and 4 (Table 1). Release of sesquiterpene compounds by BAW damaged plants was equivalent to that released by plants under XCVP3/BAW attack, but higher than those of XCVP3-infected and control plants. Emission of sesquiterpenes by plants under XCVP3 infection alone was equivalent to that of the controls. Production of LOX products was most prevalent in the emissions of plants exposed to BAW damage alone or in combination with either bacterium (Table 1). Methyl salicylate was released by plants exposed to infection by either bacterium alone or

Table 1 Biosynthetic classes of volatiles emitted over a four day period by pepper plants subjected to different BAW/*Xanthomonas* treatments

Treatment	Volatile release (ng plant ⁻¹ h ⁻¹) ^a			
	MonoT ^b	LOX ^b	SesqT ^b	Methsal ^b
<i>Day 1</i>				
Control	242 ± 15.8a	2 ± 2.0a	13 ± 6.2a	0 ± 0.0a
XCVP3	299 ± 26.1a	3 ± 2.1a	39 ± 16.4a	16 ± 10.5ab
XCVT1	568 ± 53.4b	8 ± 7.7a	479 ± 262.0c	67 ± 44.6bc
BAW	1739 ± 38.6c	38 ± 8.6b	78 ± 21.6b	0 ± 0.0a
XCVP3BAW	2409 ± 81.5c	42 ± 13.8b	73 ± 17.6b	21 ± 96.5ab
XCVT1BAW	894 ± 45.2b	51 ± 22.4b	948 ± 318.9c	184 ± 73.4c
<i>Day 2</i>				
Control	249 ± 13.3a	2 ± 1.8a	8 ± 3.9a	0 ± 0.0a
XCVP3	271 ± 20.4a	9 ± 8.8a	19 ± 6.9a	5 ± 2.3a
XCVT1	429 ± 31.1b	6 ± 3.4a	75 ± 39.0b	111 ± 41.5b
BAW	5873 ± 185.9d	142 ± 91.6b	84 ± 22.5b	0 ± 0.0a
XCVP3BAW	10,373 ± 394.0e	151 ± 65.7b	147 ± 52.1bc	38 ± 3.8b
XCVT1BAW	1390 ± 44.1c	39 ± 17.5ab	308 ± 83.9c	71 ± 38.8c
<i>Day 3</i>				
Control	325 ± 24.7a	7 ± 6.8a	9 ± 4.3a	0 ± 0.0a
XCVP3	270 ± 15.8a	4 ± 2.0a	20 ± 7.8a	154 ± 21.6bc
XCVT1	787 ± 73.3b	15 ± 14.5a	46 ± 16.4a	113 ± 59.6b
BAW	4718 ± 203.4c	178 ± 91.6b	130 ± 62.6b	0 ± 0.0a
XCVP3BAW	8970 ± 249.0e	253 ± 96.1b	143 ± 38.9b	33 ± 12.2b
XCVT1BAW	3571 ± 147.4d	167 ± 69.5b	554 ± 156.8c	510 ± 112.5c
<i>Day 4</i>				
Control	133 ± 9.4a	7 ± 6.3a	9 ± 3.6a	0 ± 0.0a
XCVP3	319 ± 28.4b	2 ± 1.5a	14 ± 4.8a	198 ± 16.7b
XCVT1	1340 ± 78.5c	27 ± 14.8b	50 ± 29.1a	62 ± 43.2ab
BAW	7983 ± 345.3d	368 ± 191.9c	150 ± 53.4b	0 ± 0.0a
XCVP3BAW	10,533 ± 409.6d	317 ± 198.6c	191 ± 81.2b	2936 ± 1458.9d
XCVT1BAW	3601 ± 181.8c	171 ± 118.5c	678 ± 317.2c	472 ± 109.1c

MonoT: Monoterpenes; SesqT: sesquiterpenes; LOX: lipoxygenase products; Methsal: methyl salicylate.

^a Mean ± SE for emissions during period 2 (12:00–6:00 P.M.).

^b Values within days followed by different letters denote significant differences in emission of compound classes between treatments.

in combination with herbivore damage (Table 1). However, plants under the incompatible bacterial treatment started releasing this compound on day 1 (XCVT1/BAW) or day 2 (XCVT1), whereas plants under the XCVP3 treatment started releasing methyl salicylate on day 3 (Table 1). Furthermore, the amounts of methyl salicylate emitted by plants infected with the incompatible *Xanthomonas* were substantially higher than those emitted by plants infected with the compatible strain during the first 2 days of sampling (Table 1). A sharp burst in the release of this compound by plants under the XCVP3/BAW treatment on day 4 is noteworthy (Table 1).

Healthy (uninfected/undamaged; control) plants emitted relatively small VOC amounts throughout the sampling period (Fig. 1, Table 2). In contrast, plants infected with XCVT1, the incompatible strain, released amounts of volatiles that were higher than those emitted by XCVP3-infected plants and healthy control plants starting on the first day of collection

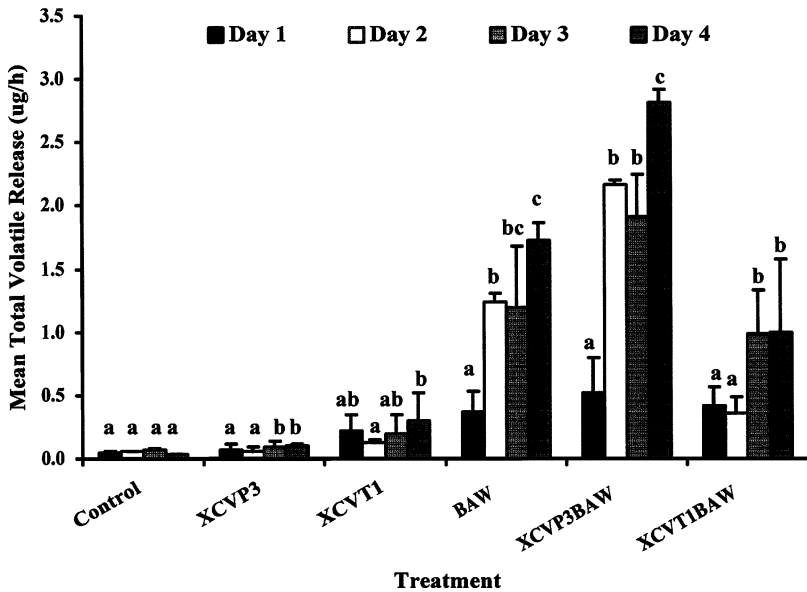


Fig. 1 Mean total volatile emissions from pepper plants for days 1–4 after bacterial inoculation. Bars within treatments headed by different letters denote significant differences between days (Tukey's mean separation test, $P = 0.05$). Error bars denote 1 SE

(Fig. 1). Emission of VOCs by plants exposed to the simultaneous bacterial and BAW challenge were higher than that of healthy plants or plants challenged by either bacterium alone (Fig. 1). Plants exposed to the XCV3/BAW also released higher amounts of VOC than healthy plants exposed to BAW damage starting on the second day of collection, which contrasts sharply with amounts released by XCV1/BAW-treated plants (Fig. 1).

Pepper plants exposed to the different bacteria/BAW treatments emitted distinct blends of compounds consisting of monoterpenes, homoterpenes, lipoxygenase products, linalool, methyl salicylate, and *cis*-jasmones. Pepper plants exposed to BAW feeding alone released a more complex VOC blend and a greater quantity of total volatiles, compared to healthy plants, or plants infected with either bacterium alone, starting on day 2 and persisting throughout the duration of the experiment (Table 1, 2). The VOC blend released by BAW damaged plants consisted of many compounds reported previously as induced by BAW feeding on other plant systems (Turlings et al., 1991; Turlings and Tumlinson, 1991; Röse et al., 1996; Cardoza et al., 2002) in addition to *Z*-3-hexenyl propionate, 2-methyl-hexyl-butyrate, 2-phenyl-ethyl-formate, *Z*-3-hexenyl-valerate, *Z*-3-hexenyl caproate, *Z*-3-hexenyl-hexenoate, β -elemene, α -selinene, and *Z*-3-hexenyl phenyl acetate (Table 2). Emission of VOC by plants infected with XCV3, the compatible strain, was comparable to that of control plants through the third day after bacterial inoculation. However, on day 4 after inoculation, XCV3-infected plants released higher amounts of β -ocimene, methyl salicylate, and α -selinene compared to healthy control plants (Table 2). The VOC profile of XCV1-infected plants was for the most part composed of α -pinene, limonene, β -ocimene, *E*-2-hexenyl butyrate, β -elemene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Table 2). Plants under the simultaneous XCV3/BAW challenge released a blend consisting mainly of *Z*-3-hexenyl acetate, β -myrcene, β -ocimene, *Z*-3-hexenyl propionate, methyl salicylate, and 2-

Table 2 Volatile emissions (mean \pm SE) from pepper plants under different bacteria/BAW treatments

Compound	Mean total volatile release (ng plant ⁻¹ h ⁻¹) ^{a,b}					
	Control	BAW	XCVP3	XCVP3+BAW	XCVT1	XCVT1+BAW
<i>Monoterpenes</i>						
α -Pinene	72 \pm 45.8	123 \pm 48.8	128 \pm 89.8	44 \pm 13.3	848 \pm 426.9	166 \pm 71.1
β -Pinene	17 \pm 7.7	38 \pm 13.1	14 \pm 4.6	17 \pm 12.0	89 \pm 3.5	23 \pm 7.9
β -Myrcene	0 \pm 0.0	1542 \pm 569.9	0 \pm 0.0	737 \pm 375.0	0 \pm 0.0	295 \pm 136.6
Limonene	8 \pm 3.8	30 \pm 30.0	3 \pm 1.5	40 \pm 36.5	129 \pm 77.5	22 \pm 9.2
Eucalyptol	0 \pm 0.0	101 \pm 31.6	3 \pm 2.3	127 \pm 44.71	34 \pm 13.8	47 \pm 21.8
β -Ocimene	22 \pm 11.3	5358 \pm 1660.1	158 \pm 124.2	9210 \pm 2629.7	155 \pm 75.8	2766 \pm 1082.1
Linalool	14 \pm 6.0	622 \pm 368.1	13 \pm 3.6	126 \pm 100.0	43 \pm 8.8	165 \pm 92.9
<i>Homoterpenes</i>						
<i>E</i> -4,8 Dimethyl-1,3,7-nonatriene	0 \pm 0.0	169 \pm 40.8	1 \pm 1.0	233 \pm 65.0	41 \pm 21.8	114 \pm 32.3
(<i>E</i>)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	20 \pm 7.9	423 \pm 130.0	44 \pm 15.3	642 \pm 191.2	149 \pm 52.1	2998 \pm 1364.4
<i>Sesquiterpenes</i>						
β -Caryophyllene	11 \pm 4.0	60 \pm 23.7	4 \pm 2.3	53 \pm 37.0	17 \pm 8.1	21 \pm 8.4
α -Humulene	36 \pm 12.2	138 \pm 44.8	33 \pm 13.8	99 \pm 37.6	34 \pm 28.2	123 \pm 45.2
β -Farnesene	6 \pm 4.4	50 \pm 15.3	7 \pm 5.0	86.7 \pm 25.3	20 \pm 12.8	113 \pm 41.5
Cadinene	0 \pm 0.0	35 \pm 10.1	1 \pm 0.5	53 \pm 16.0	12 \pm 2.8	304 \pm 252.8
β -Selinene	0 \pm 0.0	13 \pm 6.4	1 \pm 1.1	140 \pm 142.9	6 \pm 2.22	380 \pm 159.7
β -Elemene	0 \pm 0.0	308 \pm 92.4	5 \pm 2.7	493 \pm 219.8	254 \pm 193.4	3183 \pm 1505.6
α -Selinene	13 \pm 5.3	185 \pm 172.5	48 \pm 8.0	33 \pm 14.8	31 \pm 9.5	77 \pm 23.3
α -Farnesene	0 \pm 0.0	10 \pm 4.2	0 \pm 0.0	8 \pm 7.0	0 \pm 0.0	9 \pm 4.1
Nerolidol	9 \pm 3.3	92 \pm 31.1	8 \pm 2.64	123 \pm 60.6	11 \pm 2.9	110 \pm 35.9

<i>Lipoxygenase products</i>						
<i>E</i> -2-Hexenal	0 ± 0.0	427 ± 184.6	5 ± 4.9	351 ± 115.94	11 ± 4.41	56 ± 28.4
<i>Z</i> -3-Hexen-1-ol	3 ± 1.9	134 ± 91.7	3 ± 3.2	68 ± 74.11	19 ± 7.5	97 ± 35.5
<i>Z</i> -3-Hexenyl acetate	4 ± 2.8	23 ± 4.8	2 ± 1.2	1391 ± 1495.7	59 ± 23.7	14 ± 6.1
<i>E</i> -2-Hexenyl acetate	1 ± 1.3	647 ± 501.3	3 ± 2.0	360.4 ± 219.6	71 ± 46.9	114 ± 88.0
<i>Z</i> -3-Hexenyl propionate	0 ± 0.0	1199 ± 503.6	0 ± 0.0	1454 ± 515.17	0 ± 0.0	260 ± 176.1
<i>E</i> -2-Hexenyl butyrate	2 ± 2.6	1694 ± 644.9	2 ± 1.9	159 ± 133.6	117 ± 67.0	377 ± 174.4
<i>Z</i> -3-Hexenyl butyrate	0 ± 0.0	551 ± 428.7	1 ± 0.63	82 ± 24.0	99 ± 63.8	21 ± 14.2
<i>Z</i> -3-Hexenyl isobutyrate	3 ± 2.1	43 ± 16.0	6 ± 3.2	64 ± 24.1	20 ± 1.6	17 ± 5.7
2-Methyl-hexyl-butyrate	11 ± 3.7	357 ± 137.6	5 ± 3.0	679 ± 400.2	8 ± 1.6	63 ± 40.5
2-Phenyl-ethyl-formate	4 ± 2.8	261 ± 154.7	2 ± 2.3	45 ± 20.5	8 ± 4.6	47 ± 37.4
<i>Z</i> -3-hexenyl valerate	1 ± 1.1	88 ± 35.9	1 ± 0.39	76 ± 42.9	3 ± 1.4	31 ± 27.7
<i>E</i> -2-Hexenyl valerate	0 ± 0.0	0 ± 0.0	3 ± 1.5	4 ± 3.1	4 ± 1.2	4 ± 4.1
<i>Z</i> -3-Hexenyl tiglate	67 ± 73.0	90 ± 62.3	0 ± 0.0	25 ± 10.0	0 ± 0.0	23 ± 16.1
<i>Z</i> -3-hexenyl caproate	0 ± 0.0	31 ± 30.8	0 ± 0.0	0 ± 0.0	0 ± 0.0	930 ± 839.6
<i>Z</i> -3-Hexenyl hexenoate	0 ± 0.0	153 ± 71.2	0 ± 0.0	311 ± 91.4	8 ± 3.5	542 ± 350.3
<i>Z</i> -3-Hexenyl-phenyl acetate	8 ± 8.8	202 ± 202.0	0 ± 0.0	13 ± 7.5	8 ± 9.2	148 ± 51.3
<i>cis</i> -Jasmone	5 ± 2.3	334 ± 56.3	2 ± 1.7	375 ± 141.7	14 ± 7.1	142 ± 48.4
<i>Amino acid derivatives</i>						
Methyl salicylate	0 ± 0.0	0 ± 0.0	198 ± 22.4	2936 ± 1464.3	62 ± 20.8	472 ± 81.4
Indole	7 ± 3.6	148 ± 21.1	3 ± 1.8	109 ± 53.7	0 ± 0.0	41 ± 25.7

Values for day 4 period 2 (12:00–6:00 P.M.).

^a Mean ± SE for 6 replicates per treatment.

^b Underlined boldface within columns indicate the six highest emissions within a treatment.

methyl-hexyl butyrate. The VOC profile of plants under the XCVT1/BAW treatment consisted mostly of β -ocimene, methyl salicylate, Z-3-hexenyl caproate, Z-3-hexenyl hexenoate, β -elemene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Table 2). The relatively large amounts of sesquiterpene compounds released by XCVT1/BAW-treated plants starting on the first day of collection is particularly interesting (Table 1, 2). It is also worth noting that, on the first day of collection, the amounts of sesquiterpenes released by plants under the XCVT1 infection alone were similarly higher than those of other treatments, except XCVT1/BAW.

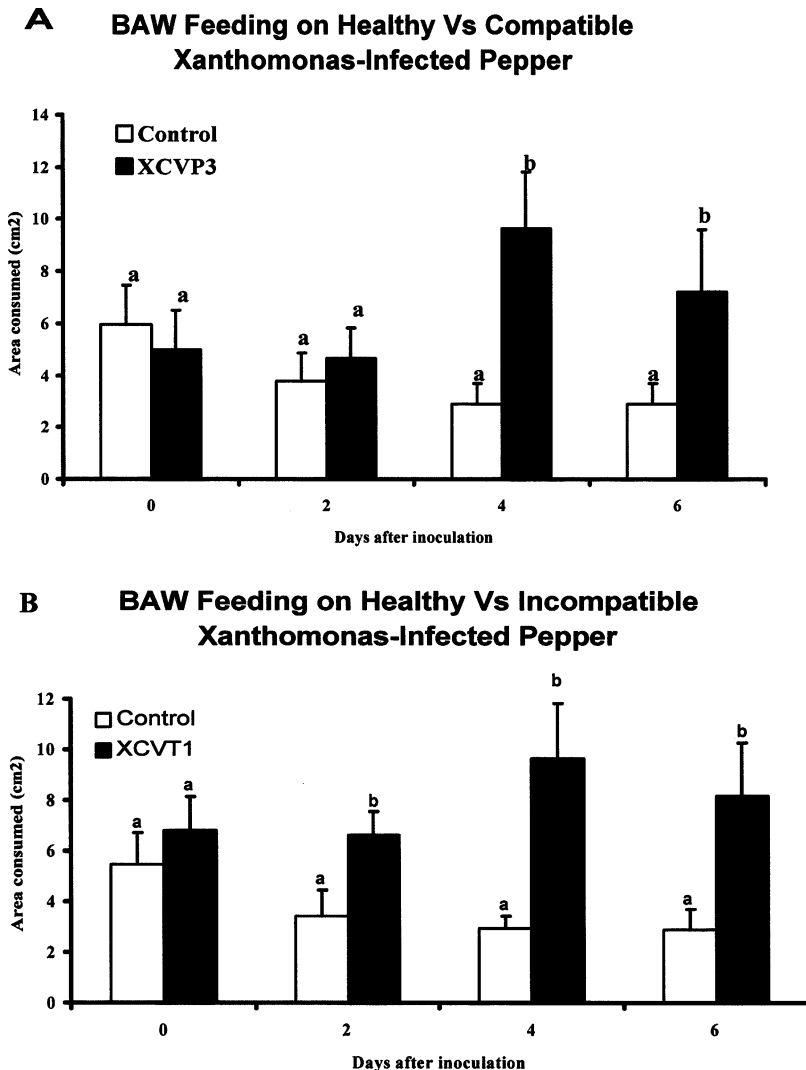


Fig. 2 Mean leaf area consumed by third instar *Spodoptera exigua* larvae in paired-choice tests with healthy (white bars) and *Xanthomonas* (thatched bars) plants at 0, 2, 4, and 6 d after bacterial inoculation. (a) Compatible (XCVP3) infection and (b) incompatible (XCVT1) infection. Error bars denote 1 SE and bars headed by different letters denote significant differences within days (paired *t* test, $P < 0.05$)

Effect of Bacterial Infection on BAW Larval Feeding and Performance

In a choice experiment, feeding by BAW on healthy and XCVP3 plants did not differ significantly on days 0 and 2 after inoculation (Fig. 2a). However, on day 4 and 6 after inoculation, there was a preference by the insects for XCVP3-infected leaves over healthy ones (Fig. 2a). When given a choice between healthy and XCVT1-infected plants, insects showed a significant preference for the latter starting on day 2. This preference was sustained until the end of the experiment (Fig. 2b). Pupal weights and time to pupation did not differ significantly between insects reared on healthy or bacteria-infected plants, regardless of bacterial strain. Combined pupal weights averaged 89.1 ± 16.80 (SD) mg. Mean (\pm SD) developmental time was 5.9 ± 1.24 for the combined treatments. Insects reared on plants infected by the two bacterial strains had significantly higher survival rates than those reared on uninfected plants ($F = 20.4$; $df = 2, 99$; $P = 0.0001$) Mean percent BAW survival (\pm SD) was 61.1 ± 16.01 on controls; 80.7 ± 11.29 on XCVP3 and 82.4 ± 15.67 on XCVT1.

Discussion

Secondary compounds produced by plants in response to insect and pathogen attack include low-molecular-weight VOC released into the plant's immediate air space. Insect herbivore-induced VOC attract natural enemies of the insects and may directly affect the performance of the organism responsible for the attack (Cardoza et al., 2002, 2003a,b). Similarly, pathogen-induced volatile substances may directly hinder pathogen development (Zeringue and McCormick, 1989, 1990; Zeringue et al., 1996; Cardoza et al., 2002). Previously, we reported that different stress factors induced the release of different volatile profiles (Cardoza et al., 2002; Huang et al., 2003). We showed that VOC release by peanut and tobacco plants is specific for compatible and incompatible pathogen infection, and for insect herbivore damage on the same plant system (Cardoza et al., 2002; Huang et al., 2003). Volatile profiles are also affected by the combination of factors afflicting the plant at any given moment (Cardoza et al., 2002). We further our contribution to this subject by evaluating the total volatile emission by pepper plants in response to a compatible and an incompatible pathogen infection alone or in combination with insect damage upon the same host plant. The effect of such pathogen infections on the feeding preference, survival, development, and weight gain of BAW on plants was also evaluated.

Our data show that the pepper plants' VOC emissions in response to damage by a herbivorous insect is significantly, and differentially, affected by inoculation with compatible and incompatible strains of a foliar pathogen. Plants exposed to the incompatible bacterial challenge emitted higher diversity and quantities of sesquiterpenes when exposed to BAW feeding than plants under the compatible bacterial challenge. Furthermore, herbivore-induced volatile emissions by plants are affected by bacterial infection and this interference depends on how soon the plant is able to detect the presence of the pathogen. Pepper plants exposed to the simultaneous XCVT1/BAW damage release a qualitatively different profile and lower amounts of BAW-induced compounds as soon as the second day after inoculation, whereas plants exposed to the XCVP3/BAW treatment released a similar profile, although higher amounts of some VOCs throughout the experiment compared to plants exposed to BAW damage alone (Table 1).

Our results also indicate that physiological changes induced in plants by a pathogen's development result in an increased phagostimulation of the insects. This phagostimulation may be attributable to reduced levels of defensive compounds in the infected plants. This

possibility is reinforced by the fact that the survival of the insects was higher when fed infected *versus* healthy pepper plants. Also, since insects reared on infected plants did not develop significantly faster, nor did they gain more weight, than those reared on healthy plants, the possibility of increased nutritional quality caused by the pathogen infection on the plant tissue is precluded. Our data are similar to previously reported findings, in that plant infection by a pathogen resulted in increased feeding and/or performance by an insect (Cardoza et al., 2002; Cui et al., 2002). The increase in insect feeding and performance on infected plants may have come as a consequence of biochemical changes induced by bacterial infection in the plants.

In recent years, there has been great interest in the potential applications of chemical elicitors to “vaccinate” plants to make them immune to attack or to “prime” them so their defense mechanisms are readily activated upon the first perception of pest damage. While chemical elicitation of plant defenses offers a promising alternative to environmentally harsh conventional pest control methods, caution needs to be exerted for there is a substantial body of evidence showing that the biochemical pathways involved in plant defense against pathogens and herbivores may interfere with one another. For example, recent studies have suggested that the plant pathway leading to jasmonate production and the salicylic acid pathway are involved in direct plant defense against pathogen invasion (Wasternack and Parthier, 1997; Thomma et al., 1998), and that the Jasmonic Acid (JA) and Salicylic Acid (SA) biochemical pathways may interfere with one another (Karban et al., 1987; Fidantsef et al., 1999; Stout et al., 1999; Bostock, 1999; Felton et al., 1999). This theory has been substantiated by findings that some insect species prefer feeding on plants that are either infected by pathogens or induced with SA analog (Felton et al., 1999; Fidantsef et al., 1999; Stout et al., 1999; Thaler et al., 1999; Cui et al., 2002). Another fact supporting this theory is that acetyl salicylic acid and SA inhibit lipoxygenase-induced plant defenses by preventing JA accumulation (Peña-Cortes et al., 1993; Doares et al., 1995). Since we found that plant inoculation with either pathogen enhanced the development and survival of insects fed on infected plant tissue, it appears that the signaling cascade in response to pathogen infection in our system interfered with production of both direct and indirect defenses against the herbivore in this particular system. Additionally, reduction in plant emission of VOCs in response to BAW feeding seem to correlate with the timing of plant detection of each of the pathogen systems tested herein. Plant resistance to pathogens is governed by “gene-for-gene” interactions so that when a pathogen has an avirulence (*avr*) gene and a plant has the corresponding resistance (*R*) gene, the plant can immediately detect the pathogen and respond defensively (Hahn, 1996; Hammond-Kosack and Jones, 1997; Dangl and Jones, 2001). When the interaction ends in pathogen establishment and disease development, the interaction is considered compatible. If the pathogen fails to establish because of resistance from the plant, and no disease develops, the interaction is incompatible. Incompatible pathogen interactions induce defensive responses in plants at a much faster rate than compatible pathogen one. Thus, the reduced amount of BAW-induced VOC in XCVT1-infected plants may be attributable to plant resource allocation to fight off the pathogen.

Although studies addressing the issue of potential conflicts in plant defense responses to pathogens and herbivores now abound, to our knowledge, this is only the second report to evaluate VOC emissions in live plants in response to simultaneous pathogen/herbivore challenge. It is the first one to do so with different pathogen compatibility systems and at different times during the disease development process. We expect that studies such as this will enhance our understanding of the regulation of plant defenses in response to different aggressors and will lead to the development and implementation of methods for plant protection that use defense priming compounds. The hierarchy governing plant defense responses when under

attack by pathogenic and herbivorous organisms merits further attention. Similarly, the potential role of plant hormones and their interactions in biochemical cascades leading to plant volatile induction by multiple herbivorous organisms needs to be investigated.

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phenotypic characters in a concerted way, being an important evolutionary factor for bacterial survival and host infection (Whitehead et al., 2001; Fuqua and Greenberg, 1998). In Gram-negative bacteria, the main signaling substances are the acyl-homoserine lactones (acyl-HSLs). Production of acyl-HSLs is widespread, being present in bacterial species found in different environments, as in *Vibrio fischeri* [*N*-(3-oxohexanoyl)-HSL], a marine bioluminescent bacterium, in *Pseudomonas aeruginosa* [*N*-butanoyl-HSL and *N*-(3-oxododecanoyl)-HSL], a human pathogen, and in *Rhizobium leguminosarum* [*N*-(3-(*R*)-hydroxy-7-*cis*-tetradecenoyl)-(*S*)-HSL], a rhizosphere symbiont (Eberhard et al., 1981; Pearson et al., 1994; Lithgow et al., 2000). The molecular basis of these communication mechanisms involves interaction between the signaling substance and a transcriptional regulator protein. An activated protein then binds to specific DNA regions, regulating the expression of genes important to multicellular behavior (Fuqua and Greenberg, 1998). The main class of genes responsible for acyl-HSL synthesis and detection are called *luxI* and *luxR* homologues, respectively (Engebrecht and Silverman, 1984).

Similar communication mechanisms are also described for plant pathogenic bacteria. In *Pectobacterium carotovorum*, the production of plant-cell-wall degrading enzymes and the antibiotic carbapenem are under the control of the signaling substance (*S*)-(-)-*N*-(3-oxohexanoyl)-HSL (Bainton et al., 1992; Jones et al., 1993). The same signaling substance is produced by the maize pathogen *Pantoea stewartii*, controlling the expression of an exopolysaccharide (EPS) that protects bacterial cells from host defense mechanisms. Also, this EPS obstructs fluid circulation in maize tissues, causing necrosis and cell death in leaves (Bodman and Farrand, 1995). However, in *P. stewartii*, the absolute configuration of the signaling substance was not determined. These examples show the importance of quorum-sensing mechanisms for successful host colonization by phytopathogenic bacteria. Quenching this communication mechanism is considered a promising avenue to control bacterial infections (Suga and Smith, 2003).

The absolute configurations of signaling substances are crucial for their biological activity. In the first work describing the chemical characterization of a signaling substance from a Gram-negative bacterium, the racemic synthetic compound (\pm)-*N*-(3-oxo-hexanoyl)-HSL was less active than the natural product in triggering *V. fischeri* bioluminescence. At that time, the smaller induction capacity was attributed to a less active enantiomer present in the racemic mixture (Eberhard et al., 1981). In *P. carotovorum*, synthetic (*R*)-*N*-(3-oxohexanoyl)-HSL was 90% less active than the natural enantiomer (*S*) during regulation of carbapenem antibiotic biosynthesis (Chhabra et al., 1993). In spite of the relevance of absolute configuration for biological activity and the large number of Gram-negative bacteria studied, the absolute configuration of acyl-HSLs has seldom been directly accessed from the natural products (Lithgow et al., 2000; Bainton et al., 1992; Pomini et al., 2005).

Pantoea ananatis (Serrano, 1928), a phytopathogen encountered worldwide, causes infection and losses in a wide variety of commercially important crops, such as sudangrass (Azad et al., 2000), pineapple (Serrano, 1928), sweet onion (Walcott et al., 2002), eucalyptus (Coutinho et al., 2002), and rice (Cothier et al., 2004), and has been identified as one of the bacteria in “white spot disease” of maize in Brazil (Paccola-Meirelles et al., 2001). In this work, the acyl-HSLs produced by *P. ananatis* CCT 6481^T (=ATCC 33244 type strain) were determined by using gas chromatography-mass spectrometry (GC-MS). The absolute configuration of the major acyl-HSL constituent was established with gas chromatography-flame ionization detection (GC-FID) equipped with a fused silica capillary column with a chiral stationary phase. Bioactivities of extracts, synthetic products, and fractions were qualitatively accessed by using the bioreporter *Agrobacterium tumefaciens* NTL4(pZLR4) in β -galactosidase expression assays.

Methods and Materials

General

Fourier-transform infrared (FTIR) spectra were obtained with a Bomem MB Michelson spectrometer, using KBr (Merck) as sample support. Silica gel for CC (0.035–0.070 mm) was from Merck. Thin layer chromatography (TLC) analyses were done on silica gel 60 F₂₅₄ plates (Merck) and visualized by exposure to UV light (254 nm) or spraying with an acidic *p*-anisaldehyde solution. Nuclear magnetic resonance (NMR) spectra were acquired by using a Varian Inova spectrometer, operating at 499.88 MHz for ¹H NMR and 125.71 MHz for ¹³C NMR or, alternatively, with a Varian Gemini spectrometer operating at 300.07 MHz for ¹H NMR and 75.45 MHz for ¹³C NMR. CDCl₃ was used as solvent and TMS as internal reference (δ_{H} and δ_{C} 0.0). Chemical shifts δ are expressed in ppm and coupling constants *J* in Hz. Optical rotation was measured on a Perkin-Elmer 341 polarimeter.

Bacteria and Media

P. ananatis Serrano CCT 6481^T (= ATCC 33244 type strain) was isolated from pineapple (*Ananas comosus*) 1965, Brazil and maintained on solid nutrient broth (NB, Oxoid, 20 g/l) medium. The indicator strain *A. tumefaciens* NTL4(pZLR4) was maintained on solid Luria–Bertani (LB) medium [1% peptone (Oxoid), 0.5% NaCl, and 0.5% yeast extract (Oxoid)], supplemented with gentamicin (50 $\mu\text{g/ml}$). Solid media were prepared with 2% agar (Oxoid). 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) was purchased from Sigma.

Extraction and Fractionation of Acyl-HSLs

P. ananatis CCT 6481^T was grown in test tubes containing liquid NB medium (10 ml), incubated at 28°C for 24 hr, then transferred to NB medium (1 l) to be incubated at 28°C under shaking at 150 rpm. After 24 hr, the culture medium was centrifuged at 5000 rpm for 20 min at 18°C. The centrifuged medium was extracted with ethyl acetate (3 \times 500 ml). The combined organic phases (1.5 l) were washed with distilled water (1 \times 500 ml) and evaporated under reduced pressure at 40°C. The whole procedure was repeated eight times yielding a crude extract (approximately 0.70 g), which was separated by silica column chromatography (15 g), eluted with hexane, dichloromethane, and ethyl acetate in increasing polarity, recovering 80 fractions of 50 ml, monitored by TLC. Similar fractions were combined and analyzed by GC-MS.

Bioassay with Reporter *A. tumefaciens* NTL4(pZLR4)

Inoculum of *A. tumefaciens* NTL4(pZLR4) was prepared in a test tube with LB liquid medium (2 ml). It was maintained at 28°C for 24 hr, until a cell concentration of 9×10^8 cells/ml, according to McFarland scale. Bioassays were performed in test tubes containing LB liquid medium (2 ml) with 20 μl inoculum of *A. tumefaciens* and 20 μl X-Gal (stock solution at 50 mg/ml in DMF). Biological activities were accessed by using 20 μl of ethanolic solutions (2 mg/ml) of each synthetic product [(\pm)-*N*-hexanoyl-HSL, (*S*)-(-)-*N*-hexanoyl-HSL, (\pm)-*N*-heptanoyl-HSL, and (\pm)-*N*-octanoyl-HSL], ethyl acetate extract from *P. ananatis* cultivation medium and the fractions. Three blanks were performed: (1) with

ethanol (20 μ l), (2) ethyl acetate extract from nutrient broth medium, and (3) with *A. tumefaciens* in the absence of other substances. Tests were performed in duplicate, by incubating the test tubes at 30°C for 24 hr.

GC-MS Analyses of Fractions from *P. ananatis* Cultivation Media

GC-MS analyses were carried out on an HP 6890/5973 instrument, equipped with a 30 m \times 0.25 mm \times 0.25 μ m i.d. HP5 fused silica capillary column. Mass spectra were recorded over the 40–450 amu range at 3.54 scans/sec, with an ionization energy of 70 eV. Helium was the carrier gas at a flow rate of 1 ml/min. The injector temperature was maintained at 250°C. The initial oven temperature was 100°C and was programmed to increase to 290°C at 10°C/min, then held for 10 min. One μ l samples were injected, without splitting.

Acyl-HSL Synthesis

Acyl-HSLs were synthesized as previously described (Chhabra et al., 1993). Briefly, to a round-bottom flask containing distilled water (2.5 ml), triethylamine (1.05×10^{-4} mol), α -amino- γ -butyrolactone hydrobromide [racemic or (*S*)-(-), 1.05×10^{-4} mol], and hexanoic, heptanoic, or octanoic acids (1.57×10^{-4} mol) were added. The reaction medium was stirred at room temperature overnight followed by extract ion with ethyl acetate (3 \times 10 ml). The combined organic phases were washed with aqueous solutions of potassium bisulfate 1 mol/l (2 \times 6 ml), sodium bicarbonate 5% (1 \times 6 ml), and saturated sodium chloride (1 \times 6 ml). The organic layer was dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness under reduced pressure. Pure acyl-HSLs were obtained as white crystals.

Chiral GC-FID Analyses

Chiral gas chromatographic analyses were carried out on an HP 6890 instrument with FID, equipped with a 25.0 m \times 250.0 μ m \times 25.0 μ m chiral capillary column Chrompack CP chiral-sil-dex coating 7502. The initial oven temperature was 50°C and programmed to increase at 2°C/min to 180°C, and then maintained for 5 min. Hydrogen was the carrier gas at a flow rate of 1 ml/min. The injector and detector (FID) temperatures were kept at 220°C and 240°C, respectively. One- μ l samples were injected, with a 1:100 split ratio.

Results

Detection of Natural Acyl-HSLs

Initially, the presence of acyl-HSLs in *P. ananatis* CCT 6481^T extracts was qualitatively evaluated by using the bioreporter *A. tumefaciens* NTL4(pZLR4) (described later). In the present case, extracts from *P. ananatis* provided positive biological activity, stimulating more accurate chemical investigations.

Cell-free extracts from 8 l of *P. ananatis* cultivation medium were obtained and purified by using column chromatography. Similar fractions were grouped based on TLC analysis. Acyl-HSL monitoring usually relies on bioassays that use specific reporters. However, a literature survey indicated that these reporters have limitations for detecting some acyl-HSLs structurally distinct from those produced by the wild-type species from which the

reporter was genetically constructed (Cha et al., 1998; Ravn et al., 2001). To avoid the use of several reporters with different specificities, fractions from *P. ananatis* were analyzed by GC-electron impact mass spectrometry. This is possible because of the standard fragmentation pattern of acyl-HSLs, displaying an intense peak at m/z 143 arising from a McLafferty rearrangement of the acyl moiety. Signals at m/z 100, 101, and 102 are usually present in smaller relative abundance and are derived from the HSL (m/z 100) moiety (Pomini et al., 2005). This methodology allowed the chemical identification of *N*-hexanoyl-HSL (major), *N*-heptanoyl-HSL (traces), and *N*-octanoyl-HSL (traces) from *P. ananatis* (Fig. 1). The presence of *N*-octanoyl-HSL was evaluated with GC-MS operating in the single ion monitoring mode (SIM), for better sensitivity.

N-Hexanoyl-HSL

GC-MS (EI, 70 eV) m/z (%): 199 (M^+ , 2), 170 (5), 156 (12), 143 (100), 125 (21), 115 (7), 102 (12), 101 (15), 100 (10), 99 (25), 57 (62), 43 (51).

N-Heptanoyl-HSL

GC-MS (EI, 70 eV) m/z (%): 213 (M^+ , 3), 170 (5), 156 (15), 143 (100), 125 (22), 113 (16), 102 (11), 101 (15), 85 (19), 57 (59), 43 (47).

N-Octanoyl-HSL

GC-MS (EI, 70 eV, SIM) m/z (%): 227 (M^+ , 4), 170 (4), 156 (18), 143 (100), 128 (8), 127 (10), 125 (19), 102 (12), 101 (12), 85 (6), 57 (48).

Synthesis of Acyl-HSLs

The low amounts of natural acyl-HSLs prevented isolation and/or further NMR spectroscopic investigations. Therefore, identification was carried out by coinjecting the

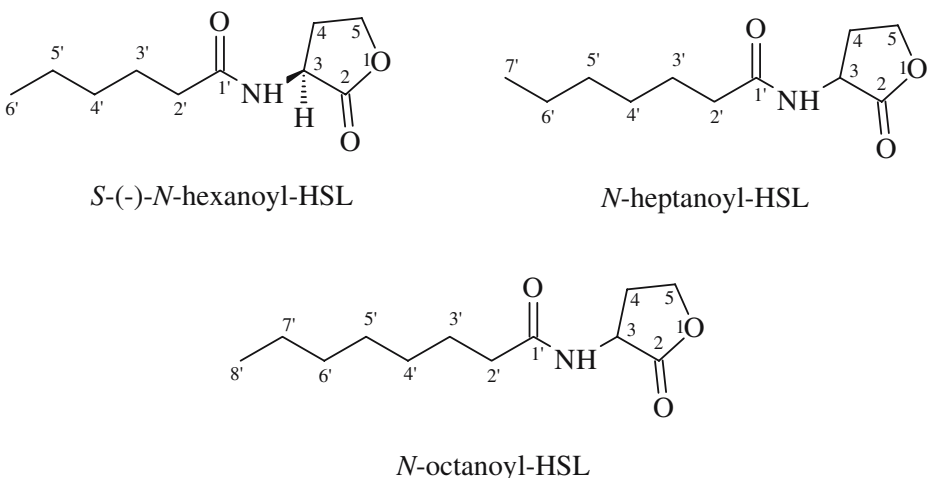


Fig. 1 Acyl-HSLs identified from *Pantoea ananatis* cell-free extracts

natural products and synthetic substances onto GC-MS. Synthetic products were obtained through a one-step, simple procedure based on the production of an acid anhydride from the corresponding carboxylic acid, in the presence of a water-soluble carbodiimide derivative. α -Amino- γ -butyrolactone hydrobromide was then acylated by these *in situ* generated anhydrides, under mild basic conditions. Purification was facilitated because of the higher affinity of the acyl-HSLs than the reactants for organic solvents (Chhabra et al., 1993). All synthetic products were obtained in reasonable yields (~55%). They were fully characterized by spectroscopy (IR, GC-MS, ^1H NMR, and ^{13}C NMR in one and two dimensions) and by data comparison with the literature (data not shown). IR and ^1H NMR spectral data for *N*-octanoyl-HSL are provided for the first time.

(\pm)-*N*-Hexanoyl-HSL

54% yield. GC-MS (EI, 70 eV) data were identical to the natural product. IR, ^1H NMR, and ^{13}C NMR data were consistent with those previously reported (Chhabra et al., 1993; Lao et al., 1999).

(S)-(-)-*N*-Hexanoyl-HSL

54% yield. IR, GC-MS, ^{13}C NMR, and ^1H NMR data were identical to those obtained for the racemic compound. $[\alpha]_D^{20} -22.86^\circ$ (ca. 0.35 MeOH).

(\pm)-*N*-Heptanoyl-HSL

64% yield. GC-MS (IE, 70 eV) data were identical to the natural product. IR, ^1H NMR, and ^{13}C NMR data were consistent with those previously reported (Pomini et al., 2005).

(\pm)-*N*-Octanoyl-HSL

53% yield. GC-MS (IE, 70 eV) data were similar to the natural product. IR (KBr) 3316 (m), 2954 (m), 2928 (m), 1777 (s), 1644 (s), 1549 (s), 1172 (s), 1013 (m), 946 (w), 656 cm^{-1} (w). ^1H NMR (499.88 MHz, CDCl_3 , TMS) δ /ppm 0.88 (t, 3H, $J = 7.2$ Hz, H-8'), 1.29 (m, 8H, H-4', H-5', H-6', H-7'), 1.64 (quintet, 2H $J = 7.2$ Hz, H-3'), 2.15 (m, 1H, H-4), 2.26 (t, 2H, $J = 7.8$ Hz, H-2'), 2.83 (m, 1H, H-4), 4.28 (ddd, 1H, $J = 5.9, 11.1$ and 9.2 Hz, H-5), 4.46 (t, 1H, $J = 9.0$ Hz, H-5), 4.57 (ddd, 1H, $J = 5.7, 11.6$ and 8.6 Hz, H-3), 6.23 (broad s, NH). ^{13}C NMR data were consistent with those previously reported (Lao et al., 1999).

Bioassays with Reporter *A. tumefaciens* NTL4(pZLR4)

A. tumefaciens is a phytopathogen that causes crown gall tumor disease in several plant species. This bacterium exploits quorum-sensing communication mechanisms to control the expression of genes associated with the infection establishment, including plasmid conjugal transfer regulation (Piper et al., 1993). The mutant NTL4(pZLR4) does not synthesize the cognate signal *N*-(3-oxo-octanoyl)-HSL (Zhang et al., 1993) produced by the wild type strain, but can detect several acyl-HSLs. The detection mechanism consists of a plasmid (for a detailed scheme, see Cha et al., 1998) that contains a *traG::lacZ* fusion and the *traR* gene, which codifies the transcriptional regulator TraR. The protein TraR binds to exogenously added acyl-HSLs present in extracts, fractions, produced by other bacteria *in vivo*, or even synthetic products. The complex acyl-HSL-TraR regulates *lacZ* gene

expression, generating β -galactosidase enzymes that metabolize the added X-Gal reagent. X-Gal enzymatic hydrolysis produces a blue-colored indigo derivative, indicating a positive bioassay.

The ethyl acetate extract from *P. ananatis* cultivation media (1 l) provided positive biological activity. The same procedure was applied to the nutrient broth medium extracts, producing negative results and indicating that active metabolites came from microorganism metabolism. The biological activity of synthetic and natural products with bioreporter *A. tumefaciens* NTL4(pZLR4) was also evaluated, and the positive results corroborated the activity observed with extracts from *P. ananatis* cultivation media.

Absolute Configuration Determination

The optimum analytical conditions for the enantiomeric discrimination of synthetic (\pm)-*N*-hexanoyl-HSL (two peaks in 1:1 ratio) were established with GC-FID equipped with a fused silica Chrompack β -cyclodextrin column. The (*R*)- and (*S*)-*N*-hexanoyl-HSL retention times were accessed by comparing the racemic mixture to the synthetic (*S*)-(-)-*N*-hexanoyl-HSL. The absolute configuration of natural acyl-HSL was determined as (*S*)-(-)-*N*-hexanoyl-HSL by retention time comparison and coinjection with synthetic (\pm)-*N*-hexanoyl-HSL (Fig. 2).

Discussion

The present communication reports the occurrence of three acyl-HSLs for the phytopathogen *P. ananatis*, previously not reported for the *Pantoea* genus. These belong to an important class of signaling substances reported in several other Gram-negative bacteria. Interestingly, the identified metabolites are short-chain acyl-HSLs, similar to those detected in other species of *Pantoea*, *Pectobacterium*, and *Erwinia* genera (Bainton et al., 1992; Bodman and Farrand 1995; Pomini et al., 2005). These three genera are taxonomically closely related and were previously a part of only one genus, *Erwinia* (Hauben et al., 1998). It is now evident that the similarities are not only morphological, but also chemical. For comparison, in distantly related *Rhizobium* and *Sinorhizobium* genera cell communication employs long-chain acyl-HSLs, ranging from dodecanoyl to *N*-octadecanoyl-HSL (Marketon et al., 2002).

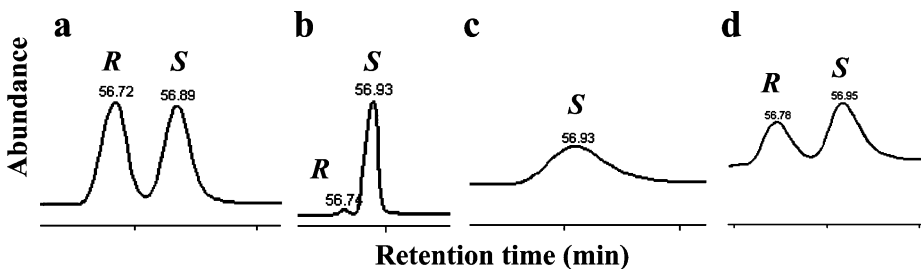


Fig. 2 Absolute configuration determination of natural (*S*)-(-)-*N*-hexanoyl-HSL using the GC(chiral)-FID methodology. (a) Enantiomeric discrimination of a synthetic racemic mixture [(*R*) enantiomer at 56.72 min and (*S*) enantiomer at 56.89 min]. (b) Analysis of synthetic (*S*) enantiomer (92% ee), at 56.93 min. (c) Analysis of natural (*S*)-(-)-*N*-hexanoyl-HSL from *P. ananatis* [(*S*) enantiomer at 56.93 min, >99% ee]. (d) Coinjection of natural product and synthetic racemic mixture, with increment in the relative abundance of the (*S*) enantiomer (56.95 min) in comparison with the (*R*) enantiomer (56.78 min)

Another interesting feature observed is the first occurrence of *N*-heptanoyl in the *Pantoea* genus. Odd-chain fatty acid derivatives such as *N*-heptanoyl-HSL are rare in nature. However, this metabolite was previously reported in *R. leguminosarum* (Lithgow et al., 2000) and *Erwinia psidii* (Pomini et al., 2005) using spectroscopic methods. Clues of its occurrence were noted in the fish pathogen *Edwardsiella tarda* (Morohoshi et al., 2004) and in *Serratia marcescens* (Hornig et al., 2002), using biorevelation procedures. Detection of this metabolite in *P. ananatis* increases its importance in quorum-sensing studies, and indicates the necessity of determining if propionate is in fact the acyl side chain biosynthetic initiator, as previously hypothesized (Whithers et al., 2001). However, in all reported cases, this substance was produced by the microorganisms in low amounts, which is an important drawback for accurate biosynthetic studies.

Prospecting of acyl-HSLs signaling substances is usually carried out by using biological reporters. These are genetically constructed microorganisms that contain a transcriptional regulator linking the expression of an observable phenotype to the presence of exogenous acyl-HSLs. The phenotypes include, for example, blue coloration in β -galactosidase assays [as in *A. tumefaciens* NTL4(pZLR4) employed here], bioluminescence, swarm motility, or production of colored antibiotics (Cha et al. 1998; Blosser and Gray 2000; Ravn et al., 2001). The use of bioreporters is crucial to understand the biological mechanisms involved, and is advantageous in high-throughput screenings of acyl-HSL producers. However, this methodology does not accurately identify the signaling substances. Protocols based on bioautography with TLC do not provide conclusive structural information on the acyl-HSLs produced. In addition, the bioreporter response to specific classes of acyl-HSLs (specially those closely related to the cognate signal produced by the wild-type strain from which the reporter was built) is a characteristic that leads to high sensitivity, but also to false negative responses when dealing with unusual acyl-HSLs. Thus, the use of several reporters of different specificities are required during bioguided studies (Cha et al., 1998; Ravn et al., 2001). An interesting example was reported in a study of signaling substances from *Erwinia amylovora*, where highly sensitive bioreporters were crucial to detect acyl-HSLs present in low amounts in infected pea tissues. However, the biorevelation methodology used could not elucidate whether the acyl-HSL produced by the microorganism was a 3-oxo or 3-hydroxy derivative of *N*-hexanoyl-HSL (Venturi et al., 2004). Thus, GC-MS methods seem to be more appropriate for correct identification of natural acyl-HSLs because the sensitivity is reasonable and the technique allows coinjection and mass spectral data comparison with synthetic compounds. The low molar mass and reasonable volatility of acyl-HSLs also makes GC-MS the method of choice for their analysis.

In spite of the great interest in quorum-sensing and the large number of bacteria under investigation, only few acyl-HSLs have been characterized at the absolute configuration level (Bainton et al., 1992; Lithgow et al., 2000). The absolute configuration of the major constituent, (*S*)-(-)-*N*-hexanoyl-HSL, produced by *P. ananatis* was accessed by using on-line GC-FID with a chiral stationary phase. This protocol is efficient when acyl-HSLs are present in complex mixtures (Fig. 2). Absolute configurations of the other two acyl-HSLs identified could not be determined because of their small quantities.

It should be mentioned that, up to now, all natural acyl-HSLs possess (*S*) stereochemistry (Bainton et al., 1992; Lithgow et al., 2000; Pomini et al., 2005). The biosynthetic origin of these metabolites was fully investigated in the marine luminescent bacterium *V. fischeri*, where the acyl-HSL synthesizing protein LuxI was isolated and the substrates were identified. The acyl side chain came from acyl-ACP (acyl carrier protein), whereas the HSL moiety came from *S*-adenosyl-methionine (SAM) (Schaefer et al., 1996). It is likely that the conserved (*S*) configuration is derived from the (*S*) absolute configuration of the SAM

amino acid portion. However, these findings do not exclude the necessity of characterizing the absolute configurations of acyl-HSLs from other producers. Based on the differentiated activity of synthetic (*R*) enantiomers in some quorum-sensing systems (Chhabra et al., 1993) and on the hypothesis that isomerization is one of the possible quorum-quenching (disruption of quorum-sensing) mechanisms (Roche et al., 2004), the detection of an (*R*)-acyl-HSL producing bacterium or a microorganism capable of isomerizing these metabolites would furnish interesting information from the chemical ecology point of view. In this context, the chiral GC-FID protocol could play an important role.

In summary, chemical investigation of the phytopathogen *P. ananatis* allowed the characterization of three acyl-HSLs not previously reported for the *Pantoea* genus. Also, the GC techniques used allowed the chemical characterization and absolute configuration determination of acyl-HSLs produced by *P. ananatis* independent of bioreporter specificities. Further work should be done to understand the roles of these substances in communication systems and gene regulatory networks in this species.

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Keywords Pinus, *Tomicus piniperda*, *Leptographium*, *Ophiostoma*, Terpene, Enantiomer, Inoculation, Resistance, (-)-p-pinene, (-)-limonene

Introduction

Most conifer resins are complex mixtures of terpenoids (Sjöström, 1990). Several ecological functions have been attributed to the numerous known conifer terpenoids. For example, they can function as insect-attracting odors in conifers (Vité et al., 1986; Byers et al., 1989; Nordlander, 1991; Almquist et al., *in press*), or as building blocks for *in vivo* syntheses of pheromones by wood-boring beetles (Hendry et al., 1980; Byers, 1995; Martin et al., 2003) and, because of their toxicity, even in defense against insects and pathogens (Raffa et al., 1985; Delorme and Lieutier, 1990; Raffa and Smalley, 1995).

Few species of bark beetles are able to overcome plant-defensive reactions and successfully colonize living trees. These aggressive beetles are normally associated with pathogenic blue-stain fungi (Krokene and Solheim, 1998) that enter the phloem with the tunneling beetles (Francke-Grosmann, 1967). The fungi invade sapwood and kill the tree by blocking water transport (see, e.g., Christiansen et al., 1987; Solheim et al., 1992). A well-studied example of this is the infection of *Picea abies* (L.) Karsten (Norway spruce) by the Eurasian spruce bark beetle, *Ips typographus* L., and associated blue-stain fungi *Ceratocystis* and *Ophiostoma* (Hornqvist et al., 1983; Solheim, 1992; Krokene and Solheim, 1998). *Pinus sylvestris* L. (Scots pine) does not appear to be afflicted by such an aggressive tree killer, although the pine shoot beetle, *Tomicus piniperda* L., may kill weakened trees (Långström, 1983; Långström and Hellqvist, 1991; Annala et al., 1999). *T. piniperda* is associated with the blue-stain fungi *Leptographium wingfieldii* Morelet and *Ophiostoma minus* (Hedgc.) H. and P. Sydow (Lieutier et al., 1989; Gibbs and Inman, 1991; Solheim and Långström, 1991), which are able to kill *P. sylvestris* trees inoculated with doses resembling fungal densities found in beetle attacks (Solheim et al., 1992). In contrast, the lesser pine shoot beetle, *Tomicus minor* Hart., is less aggressive (Långström and Hellqvist, 1993), and its principal associated blue-stain fungus, *Ophiostoma canum* (Münch) H. and P. Sydow, was recently shown to be of low virulence (Solheim et al., 2001).

Conifer defense against insects and pathogens relies on both physical and chemical barriers associated with resinosis (Berryman, 1972; Phillips and Croteau, 1999). In Pinaceae, there are two types of defense reaction: constitutive and induced resinosis. In constitutive defense, preformed resin is exuded in response to attacks by beetles or fungi, whereas in induced defense, resinous compounds are synthesized around the attack site. These processes have been studied in Norway spruce (*Picea abies*) (Evensen et al., 2000) and in *P. sylvestris* (Lieutier et al., 1991a,b, 1995; Långström et al., 1992). The existence of “acquired resistance” following a sublethal challenge (by inoculation of a sublethal dose of fungi) to the defense system was recently demonstrated in *P. abies* (Christiansen et al., 1999a,b; Krokene et al., 1999), in *P. sylvestris* (Krokene et al., 2000), and in Monterey Pine (*Pinus radiata* D. Don) (Reglinski et al., 1998). True firs (*Abies* spp.) synthesize new resin in concretized resin blisters and display a low constitutive production of terpenoids, whereas spruces (*Picea* spp.) and pines (*Pinus* spp.) have well-developed systems of resin ducts (Lewinsohn et al., 1991; Franceschi et al., 1998, 2000; Nagy et al., 2000). *Pinus* species are believed to sustain high levels of constitutive resin production, which is independent of enzyme activity associated with the wounding of tissues (Savage et al., 1994; Phillips et al., 1999). However, *de novo* syntheses of terpenoids and phenols are observed in *P. sylvestris* after insect attack and fungal infection (Lieutier et al., 1991a,b, 1995; Långström et al., 1992; Bois and Lieutier, 1999). *Abies grandis* (Dougl.) Lindl. (grand fir) has been the

subject of extensive research because of its apparent, induced biosynthesis of terpenoids after wounding (Bohlmann et al., 1999; Phillips and Croteau, 1999). The isolation, functional characterization, and gene expression of terpene synthases have contributed to the understanding of mechanisms in the induction of plant defense.

The chemical basis of the conifer defense reactions in response to attacks by bark beetles and associated blue-stain fungi is still not fully understood. Resistance towards virulent pathogens or beetles (where they are the primary killers) can be seen as an accumulation of terpenes, as a simple flushing of resin at the reaction site, or as a change in the constitutive resin composition towards a blend more toxic to invaders. Delorme and Lieutier (1990) have shown that the relative composition of various monoterpenes changes *after* fungal inoculation in resin pockets as well as in reacting phloem in *P. sylvestris*. Various beetle-associated fungi [e.g., *Ophiostoma brunneo-ciliatum* Math.-Käärk, *O. ips* (Rumb.) Nannf. and *L. wingfieldii*] induce resin production, resulting in clear differences between wounded and unwounded phloem. However, the resin in unwounded tissue differs from the preformed resin (Delorme and Lieutier, 1990). *T. piniperda* has been shown to induce resin acid production in *P. sylvestris*, by crystallization of resin acid around the attack area, but no corresponding changes elsewhere in the trunk (Långström et al., 1992).

Previous investigations of monoterpene hydrocarbons that constitute more than 50% of the resin have revealed a wide diversity in *Pinus* species and *Picea abies*, with respect to both enantiomeric compositions and relative amounts. Broad chemical diversity is seen between and within species, as well as in different tissues from the same specimen (Borg-Karlson et al., 1993; Fäldt et al., 2001; Persson et al., 1993, 1996; Sjödin et al., 1993, 1996, 2000). A high level of chemical diversity is argued to be highly beneficial for plants, as variation in terpene composition among trees of the same species probably makes it difficult for a herbivore or phytopathogen to develop resistance to specific compounds (Sturgeon, 1979).

This study is part of our research on chemical markers of resistance in conifers. The biological results related to this study have been published (Krokene et al., 2000) and are discussed below in their chemical context. The aims were: (1) to identify the monoterpenes active within the defense reaction zone against: (a) a highly virulent fungus, (b) a weakly virulent fungus, and (c) a sterile inoculation (wounding only), and thereby to find out how specific the induced defense reaction is; (2) to identify the change in monoterpene compositions after defense responses to the above inoculations in areas beyond the visible reaction zone (induced systemic reaction); (3) to identify the monoterpenes present after defense responses to mass inoculations within as well as beyond the trunk area treated, in order to compare the induced defense reactions in trees with normal and elevated levels of resistance.

Methods and Materials

Biological Material

In spring 1999, 32 pine trees of similar size [85 ± 3 mm (mean \pm SD) diam at a height of 1.3 m] were selected from a single stand at Hökensås in southwestern Sweden. The stand had been severely defoliated (ca. 90–100% of the needle biomass removed) by the pine looper, *Bupalus piniaria* (Lepidoptera, Geometridae) in 1996 (Långström et al., 1999). The trees were estimated to have about 30–50% of full foliage, and had smaller-than-normal shoots of the age classes 1997 and 1998. In addition, four undefoliated trees (60–70 mm in diam) were selected in an adjoining young pine stand.

On May 25–26, 1999, four groups of eight trees each (32 trees in total) were randomly subjected to one of four pretreatments (I to IV). Each pretreatment consisted of a low dosage (50 inoculations m^{-2}) of one of the following inoculants: (I) *L. wingfieldii* (LW) (isolate NISK 89-369/11), (II) *O. canum* (OC) (isolate NISK 97-33/47), (III) sterile agar (W), and (IV) no pretreatment—as a control group (TC). The rationale for these pretreatments is described by Krokene et al. (2000). Inoculations were done with a 5-mm cork borer by using a standard. A bark plug was removed and agar with or without mycelium was inserted into the hole and the bark plug was replaced. Pretreatment inoculations were performed in a 60-cm band around each tree trunk at breast height, and the dose corresponded to 10 plugs per tree. Group IV trees, as well as four extra trees in the same stand were left for use as an extra control group (c) together with the four reference trees from the adjoining stand (c0). In total, 24 trees were used for the chemical analyses.

Four wk after pretreatment, the 32 trees (LW, OC, W, TC) were mass-inoculated with *L. wingfieldii* at a density of 800 inoculations m^{-2} in the same trunk areas as the pretreatment inoculations, in a 60-cm band around each trunk at breast height, in order to assess tree resistance. Trees were felled on September 27, 1999. The lower trunks of half of the trees (16 trees in all) were cut and used for further analyses of defense reactions and fungal performance (see Krokene et al., 2000 for more details).

Chemical Sampling

Four of each pretreatment plus the eight extra trees (c0) and (c) (24 in all) were selected for chemical sampling as described below. All samples were taken with a 5-mm cork borer, which removed a 5-mm sample plug containing cortex, phloem, and some xylem. The cortex and xylem residues in the plug were removed immediately after sampling, before the monoterpenes in the phloem only were extracted with hexane (1 ml, using tetradecane as the internal standard for quantification at a concentration of $0.1 \mu l ml^{-1}$ hexane). At each sampling occasion, ten or five phloem plugs [diam 5 mm, approx. 0.1 g (10 plugs) and 0.05 g (5 plugs)] were taken from each sampling site in each tree. Samples were taken as follows: Day 0 (the pretreatment day): phloem was collected and extracted from 10 plugs per tree in order to have an undisturbed starting sample (constitutive phloem resin, “0” in Fig. 1). No samples were taken from the untreated controls (TC) to avoid triggering any chemical reactions, but the extra controls (c) and the young reference trees were sampled (c0) (see Biological Material; Fig. 1 and Table 1). Day 1: samples were taken 5 mm above the pretreatment inoculation holes in LW, OC, and W trees (“1” in Fig. 1). Again, no samples were taken from the untreated controls (TC) (Table 1). Day 28: (mass inoculation day), new samples were taken 5 mm below the pretreatment inoculation holes, designated area “1” in Fig. 1, and in random positions above the inoculated trunk area (>20 cm above any pretreatment inoculation hole), designated area “2” in Fig. 1, in the LW, OC, and W trees. Samples were also taken from the untreated controls (TC) for the first time, and at random positions in the extra controls (c) and young reference trees (c0) (Fig. 1 and Table 1). Day 124: the 16 mass-inoculated trees were all sampled at random positions within the inoculation area designated “1” in Fig. 1, above the inoculation area (>20 cm) designated area “2” in Fig. 1, and outside the visible phenolic reaction zone, designated area “3” in Fig. 1. Samples were also taken from area designated “2” in Fig. 1 (2) in the extra controls (c) and from reference trees (c0) (Fig. 1 and Table 1).

All samples were extracted in hexane at room temperature for 48 hr. The phloem was removed and the extract was divided into two parts, one of which was filtered through SiO_2

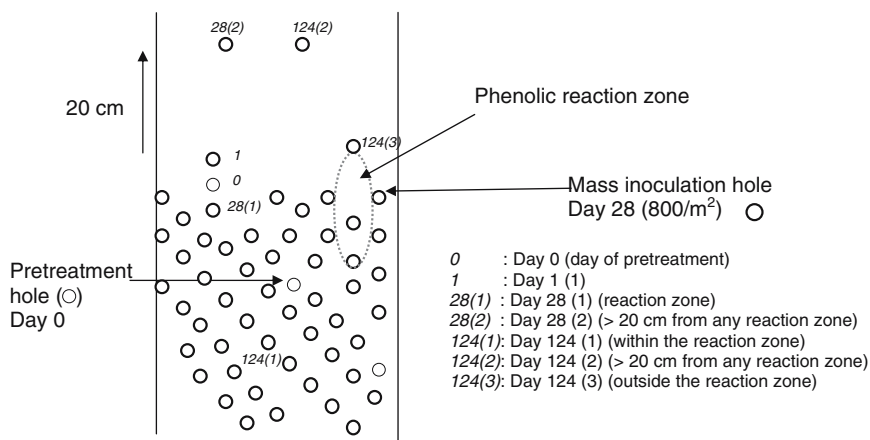


Fig. 1 Schematic representation of the localization of the samples taken on the Scots pine trunk. Trees were pretreated day 0, mass-inoculated on day 28, and harvested on day 124. Phloem samples were obtained (at all occasions) with a cork borer (5 mm diam), and extracted in hexane after removal of xylem and cortex. See also Table 1 for further information

(100 mg) before analysis and the other was stored at -24°C . The residual phloem was dried at 150°C for >3 hr and weighed.

Chemical Analyses

All samples were analyzed with a two-dimensional gas-chromatographic system (2D GC) that employed two Varian 3400 gas chromatographs, as previously described by Borg-Karlson et al. (1993). In the first GC (for nonenantiomeric separation), a DB-WAX column was used (polyethylene glycol column of 30 m length, 0.25 mm inside diam, and 0.25 μm film thickness, J&W ScientificTM). The temperature program started at 42°C for 1 min, increasing to 70°C at a rate of $3^{\circ}\text{C min}^{-1}$, to 160°C at a rate of $10^{\circ}\text{C min}^{-1}$, to 200°C at a rate of $20^{\circ}\text{C min}^{-1}$, and then remaining at 200°C for 20 min. A split/splitless injector (6 s splitless) was used at a temperature of 180°C . The detector temperature was 200°C . Chiral monoterpene hydrocarbon fractions were “cut” by pneumatic valves, controlled by relays. By changing the relays, and, thereby the position of the valve, it was possible to choose a path for the compounds that led either to the detector in the first GC or through the first to

Table 1 Sampling scheme (see also Fig. 1)

Sample	Position	Days	Purpose
Constitutive resin		0(LW, OC, W, c, c0), 28 (TC)	To examine constitutive phloem resin chemistry
(1)	5 mm above pretreatment holes	1, 28, 124	To examine reaction zone chemistry
(2)	>20 cm from the nearest reaction zone	28, 124	To examine chemistry in uninjured phloem
(3)	Outside the visible phenolic reaction zone	124	To examine chemistry outside the visible phenolic reaction zone

the second GC. Two chiral columns were installed in the second GC oven, both of them being used during the same run (operated by the relay control). Column number 1 was a Cyclodex B (permethyl- β -cyclodextrin/DB-1701) column of 30 m length, inside diam of 0.25 mm, and a film thickness of 0.25 μm (J&W ScientificTM), and column 2 was a Chirasil-DEX CB (permethyl- β -cyclodextrin, chemically bound to a polydimethyl siloxane) column of 25 m length, 0.25 mm inside diam, and 0.25 μm film thickness (ChrompackTM). The temperature program started at 55°C for 15 min, increasing to 77°C at 1°C min⁻¹, and then remaining constant for 8 min. The detector temperature was 160°C. The chiral monoterpenes analyzed were α -pinene, camphene (when present in sufficient amounts), β -pinene, sabinene, limonene, and β -phellandrene. The amount of camphene in some of the samples was not large enough to determine the enantiomeric composition. 3-Carene was considered present only as the (+)-enantiomer, being the only enantiomer present in all our previous studies on monoterpene hydrocarbons from Scots pine (Sjödin et al., 1996, 2000; Fäldt et al., 2001).

All samples were analyzed with a gas chromatograph mass spectrometer (GC-MS) (Varian 3400 GC connected to a Finnigan SSQ 7000 MS) for identification and quantification of the monoterpenes. A DB-WAX column was used (J&W ScientificTM; 30 m, 0.25 mm id, and 0.25 μm film thickness). The temperature program was set as follows: 40°C for 4 min, increasing to 200°C at a rate of 4°C min⁻¹, to 220°C at a rate of 20°C min⁻¹, and then remaining constant at 220°C for 10 min. A Finnigan autoinjector was used with a split/splitless injector (6 s splitless) at 210°C for assuring the repeatability. Helium was used as the carrier gas, the transfer line was 250°C, and the ion source 150°C. The compounds were identified by means of their retention times (GC), or by comparison with either natural or synthetic, available reference samples (for (+)-*b*-pinene; see Valterová et al., 1992). The order of elution of monoterpene hydrocarbons on the Chirasil-Dex column was the same as on the Cyclodex B column (Borg-Karlson et al., 1993).

The *absolute amounts* of compounds extracted were calculated according to the formula provided below [Eq. (1)]. The tetradecane concentration used was 0.1 $\mu\text{l ml}^{-1}$ hexane, and the volume of hexane added to each of the samples was 1 ml. Therefore, the mass of the compound could be calculated, by using the following equations:

$$\begin{aligned} n_x(\text{mmol}) &= A_x/A_{C_{14}} * rf * n_{14}; \text{ and } m_x(\text{mg}) \\ &= A_x/A_{C_{14}} * rf * 0.000385 * 136 \end{aligned} \quad (1)$$

where n_x is the molar amount of monoterpene hydrocarbon (x); A_x is the peak area of monoterpene hydrocarbon (x) (GC-MS data); $A_{C_{14}}$ is the area of tetradecane (GC-MS data); rf is the response factor for monoterpene hydrocarbons (acyclic, 1.71; cyclic, 1.53; and bicyclic, 1.72) based on samples injected into the GC-MS containing exactly the same molar concentration of acyclic (myrcene), cyclic (limonene), and bicyclic (α -pinene) monoterpene hydrocarbons as well as tetradecane; n_{14} is the known molar concentration of tetradecane in all samples (0.000385 mmol ml⁻¹); m_x is the calculated mass of the monoterpene hydrocarbon (mg). Standard solutions (with increased concentrations) were injected by the autoinjector. The mass of the compound calculated by means of Eq. (1) was then divided by the dry weight (dw) of each sample to achieve the final expression in mg g dw⁻¹.

The *proportions* (relative amounts) were calculated as the ratio of the area of each peak to the sum of all the areas of monoterpene hydrocarbons in a defined GC fraction (including the enantiomeric ratio of the analyzed chiral constituents) retained from tricyclene to terpinolene, and expressed in percentages. *Enantiomeric composition* was defined as the GC

peak area of the (–)-enantiomer divided by the sum of GC peak areas of both enantiomers, multiplied by 100.

Results

Biological Results

As the biological results have been reported elsewhere (Krokene et al., 2000), we only summarize here that all pretreatments resulted in induced resistance. This reaction was more pronounced after pretreatment with the aggressive fungus (LW) than with OC or the sterile inoculation. Furthermore, we demonstrated that wounding alone produced a small reaction zone, only extending ca. 10 mm vertically from the inoculation point after ca. 11 wk. The corresponding length for inoculations with *O. canum* was ca. 25 and with *L. wingfieldii* ca. 150 mm (Solheim et al., 2001). Lesion size after inoculation with *L. wingfieldii* was 2, 30, and 60 mm after 1, 2, and 4 wk, respectively (Långström et al., 1993). Thus, the samples taken in “area 1” (cf. Fig. 1) were inside the reaction zones on day 28 and day 124, whereas all other samples were taken outside of the reaction zones.

Chemical Results

Absolute Amounts. Day 1. The absolute amounts of the main monoterpene hydrocarbons showed a 3- to 5-fold increase at the site of inoculation as early as 1 d after pretreatment, without any notable differences between the various treatments (Fig. 2). At this early stage,

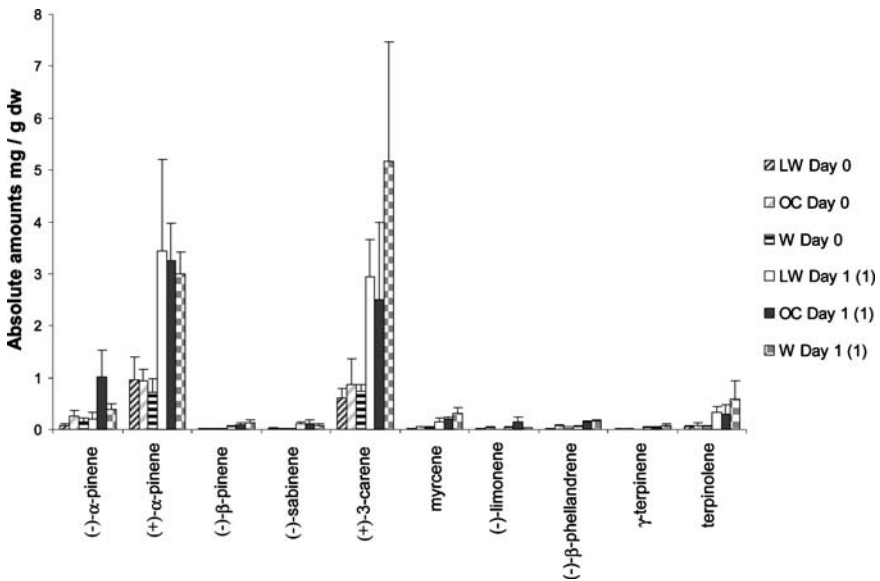


Fig. 2 Absolute amounts of monoterpene hydrocarbons in the phloem of *Pinus sylvestris* before inoculation (day 0) and 1 d after inoculation (day 1) (mean values from four trees in mg/g dry weight). Generally, there is a 2- to 3-fold increase in the main monoterpene hydrocarbons 1 d after pretreatment. Pretreatment designations: LW, *Leptographium wingfieldii*; OC, *Ophiostoma canum*; W, sterile agar. The numbers in brackets refer to the places of the samplings and are described in Fig. 1 and Table 1

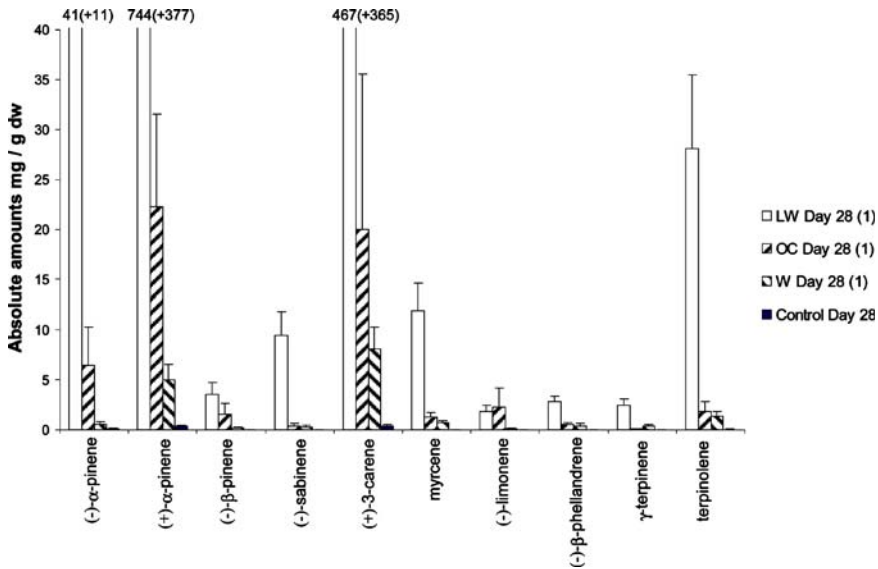


Fig. 3 Absolute amounts of monoterpene hydrocarbons in the phloem of *P. sylvestris* on the day of mass inoculation (day 28) (mean values from four trees in mg/g dry weight). Note that (–)-α-pinene, (+)-α-pinene, and (+)-3-carene increase to 41, 744, and 467 mg/g dry weight, respectively. Samples were taken locally from the reaction zone phloem. Pretreatment designations: LW, *Leptographium wingfieldii*; OC, *Ophiostoma canum*; W, sterile agar

the exudation of resin was caused by generalized wounding, and was not expected to be specific for any of the particular fungal inoculants or wounding techniques. Day 28. After 4 wk, the increase in absolute amounts of monoterpenes was more pronounced in trees inoculated with *L. wingfieldii* (LW) than in trees inoculated with *O. canum* (OC) or wounded only (W) (Fig. 3), measured in the hypersensitive reaction zone of the phloem (at site 28 (1), Fig. 1). Comparing the LW and OC trees, the increase in (+)-α-pinene was more pronounced than the increase in (–)-α-pinene (Fig. 3) for LW trees. By day 124, 3 mo after the first inoculations (pretreatments), there was an obvious accumulation of resin within the area of inoculation [Fig. 4, 124 (1)]. However, the absolute amount of monoterpenes found in the reaction zone of the LW trees were generally lower than those seen on day 28 (Fig. 3) as opposed to the OC and TC trees, where the amounts were higher after 124 d, which can be attributable to leakage through the heavy damage tissues by fungi. When compared with the composition before pretreatment (day 0), the chemical changes in the phloem sampled >20 cm from the nearest reaction zone were more pronounced at day 124 than at day 28 (Fig. 5). The main components, (+)-α-pinene and (+)-3-carene, both decreased in all treatment and control groups. In contrast, the amounts of (–)-β-pinene and (–)-limonene increased in all treatments, but in none of the controls (Fig. 5).

Relative Amounts

The relative amounts of various monoterpenes did not show any marked differences between the constitutive resin (sampled day 0) and the resin sampled in the phloem >20 cm from the nearest reaction zone after 28 d (Fig. 6a and b). There was no clear difference between the treatments. However, after mass inoculation and intense fungal growth, the

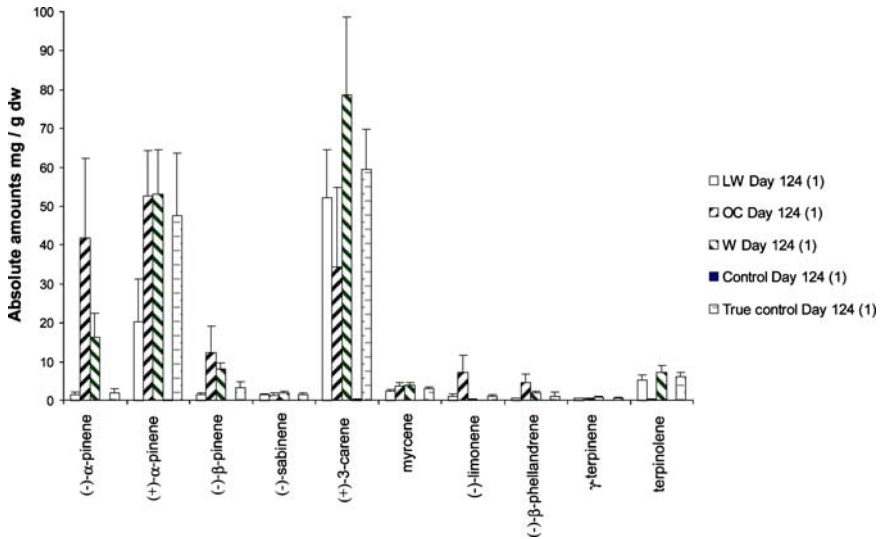


Fig. 4 Absolute amounts of monoterpene hydrocarbons in the phloem of *P. sylvestris* on day 124 (after fungal growth) from four trees in mg/g dry weight). Samples were taken locally from the reaction zone phloem. Pretreatment designations: LW, *Leptographium wingfieldii*; OC, *Ophiostoma canum*; W, sterile agar; TC, true controls, no pretreatment. The numbers in brackets refer to the places of the samplings and are described in Fig. 1 and Table 1

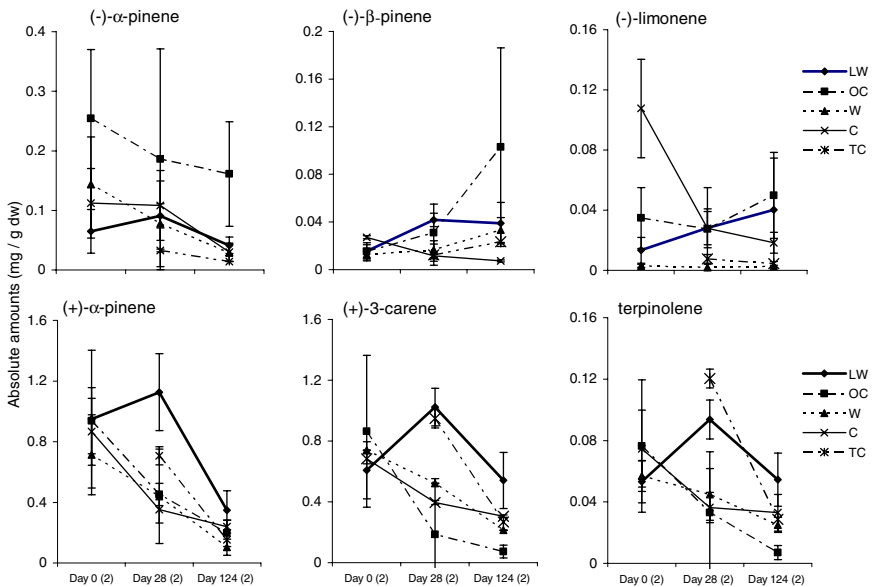
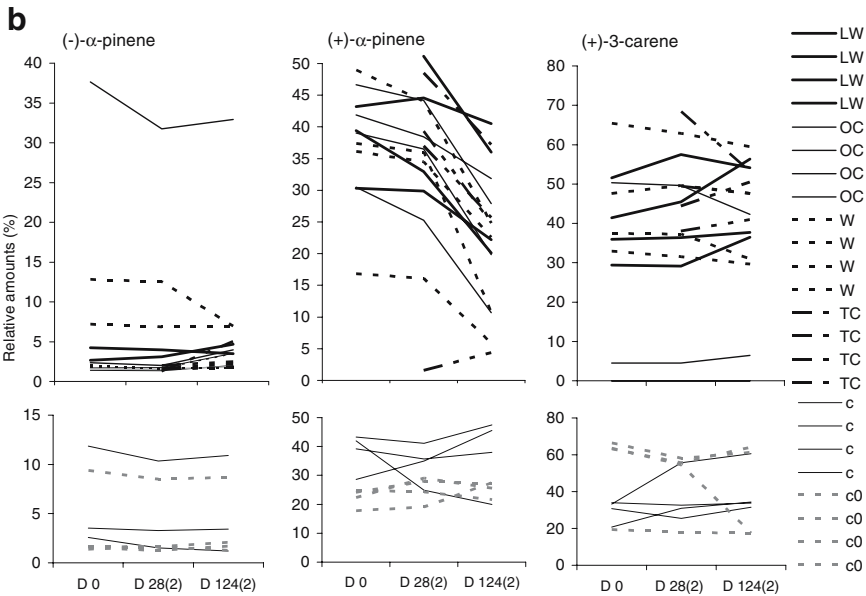
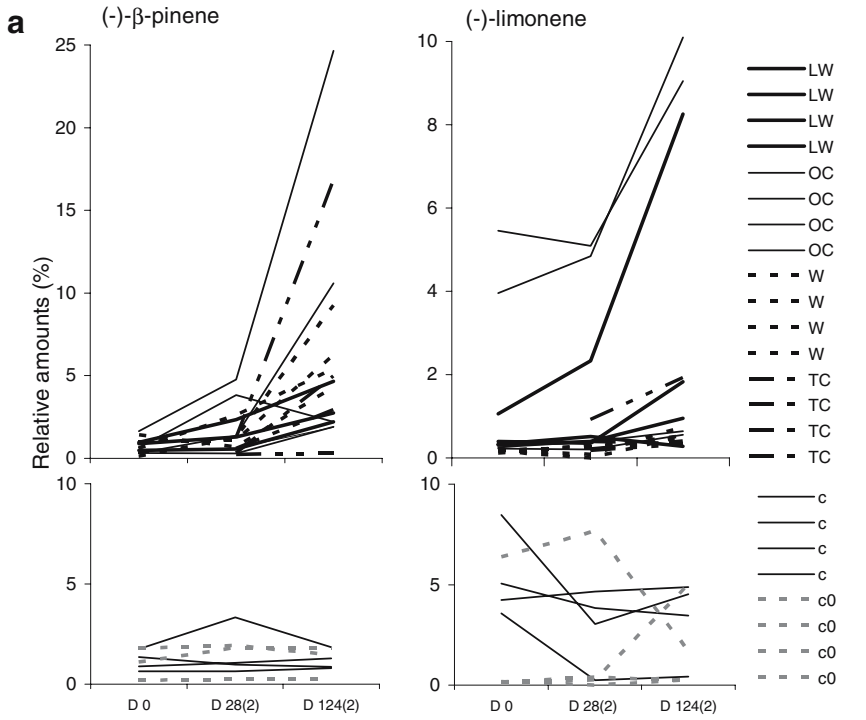


Fig. 5 Absolute amounts of $(-)\alpha$ -pinene, $(-)\beta$ -pinene, $(-)$ -limonene, $(+)\alpha$ -pinene, $(+)\text{-}3$ -carene, and terpinolene in the phloem of *P. sylvestris* before inoculation (day 0), on the day of mass inoculation (day 28), and on day 124 (after fungal growth) (mean values from four trees in mg/g dry weight). Note the difference among the constituents throughout the season as well as after fungal growth. The numbers in brackets refer to the places of the samplings and are described in Fig. 1 and Table 1. The samples (after 28 and 124 d) have been taken >20 cm from the nearest inoculation hole



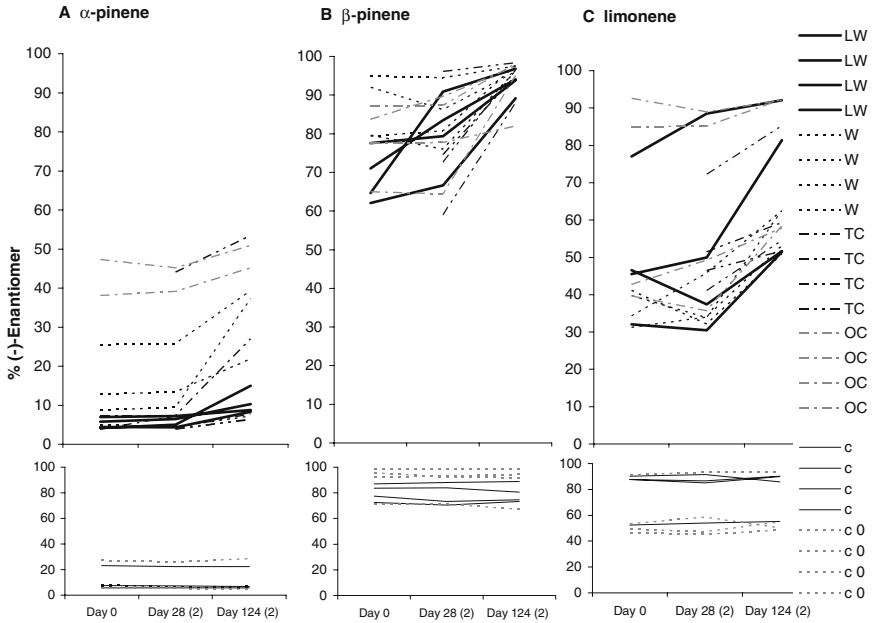


Fig. 7 Changes in enantiomeric compositions of α -pinene (A), β -pinene (B), and limonene (C) for individual trees. Upper diagrams refer to samples taken from treated trees and lower diagrams refer to samples taken from untreated control trees. The (-)-enantiomers increase after pretreatments, but is more obvious after fungal growth of the mass-inoculates. Pretreatment designations: LW, *Leptographium wingfieldii*; W, sterile agar; OC, *Ophiostoma canum*; TC, true controls, trees without any pretreatment. Note that c designates extra controls in the area and c0 designates reference trees outside the test area. The reactions of four trees of each treatment are shown and each tree is connected with a line. The numbers in brackets refer to the places of the samplings on the tree and are described in Fig. 1 and Table 1

relative amounts in the treated groups of trees differed from the control trees as well as from the constitutive resin (day 0) in the treated trees. The most striking differences were found after 124 d with increases in (-)-limonene and (-)- β -pinene (Fig. 6a), and a decrease (or lack of change) in (-)- α -pinene, (+)- α -pinene, and (+)-3-carene (Fig. 6b).

Enantiomeric Composition

Large individual responses on fungal inoculations were observed and, therefore, we present individual reactions in Fig. 7. The (-)-enantiomers of β -pinene and limonene already increased slightly in phloem sampled >20 cm from the reaction zone in the LW trees before mass inoculation, 28 d after pretreatment (Fig. 7), which could be an indication of induced biosynthesis. However, the pronounced increase in the enantiomeric composition of α -

◀ **Fig. 6** Relative amounts of (-)- β -pinene, (-)-limonene (A), and (-)- α -pinene, (+)- α -pinene, and (+)-3-carene (B) in the constitutive resin (day 0) and in the phloem sampled >20 cm from the nearest reaction zone after 28 d (2) and 124 d (2). The relative amounts of (-)- β -pinene and (-)-limonene are increasing after fungal growth, whereas the relative amounts of (-)- α -pinene, (+)- α -pinene, and (+)-3-carene are unchanged, decreasing, or somehow altered, respectively. Pretreatment designations: LW, *Leptographium wingfieldii*; OC, *Ophiostoma canum*; W, sterile agar; TC, true controls, trees without any pretreatment. Note that c designates extra controls and c0 designates reference trees. The reactions of four trees of each treatment are shown and each tree is connected with a line. The numbers in brackets refer to the places of the samplings and are described in Fig. 1 and Table 1

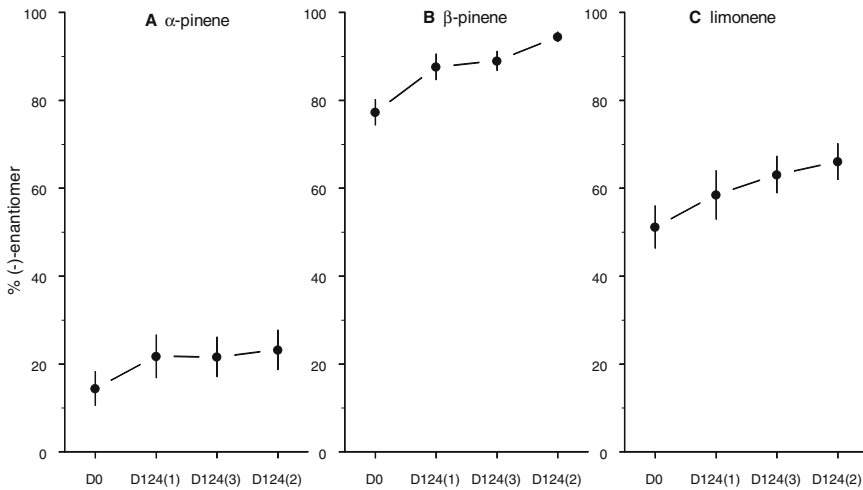


Fig. 8 Enantiomeric compositions of α -pinene (A), β -pinene (B), and limonene (C) in phloem samples sampled at increasing distances from the inoculation. The proportion of (-)-enantiomer increases with distance from the inoculation for β -pinene and limonene (mean value with standard error). See Fig. 7 for the seasonal variation in extra control and reference trees. The numbers in brackets refer to the places of the samplings and are described in Fig. 1 and Table 1

pinene, β -pinene, and limonene was found in the phloem sampled at the same distance on day 124 [see 124 (2) in Fig. 7]. The enantiomeric compositions of the three terpenes increased or changed in phloem from trees stressed by fungal growth, whereas in the phloem extract of the control trees no change in enantiomeric composition of α -pinene throughout the season was found.

With increasing distance from inoculation sites, the proportions of (-)-enantiomers of β -pinene and limonene clearly rose in nearly all of the mass-inoculated trees (Fig. 8). The enantiomeric composition both in the reaction zone [day 124 (1) in Figs. 1 and 8] and in the area near the fungal growth, zone [day 124 (3) in Figs. 1 and 8], exhibited greater variation than the phloem sampled >20 cm from the nearest reaction zone [day 124 (2) in Figs. 1 and 8]. The (-)-enantiomers predominated both in sabinene and β -phellandrene, but the enantiomeric composition remained unchanged (with the exception of low-3-carene-containing trees, which contained low constitutive proportions of (-)-sabinene).

Discussion

This study has shown that mechanical wounding and fungal inoculation both resulted in reduced susceptibility to mass inoculation with LW that was manifested by reduced phloem necrosis and sapwood occlusion. As there was no difference in induced response between the pretreatments, we tend to believe that this is a general, rather than specific, response. It was not possible to determine whether the response was local or systemic as the mass inoculation was done in the same stem area as the pretreatments. In Norway spruce, where this phenomenon was first seen, the enhanced resistance was local (Krokene et al., 1999), but it seems possible that the effect may spread further with increasing time between pretreatment and mass inoculation. The mechanisms responsible for this induced resistance in conifers

are largely unknown, but formation of traumatic resin ducts and activation of phenol-rich parenchyma cells (PP cells) seem to be involved (Franceschi et al., 1998, 2000; Nagy et al., 2000).

Infection-induced responses, in the form of monoterpene production, have been previously investigated in *P. sylvestris* infected by *T. piniperda* (Lieutier et al., 1995) and associated blue-stain fungi (Delorme and Lieutier, 1990). However, an accurate study of the changes in the enantiomeric composition of the chiral constituents has, to our knowledge, not been done to date.

This study shows that the virulence of fungi causes a chemical response in trees that is most obviously close to the inoculation hole, as the quantity of monoterpenes increases up to 800-fold in LW trees, compared to 30-fold in OC trees (see (+)- α -pinene; Fig. 3).

These results, which may be the result of a lower growth rate of OC, are in accordance with the less virulent OC compared to LW (Solheim et al., 2001), and consequently to that of *T. minor* compared to *T. piniperda* (Långström and Hellqvist, 1993).

The presence of an induced systemic reaction and an allocated biosynthesis due to fungal growth is indicated by alteration in the relative amounts of (–)- α -pinene and (–)-limonene, and also by an increase in (–)- β -pinene in samples taken outside of the inoculation zone at day 124 (Fig. 6a and b). This is further confirmed by a change in enantiomeric compositions for the three constituents (Figs. 7 and 8). However, at day 28, before mass inoculation, we could not find any significant difference in the relative amounts or enantiomeric composition between pretreated trees (trees with possibly induced resistance) and trees with a normal level of resistance (TC trees).

The absolute amounts of monoterpenes in the reaction zone samples from day 28 were consistent with amounts found in previous studies on *P. sylvestris* inoculated with blue-stain fungi (Delorme and Lieutier, 1990). They showed marked qualitative and quantitative changes in the monoterpene pattern between unaffected phloem and phloem affected by different fungi (Fig. 3). We found that the most virulent fungus (LW) elicited the largest response in monoterpene production, but the tremendous variation in resin composition and amounts (e.g., low and high (+)-3-carene trees)—not only within each tree, but also between trees in this natural generated forest—prohibited us from finding a monoterpene pattern specific for the two fungal species used.

We found a minor effect of pretreatment on tree chemistry, as the enantiomeric composition in LW and TC treatment groups were in the same range at the start of mass inoculation (day 28). Changes in enantiomeric composition of TC trees to mass inoculation showed the same pattern as changes in LW trees (Fig. 7).

There was high variation in the composition of monoterpenes among trees within each pretreatment group, both before (constitutive) and after the pretreatments. While evaluating changes in relative and enantiomeric monoterpene composition, we found a high variation in the way each tree changed (Figs. 6, 7, and 8). However, there was no specific or distinctive pattern between the chemotypes. The ecological relevance of maintaining high chemodiversity of defense-related constituents in plants has been previously discussed (Sturgeon, 1979; Jones and Firn, 1991), yet susceptibility and resistance among chemotypes is poorly investigated in Scots pine.

Several of the constituents that increased after fungal infection are known to be toxic or growth-inhibiting towards fungi (Delorme and Lieutier, 1990; Klepzig et al., 1996). After the sublethal inoculations (day 28), the monoterpene profiles changed. This was most clearly seen in the enantiomeric composition of some of the chiral monoterpenes. The chemical defense responses elicited by fungal attacks and analyzed via phloem samples

taken beyond the reaction zone suggest that biosyntheses of monoterpenes took place either (1) both locally and at a distance from the reaction zone phloem, or (2) at a distance from the inoculation and reaction zone phloem, and were transported into the reaction zone phloem. Whether this transport is an active or passive process might be a key in the search for chemical markers of resistance.

Induced chemical response due to insect attack and pathogen invasion can be characterized by a change in a plant's chemical profile. This *de novo* production of compounds should benefit the plant by making it more toxic to an invader—herbivore or pathogen—as the induced production of stilbenes observed after inoculation *Ceratocystis polonica* (Viiri et al., 2001), but it may also attract predators or parasitoids of the herbivore. Induced terpene production following wounding or elicitor treatment has been established by enzyme assays (Martin et al., 2002) as well as by gene isolation, characterization, and expression in *A. grandis* (Bohlmann et al., 1999), *Picea sitchensis* (Miller et al., 2005) and *P. abies* (Fäldt et al., 2003; Martin et al., 2004). However, *Pinus* species are believed to rely on their constitutional chemical defense as there is minimal activation of biosynthesis upon wounding (Lewinsohn et al., 1991; Phillips et al., 1999). However, in this study, we have shown that fungi induce a local change in the proportion of monoterpenes, especially in the enantiomeric composition of chiral monoterpenes. In the light of recent knowledge from the products of isolated and cloned terpene synthases, we can understand the correlation between (+)-3-carene and terpinolene, as most probably the (+)-3-carene synthase isolated from Norway spruce by Fäldt et al. (2003) is also present in Scots pine. We hypothesize that a different (–)-pinene synthase is present in pine compared to spruce (Martin et al., 2004). Possibly, a (–)- β -pinene synthase is activated in Scots pine as a result of fungal infection, as the increase in (–)- α -pinene and (–)- β -pinene was not correlated (Figs. 7 and 8). Whether the changes we have observed can be regarded as a systemic induced monoterpene biosynthesis or not, has to be further analyzed, as there is no evidence for systemic enzyme activation in Scots pine.

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dominant, whereas ions for hetero-oligomers predominated in the other *Lotus* spp. Ions indicative of A-type linkages were observed in the MS of *L. americanus*. The results are discussed in terms of possible relationships between the concentration and composition of the PAs of *Lotus* spp. and ecological factors.

Keywords Proanthocyanidins · Thiolysis · Procyanidins · Prodelphinidins · HPLC · ^{13}C NMR · ESI-MS · MALDI-TOF-MS · *Lotus*

Introduction

Proanthocyanidins (PA) are a class of polymeric, polyphenolic, plant secondary metabolites that are widely distributed in the plant kingdom. These polymers encompass a wide range of structural variants. They consist of chains of flavan-3-ol (Fig. 1) units linked together through C4–C8 and/or C4–C6 linkages (B-type) and may be doubly linked with an additional C2–O–C7 linkage (A-type). In addition to these variations in interflavanoid linkages, PAs can vary in the hydroxylation pattern of the A- and B-rings, stereochemistry at the three chiral centers (C2, C3, and C4) of the C ring, and the degree of polymerization (DP). The most common PAs in forage legumes are procyanidins (PC) with a 3',4'-dihydroxy substitution of B-ring (catechin and epicatechin) and prodelphinidins (PD) with 3',4',5'-trihydroxy substitution (gallocatechin and epigallocatechin).

Proanthocyanidins are not known to play any role in physiological processes of plants. However, they are known to precipitate proteins (Hagerman and Butler, 1981) and have been studied for likely ecological roles. They have been considered as plant defenses against insect (Zucker, 1983; Ayres et al., 1997; Heil et al., 2002) and mammalian herbivores (reviewed in Iason, 2005), but their role in the dynamics of nutrient cycling (Zucker, 1983; Kraus et al.,

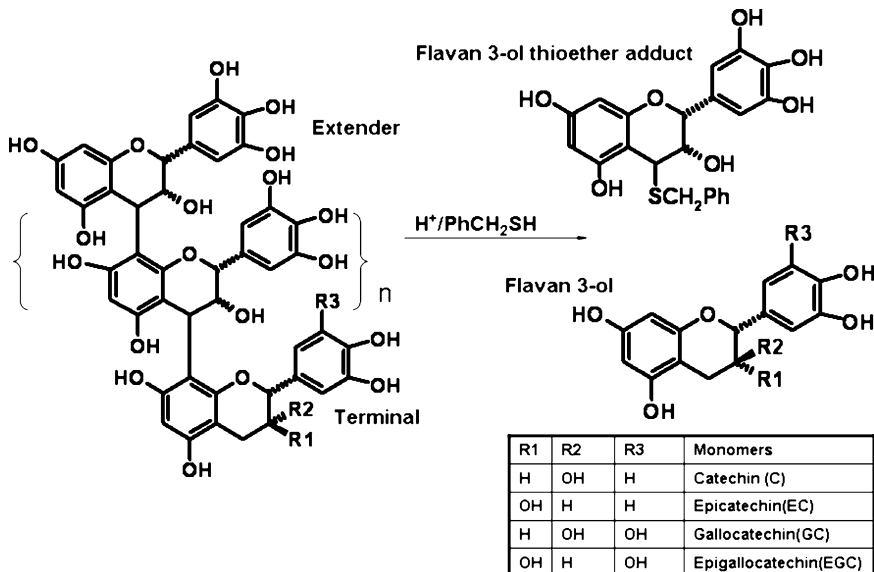


Fig. 1 Thiolytic cleavage of proanthocyanidin terminal units released as flavan-3-ols and extension units as flavan-3-ol thioether adducts

2003) and in the photoprotection of plant tissues (Close and McArthur, 2002) has also come into consideration.

In agricultural systems, high concentrations of PA [$>6\%$ of dry matter (DM)] in forage and browse plants reduce voluntary feed intake, digestibility, and animal performance of ruminants (Min et al., 2003), consistent with a defensive role. However, at lower concentrations, the protein-binding effects of PAs protect dietary protein against excessive degradation in the rumen and can have beneficial effects for ruminant herbivores (Min et al., 2003). Low to medium concentrations of PA (2–4% of DM) increase protein utilization, which contributes to increases in lactation, wool growth, and live weight gain (Waghorn et al., 1994; Wang et al., 1996). Other beneficial effects for agriculture include the control of bloat (Chiquette et al., 1988) and improved tolerance against internal parasites (Niezen et al., 1998).

Forage cultivars of *Lotus* spp. are of interest in agriculture, as they combine improved protein supply for animal production through symbiotic nitrogen fixation with the protein-protective action of PAs in the rumen. They exhibit the dichotomy described above of both beneficial and detrimental effects on feed value and animal performance (Waghorn et al., 1998; Aerts et al., 1999). The PA from *L. corniculatus* is associated with better animal performance than that from *L. pedunculatus* (Waghorn et al., 1999), and this cannot be accounted for by the lower PA content of *L. corniculatus* (above half that of *L. pedunculatus*; Terrill et al., 1992). The PA in *L. corniculatus* increased the observed absorption of amino acids from the small intestine of sheep (Waghorn et al., 1987), whereas the PA in *L. pedunculatus* (Waghorn et al., 1994) provided ruminal protein protection, but did not improve absorption.

The primary factor in the difference in activity of the two PAs may be differences in their structure (Min et al., 2003). Structural studies of *L. pedunculatus* and *L. corniculatus* PAs (Foo et al., 1996, 1997; Hedqvist et al., 2000; Meagher et al., 2004) have shown that *L. pedunculatus* PAs have a much higher prodelphinidin (PD) content. The structure of PAs as well as their concentration in the plant are important factors determining their activity and role in both agricultural systems (Min et al., 2003) and the natural environment (Zucker, 1983; Clausen et al., 1990; Ayres et al., 1997; Heil et al., 2002).

The genus *Lotus* contains many species, several of which have been used as a forage for ruminants (Papadopoulos and Kelman, 1999), and the initial motivation for this study was to identify additional *Lotus* species of potential value as forages, which might provide PAs at appropriate concentrations and with desirable properties. *Lotus* spp. are found worldwide, except for cold arctic regions and low land tropical areas of Asia, South and Central America (Kirkbride, 1999a). The largest collection of *Lotus* genetic resources is in New Zealand (Greene, 1999). A number of groupings of *Lotus* spp. have been constructed based on plant morphology (Arambarri, 2000), and within *L. corniculatus*, genotypes have been grouped based on specific characteristics or habitats (Steiner and Garcia de los Santos, 2001). However, relationships between phylogeny and geographical distribution and the phytochemistry of *Lotus* PAs have not been investigated.

In this study, we compare the chemistry of PAs of forage cultivars of *L. corniculatus*, *L. pedunculatus*, and *L. tenuis* with those of 12 additional *Lotus* spp. not currently used in agriculture. Estimates of the concentration of PAs in all 15 species are reported. In addition, the chemical characteristics of PA fractions from *L. pedunculatus* and *L. corniculatus* (including new data on a “creeping” selection of *L. corniculatus*) are compared with new findings on the composition of PAs of four additional *Lotus* species found to have a high PA content in the initial screening: *L. americanus*, *L. angustissimus*, *L. parviflorus*, and *L. suaveolens*.

We applied a number of complimentary and independent approaches to characterize the structure of the *Lotus* PA polymer mixtures. Whereas proanthocyanidin oligomers with DP as high as 5 have been isolated and characterized as single entities (Hemingway et al., 1982; Foo and Karchesy, 1991), the majority of the PA polymer in plants comprises mixtures of oligomers (DP 5–10) and higher polymeric material (DP > 10) that must be characterized collectively. PA polymer fractions prepared by Sephadex LH-20 chromatography have been characterized by acid catalyzed degradation in the presence of benzyl mercaptan coupled with high-performance liquid chromatography–photodiode array (HPLC-PDA) detection to provide information on the nature of the terminal and extension units and the mean degree of polymerization (mDP; Guyot et al., 2001; Gu et al., 2002; Taylor et al., 2003; Sivakumaran et al., 2004). Mass spectrometric methods [liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)] have been used to provide further characterization of the PAs in terms of their polydispersity and interflavanoid linkages (A- or B-types), without further isolation or chromatographic separation (Le Roux et al., 1998; Foo et al., 2000a). These estimates of composition are supported by independent nuclear magnetic resonance (NMR) estimates (Porter et al., 1982).

The results are discussed in terms of possible relationships between the concentration and composition of the PAs of *Lotus* spp. and ecological factors.

Methods and Materials

Chemical

Analytical grade acetone and dichloromethane, HPLC grade methanol, acetonitrile, and ascorbic acid were obtained from BDH Ltd., Auckland, New Zealand. Catechin, epicatechin, gallic acid, gallocatechin, and epigallocatechin were obtained from Sigma, St Louis, MO, USA. Benzyl mercaptan was obtained from Merck, Darmstadt, Germany. 2,5-Dihydroxybenzoic acid was obtained from L. Light & Co. Ltd., Colnbrook, UK. Sephadex LH-20 was obtained from Pharmacia, Sweden.

Plant Material

Herbage samples of 15 *Lotus* species were grown in pots at Grasslands Research Centre, Palmerston North, New Zealand; *L. corniculatus* (birdsfoot trefoil, cv. “Grasslands Goldie”), *L. pedunculatus* (big trefoil, cv. “Grasslands Maku”), *L. tenuis* (narrow-leaf trefoil, cv. “Esmeralda”), *L. americanus* (American birdsfoot trefoil), *L. angustissimus* (slender birdsfoot trefoil), *L. arenarius*, *L. crassifolius* (big deervetch), *L. creticus*, *L. decumbens*, *L. edulis*, *L. japonicus* (*L. corniculatus* L. var. *japonicus*), *L. ornithopodioides*, *L. parviflorus* (small-flower birdsfoot trefoil), *L. schoelleri* (*L. corniculatus* L. var. *schoelleri*), and *L. suaveolens* (hairy birdsfoot trefoil) were harvested in July 2000, and regrowth was sampled again in September 2000, freeze-dried, milled, and PA content estimated by the vanillin–HCl assay. The four species with the highest PA content were selected for further investigation, together with the established forage species *L. pedunculatus* and *L. corniculatus*. Herbage samples of 25 individual plants of each of the six *Lotus* species grown in pots were harvested in October 2001, freeze-dried, and milled. The extractable PA content of the individual plants was estimated by the BuOH–HCl assay.

Herbage samples of *L. corniculatus*, *L. pedunculatus*, *L. americanus*, *L. angustissimus*, *L. parviflorus*, and *L. suaveolens* grown in a sand-frame were harvested during December 2001 to January 2002 and were stored at -20°C for extraction. These *Lotus* spp. were grown again in the sand frame and sampled during December 2002 to January 2003 along with two additional *Lotus* spp. in a breeding program: *L. corniculatus* (birdsfoot trefoil, “Grasslands creeping selection”) and *L. tenuis* (narrow-leaf trefoil, cv. “Esmeralda”). Herbage samples harvested in summer 2002–2003 were freeze-dried, milled, and analyzed for PA content by the BuOH–HCl assay.

Colorimetric PA Assay

Plant samples harvested in 2000 were analyzed by the vanillin–HCl assay (Scalbert, 1992). Individual plants harvested in 2001 and bulk plant samples harvested in 2002–2003 were analyzed by the BuOH–HCl assay (Terrill et al., 1992). Freeze-dried plant samples (leaves and stem) were ground in a Wiley mill (1-mm screen). For the 2000 and 2001 samples, “free” PA concentrations were estimated, and for the 2002–2003 samples, a three-step extraction procedure was performed to determine free, protein-bound, and fiber-bound PA. Measurements were performed in duplicate and the mean value reported as a percentage (%) of DM. A purified PA fraction from *L. pedunculatus* was used as a standard for calibration.

Proanthocyanidin Preparative Extraction

Frozen herbage of *Lotus* spp. (600 g/l) were extracted with acetone/water (7:3; v/v) containing ascorbic acid (1 g/l) in a blender (Halldé VCM62 Varning, AB Halldé Maskiner, Kista, Sweden) for 30 min and strained through two layers of cheesecloth to remove plant material. The extract was concentrated *in vacuo* (40°C) to remove acetone and the aqueous solution defatted with dichloromethane (4×11). The aqueous layer was concentrated *in vacuo* and subsequently freeze-dried to yield an aqueous acetone PA extract.

Purification of Proanthocyanidin Fractions

Each freeze-dried aqueous acetone PA extract (6 g) was dissolved in aqueous methanol (1:1, v/v, 50 ml) and centrifuged at $4500 \times g$ for 10 min. The PA solution was applied to a Sephadex LH-20 column (Pharmacia, SR 25/45) preconditioned with aqueous methanol (1:1, v/v) and connected to a Pharmacia GradiFrac system. After loading, the column was washed with aqueous methanol (1:1, v/v; fraction 1 100 ml and fraction 2 500 ml) at a flow rate of 5 ml/min. The PA fractions were eluted with acetone/water (7:3, v/v; fraction 1 100 ml and fraction 2 500 ml) and analyzed by HPLC–PDA at 280 nm. PA fractions were characterized by the observation of a broad unresolved hump at 280 nm in the HPLC trace, combined, and concentrated *in vacuo* (40°C). The aqueous residues were freeze-dried to yield PA fractions.

The initial aqueous methanol (1:1, 100 ml) eluate of *L. americanus* was characterized as containing PAs by the presence of a broad unresolved hump in the HPLC–PDA at 280 nm. The aqueous methanol (1:1) fractions were rechromatographed on a Sephadex LH-20 (Pharmacia, SR 25/45) column. An initial wash with water (100 ml) was followed by elution with aqueous methanol (1:1, 500 ml), which yielded a high molecular weight proanthocyanidin (HMWPA) fraction, and subsequent elution with acetone/water (7:3, v/v, 500 ml) yielded a medium molecular weight proanthocyanidin (MMWPA) fraction.

Thiolysis

A method based on that described by Guyot et al. (1998) was utilized. A PA solution (4 mg/ml in methanol) was prepared for each PA fraction. A subsample (50 μ l) was placed into a vial to which 3.3% (v/v) hydrochloric acid in methanol (50 μ l) and 5% (v/v) benzyl mercaptan in methanol (100 μ l) was added. Each solution was heated to 40°C for 30 min in a heating block and cooled to room temperature. An internal standard, dihydroquercetin in water (100 μ l, 2.5×10^{-2} mg/ml solution), was added and the sample analyzed immediately by reversed phase HPLC. Concentrations of terminal flavan-3-ol units and extender flavan-3-ol thiol adducts were estimated by peak area integration at 280 nm. Responses relative to dihydroquercetin were determined from standards. Response factors to PC and PD flavan-3-ols (0.26 and 0.07, respectively) were the same as the corresponding benzylthioethers, isolated from *Dorycnium rectum* PA fractions according to Sivakumaran et al. (2004) as reported by Gu et al. (2002). Thiolysis 10- μ l subsamples were analyzed by the method of Meagher et al. (2004).

Mass Spectrometry

Electrospray ionization mass spectrometry data were acquired on a Shimadzu LC-MS QP8000 α in scan mode (m/z 250–1400) and detection in the negative ion mode using the conditions described by Meagher et al. (2004).

Matrix-assisted laser desorption/ionization time-of-flight mass spectra were acquired on a Micromass M@LDI LR time of flight mass spectrometer, equipped with delayed extraction and a N₂ laser, set at 337 nm. For positive reflectron mode spectra, an accelerating voltage of 15 kV and a reflectron voltage of 2 kV were used. The PA fractions were reconstituted in acetone/water (8:2, v/v; 0.5 mg/ml) and mixed with a matrix solution of 2,5-dihydroxybenzoic acid in acetone/water (8:2, v/v; 10 mg/ml) at a volumetric ratio of 1:1. The PA-matrix solutions were deionized on a cation exchange cartridge (Strata SCX, 100 mg, 1 ml) preconditioned with HCl (1 ml, 0.1 M), Milli-Q water (5 ml), and finally acetone/water (8:2, v/v; 2 ml). The deionized PA-matrix solutions were spiked with a NaCl solution (0.1 M, 0.5 μ l) to promote the formation of single ion adducts ($[M + Na]^+$), and the mixture (1 μ l) was applied to a stainless-steel target plate and crystallized at room temperature prior to analysis.

¹³C NMR. NMR spectra were recorded in methanol (CD₃OD) at 90 MHz using a Bruker 400 MHz instrument.

Results and Discussion

Estimation of Proanthocyanidin Concentration

Herbage samples of 15 *Lotus* species in an initial study were harvested in winter 2000, and regrowth was again sampled in spring 2000. The extractable PA content was estimated by the vanillin-HCl assay (Table 1) and ranged from 0.2 to 7.4% of DM in winter and from 0.9 to 10.9% of DM in spring. The four species with the highest PA content were selected for further investigation, together with the established forage species *L. corniculatus*, *L. pedunculatus*, and *L. tenuis*.

Extractable PA concentrations were estimated for individual plants in 2001, and extractable, protein-bound, and fiber-bound PA concentrations were estimated in bulk material

harvested during 2002 and 2003 using the BuOH–HCl assay. The estimates of PA concentration by the two methods were in reasonable agreement. Extractable PA concentrations ranged from 0.2 to 10.9% of DM (Table 1). There were variations in PA estimates between sampling dates, but on both occasions in 2000, PA concentrations were consistently lower for *L. creticus*, *L. ornithopodioides*, *L. arenarius*, *L. crassifolius*, *L. japonicus*, *L. decumbens*, *L. edulis*, *L. schoelleri*, and *L. tenuis* than for the other species. The remaining “high PA” *Lotus* spp. were retained for further examination. The PAs of these species were predominantly extractable with bound PAs comprising between 5 and 13% of DM of the total PAs in 2002–2003. At each sampling, the PA concentration was highest for *L. americanus* (9.8% DM total PA in 2002–2003), and at three of four samplings, the PA concentration was lowest for *L. corniculatus* (3.0% DM total PA in 2002–2003). PA concentrations in *L. pedunculatus*, *L. angustissimus*, *L. corniculatus* “creeping” selection, and *L. parviflorus* were intermediate (4.2–7.2% of DM total PA in 2002–2003). The PA content of the “creeping” selection of *L. corniculatus* was higher than in the standard cultivar, consistent with previous screening and selection (Rumball, unpublished observations). Only a trace amount of PA was determined in *L. tenuis* in agreement with reported values (Kelman and Tanner, 1990; Strittmatter et al., 1992; Terrill et al., 1992).

Of the *Lotus* species evaluated, the highest PA concentration (>6% of DM) was found for *L. americanus*, a bushy branched annual about 30 cm high, present in dry prairies and rangeland, used as forage (Table 1). Moderate PA concentrations (<6% of DM) were found in *L. angustissimus*, *L. parviflorus*, and *L. suaveolens*. These three species grow in habitats such as dry grassland that are accessible to grazing (Kirkbride, 1999a,b). The latter two also carry “hairy” foliage, which is generally regarded as a deterrent to predation. However, both low and high PA species are associated with Mediterranean grasslands, and there is no clear association between annual or perennial types and PA accumulation. The four species with the lowest PA concentrations (Table 1) are *L. tenuis*, *L. decumbens*, *L. japonicus*, and *L. edulis*. They are, respectively, (1) vigorous annual, (2) small and weak prostrate annual, (3) prostrate and moderately vigorous perennial, and (4) moderately vigorous short-lived species (ILDIS).

It is difficult to observe any clear ecological pattern to the occurrence of PAs in the *Lotus* spp. evaluated in this study. Although the ecological role of plant PAs has been extensively examined, a robust theoretical basis for their ecological role is not yet available. The interactions and trade-offs between plant growth and defense are complex (Stamp, 2003), and investigations of a single secondary metabolite class such as PAs provide a limited view of defensive chemistry. Thus, (Berger et al. 2003) found a complementary pattern of distribution of PAs and proteinase inhibitors in *Vicia* species. The *Lotus* spp. we have found to have low PA content may have alternative defensive chemistry or, in the case of the more vigorous species, have greater tolerance of herbivory. Considerations of plant defense would suggest that high-PA-containing plants might be found in environments with low resource availability (Coley et al., 1985). Considerations of nutrient cycling suggest that acidic and infertile soils might be favored (Kraus et al., 2003), and considerations of photoprotection suggest that they might be found at low latitudes and high altitudes (Close and McArthur, 2002). To directly address these environmental factors would require an extensive investigation of *Lotus* spp. in their natural environment that was beyond the scope of the present study.

Fractionation of Proanthocyanidins

Frozen herbage of each *Lotus* spp. was extracted with aqueous acetone. A single extraction provided sufficient material for fractionation. The composition of PA from a single

Table 1 Estimated proanthocyanidin concentration (% of Dm) in the herbage of the *Lotus* spp

<i>Lotus</i> spp.	2000		2001		2002–2003			Distribution	Season ^e	Habitats ^f
	E ^a	E ^b	E ^c (SD)	E ^d	E ^d	P ^d	F ^d			
<i>L. americanus</i>	7.4	10.9	7.1 (0.7)	9.4	0.4	0.1	9.8	Canada ^g , USA, Mexico	Annual	Forage
<i>L. pedunculatus</i>	4.9	5.5	2.9 (0.4)	6.4	0.7	0.1	7.2	Europe ^h , Turkey, Africa, USSR, Argentina, Australia	Perennial	Forage; Mediterranean grasslands
<i>L. parviflorus</i>	3.1	5.6	2.9 (0.4)	5.3	0.5	0.1	5.9	Africa ⁱ , Australia, Europe, Middle East, Azores	Annual	
<i>L. angustissimus</i>	3.7	6.4	2.9 (0.9)	4.7	0.5	0.2	5.3	China ⁱ , Europe, Middle East, Siberia, Azores	Perennial	Mediterranean grasslands
<i>L. corniculatus</i> sel.	4.7	5.1	4.0 (0.7)	4.0	0.5	0.1	4.6	Western Mediterranean basin ^h , UK, Africa	Annual	Mediterranean grasslands
<i>L. corniculatus</i>	2.2	5.5	1.3 (0.6)	2.8	0.1	0.1	3.0	Widespread in Europe ^e , China	Perennial shrub	Forage; Mediterranean grasslands; Afriomontane grassland
<i>L. creticus</i>	1.9	ND						Africa ⁱ , Australia, Europe, Middle East, Canada, Azores	Perennial	Mediterranean grasslands; Mediterranean/Sahara regional transition zone; grassland
<i>L. ornithopodioides</i>	1.3	1.7						Africa ⁱ , Europe, USSR, USA, Asia	Annual	Mediterranean woodland; Mediterranean grasslands
<i>L. arenarius</i>	0.7	1.7						Senegal ⁱ , Egypt, Morocco, Spain	Annual	Sahara regional transition zone; desert

<i>L. crassifolius</i>	0.6	ND	Western US ^l , United Arab Emirates ^l	Perennial	
<i>L. japonicus</i>	0.4	1.3	Japan ^b , Korea, China, Tibet	Perennial	Mediterranean grasslands;
<i>L. decumbens</i>	0.3	1.4	Africa, Australia, Europe	Perennial	Mediterranean/Sahara regional transition zone; grassland
<i>L. edulis</i>	0.3	0.7	Africa ^l , Europe, Middle East	Annual	Mediterranean woodland; Mediterranean grasslands
<i>L. schoelleri</i>	0.3	2.9	Ethopia ^{ik} , Kenya, Sudan	Perennial (diploid)	Afromontane grassland
<i>L. tenuis</i>	0.2	1.4	Africa ^h , Argentina, China, Middle East, New Zealand	Perennial	Forage

E = extractable PA; P = protein-bound PA; F = fiber-bound PA; T = total PA; SD = standard deviation; ND = not determined, insufficient herbage for sampling.

^a Plants, herbage harvested winter 2000.

^b Plants, herbage regrowth harvested spring 2000.

^c Individual plants (25), herbage harvested spring 2001.

^d Bulk plants, herbage harvested summer 2002–2003.

^e Nonclimbing herb.

^f International Legume Database and Information Service (ILDIS) at <http://www.ildis.org/>.

^g Steiner, 1999.

^h Kirkbride, 1999a.

ⁱ Kirkbride, 1999b.

^j USDA Natural Resource Conservation Service Plants profile.

^k Agriculture and Agri-Food Canada Taxonomy at <http://pgrc3.agr.ca>.

extraction had previously been found in the cases of *L. pedunculatus* and *L. corniculatus* (Meagher et al., 2004) and was similar to that reported following exhaustive extraction of plant material (Foo et al., 1997). The extracts were defatted and fractionated on a Sephadex LH-20 column. Low molecular weight proanthocyanidin (LMWPA) polymer fractions were recovered by elution with aqueous acetone and characterized by the presence of a broad unresolved hump at 280 nm by HPLC-PDA. The first aqueous acetone fraction containing flavanol monomers and dimer PAs was kept separate from the second polymeric PA fraction. The chemical nature of these polymer fractions was determined by ^{13}C NMR, the thiolysis degradation reaction, ESI-MS, and MALDI-TOF-MS.

In the case of *L. americanus*, a portion of the PA was not retained on the Sephadex LH-20 column and was separated into MMWPA and HMWPA fractions that were characterized separately (below). Similar observations have been reported for *L. pedunculatus* (Meagher et al., 2004) and the PA-containing plant *D. rectum* (Sivakumaran et al., 2004). The PA extracted from *L. tenuis* was not characterized as it was obtained in trace amounts.

Characterization of Proanthocyanidin Chemical Composition

The chemical composition of fractions was characterized by thiolysis and NMR. Fractions were acid hydrolyzed in the presence of benzyl mercaptan, which yielded the extension units as flavan-3-ol-4-benzylthioether adducts and terminal units as flavan-3-ols (Fig. 1). The compositional data and mDP were determined for each *Lotus* spp. PA fraction (Table 2). Catechin was the dominant terminal unit in the PA fractions from most *Lotus* species, with the exceptions of *L. parviflorus* where epicatechin was dominant and *L. suaveolens* where equal proportions of catechin and epicatechin were found. In contrast, there was more variation in the extension units: epigallocatechin was the dominant extension unit of *L. americanus*, *L. corniculatus* “creeping”, and *L. pedunculatus*, whereas epicatechin was the dominant extension unit of *L. corniculatus* and *L. angustissimus* (53 and 66% mol/mol, respectively). Epicatechin and epigallocatechin were found in equal proportions for *L. parviflorus* and *L. suaveolens* extension units. In contrast, *L. angustissimus* is the one species dominated by epicatechin in both the extender and terminal units.

Thiolysis gave a direct insight into the relative stereochemistry at C2 and C3 in the PA units. The *cis/trans* ratio of PA fractions was determined for the terminal and extension units for each species (Table 2). The terminal units were predominantly of *trans* configuration, whereas the extensions units were predominantly *cis*.

For each of the *Lotus* spp., a low molecular weight PA fraction (LMWPA) was isolated. The mDP determined by thiolysis for these fractions (Table 2) varied from 8.1 to 16. Variation in mDP of these fractions may be affected by the presence of flavan-3-ol monomers that were detected by ESI-MS (not shown in Table 3) in the LMWPA fractions of *L. angustissimus*, *L. corniculatus*, *L. parviflorus*, and *L. suaveolens*. For *L. americanus*, the mDP of the HMWPA and MMWPA fractions was estimated to be 97 and 40, respectively. These fractions were of higher mDP than the comparable fractions previously reported for *L. pedunculatus* (Meagher et al., 2004). The composition of the terminal units of the polymer fractions (LMWPA, MMWPA, and HMWPA) of *L. pedunculatus* (Meagher et al., 2004) and *L. americanus* was not significantly different between fractions of differing mDP. The epigallocatechin content of the extension units increased with the increasing mDP (Table 2) for *L. pedunculatus*, but such a trend was not as apparent for *L. americanus*.

The ^{13}C NMR spectrum of PA polymers from the *Lotus* spp. gave broad peaks, indicating the polymeric nature of the PA. Estimates of the *cis/trans* ratio and PC/PD ratio

Table 2 Comparison of chemical composition of proanthocyanidin fractions in the *Lotus* spp. as determined by thiolytic degradation

<i>Lotus</i> spp.	Terminal (%)				Extender (%)				<i>cis/trans</i>	PD/PC	mDP
	GC	EGC	C	EC	GC	EGC	C	EC			
<i>L. pedunculatus</i>											
LMWPA	26	14	46	14	16	68	4	14	76:24	80:20	12
MMWPA ^a	23	12	51	14	6	46	2	15	88:12	80:20	18
HMWPA ^a	25	11	51	13	13	72	3	13	83:17	84:16	44
<i>L. corniculatus</i>											
LMWPA	2	2	75	21	5	39	3	53	84:16	40:60	8.7
<i>L. corniculatus</i> “creeping”											
LMWPA	17	4	61	18	7	62	4	27	85:15	66:34	14
<i>L. suaveolens</i>											
LMWPA	4	4	46	47	12	41	4	43	80:20	48:52	8:1
<i>L. parviflorus</i>											
LMWPA	1	4	32	63	10	40	4	45	85:15	46:54	8.1
<i>L. americanus</i>											
LMWPA	18	2	78	3	9	59	5	27	80:20	65:35	16
MMWPA	20	0	75	2	6	66	3	25	90:10	70:30	40
HMWPA	22	0	75	3	5	75	2	19	92:08	80:20	97
<i>L. angustissimus</i>											
LMWPA	1	1	4	94	16	17	1	66	85:15	30:70	8.7

Percentage of extender and terminal units, PC and PD units given as mol/100 mol.

C = catechin, EC = epicatechin, EGC = epigallocatechin, GC = galocatechin, mDP = mean degree of polymerization, PC = procyanidin, PD = prodelfphinidin.

^a From Meagher et al. (2004).

from the NMR data based on the calculation described by Porter et al. (1982) were generally in good agreement with the estimates from thiolysis (data not shown).

The molecular weight estimation from the carbon signal intensity gave approximate values of 7–10 mDP for the *Lotus* spp. LMWPA fractions and 15–18 mDP for MMWPA fraction of *L. americanus*, somewhat lower than the estimates from thiolysis. Because of the limited dynamic range of the NMR method, calculation of a credible mDP value from the NMR data was not feasible for the HMWPA fraction, but the NMR observations were consistent with a large mean molecular weight as found by thiolysis. Similar higher molecular weight PAs with mDP ranging from 33.8 to 189 have been reported for apple (Guyot et al., 2001), grape skin (Monagas et al., 2003), and *D. rectum* (Sivakumaran et al., 2004).

Determination of Polydispersity of Proanthocyanidins.

The polydispersity of the PA fractions was analyzed by ESI-MS in negative ion mode and MALDI-TOF-MS in positive mode. Although the MS ion intensities are not a quantitative measure of oligomer species, the patterns reflect polymer heterogeneity and polydispersity. The observed singly charged $[M - H]^-$ and doubly charged $[M - 2H]^{2-}$ ions, corresponding to the molecular ion masses of PA oligomers ranging from dimer (DP2) through to heptamer (DP7) observed in negative ESI-MS, are shown in Table 3. Multiple-charged species are observed for DP > 4, as with longer chain lengths, the charge can be better

Table 3 Observed m/z values of proanthocyanidin ions in negative ESI-MS analysis of *lotus* spp. polymer fractions

DP	Ions	<i>L. pedunculatus</i>	<i>L. corniculatus</i>	<i>L. corniculatus</i> "creeping"	<i>L. suaveolens</i>	<i>L. parviflorus</i>	<i>L. americanus</i> ^a	<i>L. angustissimus</i>
2	$[M - H]^-$	593 , 609	577 , 593 , 609	577 , 593	577 , 593	577 , 593	577, 591, 593 , 607	577
3	$[M - H]^-$	881, 897 , 913	865 , 881, 897	865, 881, 897	865 , 881, 897	865 , 881, 897	865, 881, 895 , 897	865 , 881, 897
4	$[M - H]^{2-}$	584, 592, 600 , 608	576 , 584, 592	584, 592	584, 592	576, 584, 592	592, 608	576 , 584, 592
5	$[M - H]^{2-}$	720, 728, 736, 744 , 752, 760	720 , 728, 736, 744	720, 728, 744 , 752, 760	720, 728 , 736, 744	720, 728 , 736, 744	720, 727, 728, 735, 736, 744 , 752, 760	720 , 728, 736
6	$[M - H]^{2-}$	880, 896, 904 , 912	872 , 880, 888	864, 872, 888 , 896, 904, 912	864, 880 , 888, 896	864, 872, 880 , 888, 896	864, 880, 888, 896, 903, 904 , 911	864 , 872
7	$[M - H]^{2-}$	1032, 1040, 1048 , 1056	1008, 1016 , 1024, 1032, 1040, 1049	1008, 1016, 1024, 1032, 1040 , 1048, 1056	1008, 1016, 1024 , 1033	1008, 1016, 1024 , 1032, 1040, 1048	1016, 1032, 1048, 1047 , 1048, 1056, 1063, 1064	1008

Numbers in bold represent the most intense ion observed.

^a Trimer $[M - H]^-$ at m/z 895, tetramer $[M - 2H]^{2-}$ or dimer $[M - H]^-$ at m/z 607, pentamer $[M - 2H]^{2-}$, and hexamer $[M - 2H]^{2-}$ at m/z 903 were observed, supporting the presence of A-type linkages in the ESI mass spectrum (see Table 4 for explanation of the calculated molecular ion masses).

distributed, minimizing repulsive forces in the polymer chain (Foo et al., 2000b). Positive-ion MALDI-TOF-MS of the PA fractions from the seven *Lotus* spp. were acquired, and trimer (DP3) to heptamer (DP7) homo- and heteropolymers of LMWPA fractions were detected as singly charged $(M + Na)^+$ adducts (Fig. 2). Monomers (DP1) and dimers (DP2) were not detected by MALDI-TOF because of noise and matrix interference. By comparison with the estimated mDP for these LMWPA fractions (Table 2), the ion intensities are biased toward the lower oligomers, as the MALDI-TOF in reflectron mode gives lower sensitivity for the larger ions because of greater breakdown as the result of longer flight paths and the postacceleration process (Yang and Chien, 2000). The calculated molecular ion masses for ESI and MALDI-TOF ranging from trimer (DP3) to hexamer (DP6) are shown in Table 4. The MALDI-TOF-MS were enhanced by desalting by cation exchange and subsequent addition of NaCl to increase the $[M + Na]^+$ ion signal and reduce the $[M + K]^+$ ions naturally present (Ohnoshi-Kameyama et al., 1997; Krueger et al., 2003). With $[M + Na]^+$ adduct ions dominant in the spectrum, the number of hydroxyl functions present in a PA polymer unit can be identified (Table 4).

The most intense ions observed by MALDI-TOF-MS for the trimer (DP3) to heptamer (DP7) series of *Lotus* spp. LMWPA fractions (Fig. 2) are generally in agreement with the most intense ions observed by ESI-MS as singly charged trimer (DP3) species or doubly charged tetramer (DP4) to pentamer (DP5) species (Table 3). Heteropolymer ions (containing both PC and PD units) dominate, except in the case of *L. angustissimus* (PC-only units dominant). Similar heteropolymer molecular forms and ionization patterns in a variety of plants have been reported in the literature (Behrens et al., 2003; Krueger et al., 2003; Taylor et al., 2003). The data for *L. corniculatus* is consistent with those from a study of several *L. corniculatus* cultivars reported by Hedqvist et al. (2000). Ions from the polymeric HMWPA and MMWPA fractions with mDP > 17 of *L. americanus* and *L. pedunculatus* were not detected by ESI-MS or MALDI-TOF-MS.

Proanthocyanidin oligomer ions observed in the ESI mass spectrum of the *L. americanus* LMWPA fraction suggest the presence of A-type interflavan linkages (Tables 3 and 4) in that doubly charged ions were observed that were one m/z unit less than that reported by Foo et al. (1996, 1997) for B-type linkages for *Lotus* PAs. Correspondingly, in the MALDI-TOF-MS from *L. americanus* PA, a series of (singly charged) ions was observed that was two m/z units lower than the species with B-type interflavan linkages (Fig. 2f, insert), indicative of A-type linkages (Table 4). Partial thiolysis results (not shown) suggested the presence of A-type linkages in the terminal units. The A-type interflavan linkage is known in PAs from cranberry, which are terminated by A-type linkages (46%; Foo et al., 2000a; Gu et al., 2002), and cinnamon (Anderson et al., 2004), but has not been reported for forage legume PAs.

Proanthocyanidin Structure, Geography, and Ecology

These results demonstrate that PAs from *Lotus* spp. differ widely in both concentration and structure. The extractable PA fractions are structurally heterogeneous differing in terms of constituent flavan-3-ol units, mDP, and dispersion of oligomers. Whereas the relationship between PA concentrations and ecology and geography is weak (above), the PC/PD ratio in the PA fractions (Table 2) shows a relationship with the geographical distribution of species (Table 1). *Lotus* spp. dominated by PC-type units *L. angustissimus* (70%), *L. corniculatus* (60%), and *L. parviflorus* (54%) have broad distribution and are adapted to a variety of habitats and have moderate PA concentrations (<6% of DM). *Lotus* varieties adapted to warm environments tend to be more widely distributed than those adapted to colder envi-

Table 4 Calculated masses of poly flavan-3-ol ions

Oligomers	PC	PD	No. of linkages		ESI-MS		MALDI-TOF-MS [M + Na] ⁺
			A-type	B-type	[M - H] ¹⁻	[M - H] ²⁻	
Trimer	3	0	0	2	865		889
	3	0	1	1	863		887
	2	1	0	2	881		905
	2	1	1	1	879		903
	1	2	0	2	897		921
	1	2	1	1	895		919
	0	3	0	2	913		937
	0	3	1	1	911		935
Tetramer	4	0	0	3		576	1177
	4	0	1	2		575	1175
	3	1	0	3		584	1193
	3	1	1	2		583	1191
	2	2	0	3		592	1209
	2	2	1	2		591	1207
	1	3	0	3		600	1225
	1	3	1	2		599	1223
	0	4	0	3		608	1241
	0	4	1	2		607	1239
Pentamer	5	0	0	4		720	1466
	5	0	1	3		719	1464
	4	1	0	4		728	1482
	4	1	1	3		727	1480
	3	2	0	4		736	1498
	3	2	1	3		735	1496
	2	3	0	4		744	1514
	2	3	1	3		743	1512
	1	4	0	4		752	1530
	1	4	1	3		751	1528
	0	5	0	4		760	1546
	0	5	1	3		759	1544
Hexamer	6	0	0	5		864	1754
	6	0	1	4		863	1752
	5	1	0	5		872	1770
	5	1	1	4		871	1768
	4	2	0	5		880	1786
	4	2	1	4		879	1784
	3	3	0	5		888	1802
	3	3	1	4		887	1800
	2	4	0	5		896	1818
	2	4	1	4		895	1816
	1	5	0	5		904	1834
	1	5	1	4		903	1832
	0	6	0	5		912	1850
	0	6	1	4		911	1848

Mass calculations were based on the equation $2 + 288a + 304b + 23$, where 2 is the molecular weight of two additional hydrogen atoms of terminal flavan 3-ol units, a is the number of PC units, b is the number of the PD units, and 23 is the atomic weight of sodium. Formation of the one A-type linkage results in the loss of two hydrogen atoms.

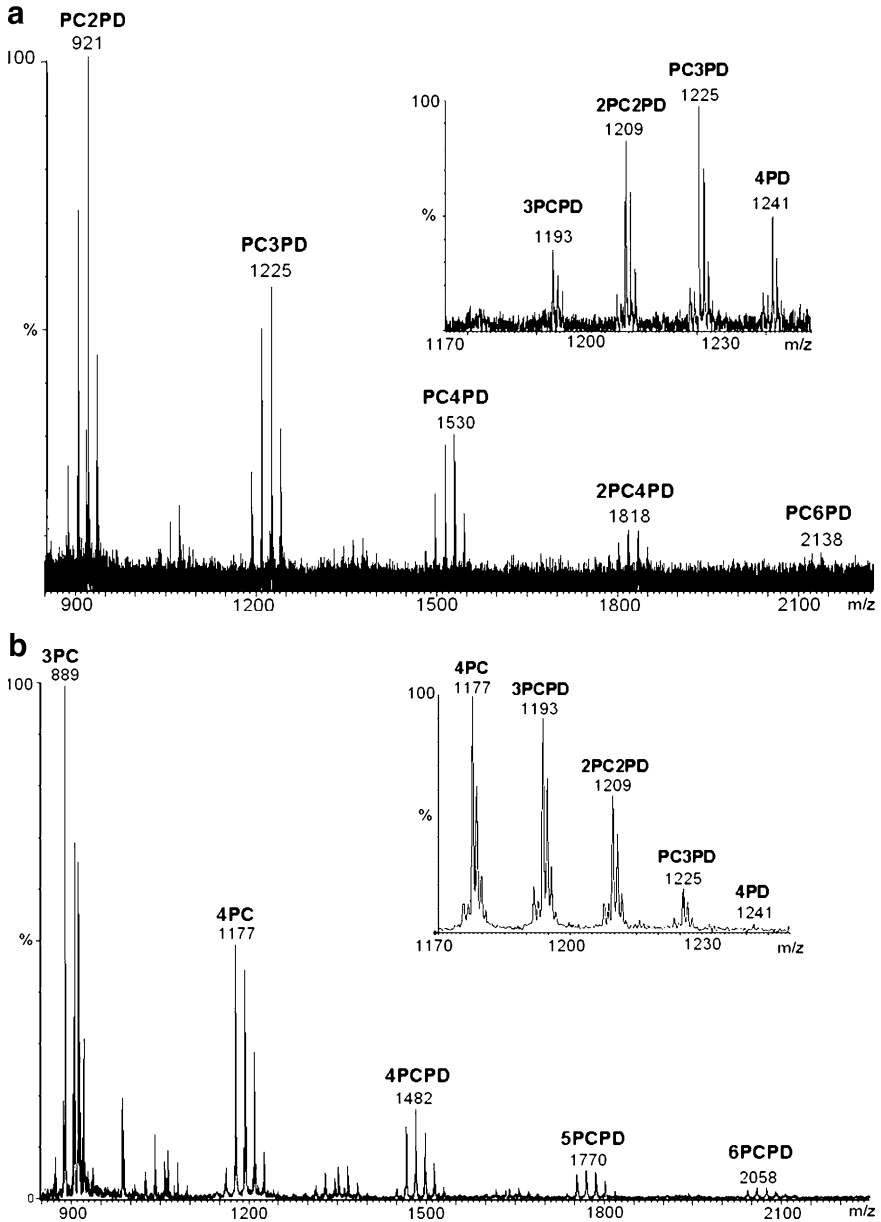


Fig. 2 MALDI-TOF-MS (positive reflectron mode) of a series of polymeric proanthocyanidin fractions from (a) *L. pedunculatus*, (b) *L. corniculatus*, (c) *L. corniculatus* “creeping”, (d) *L. suaveolens*, (e) *L. parviflorus*, (f) *L. americanus*, and (g) *L. angustissimus*. The most intense ions are shown. The inset is an enlarged spectrum of the tetramer series showing different chemical constitutions. See Table 4 for explanation of the calculated molecular ion masses

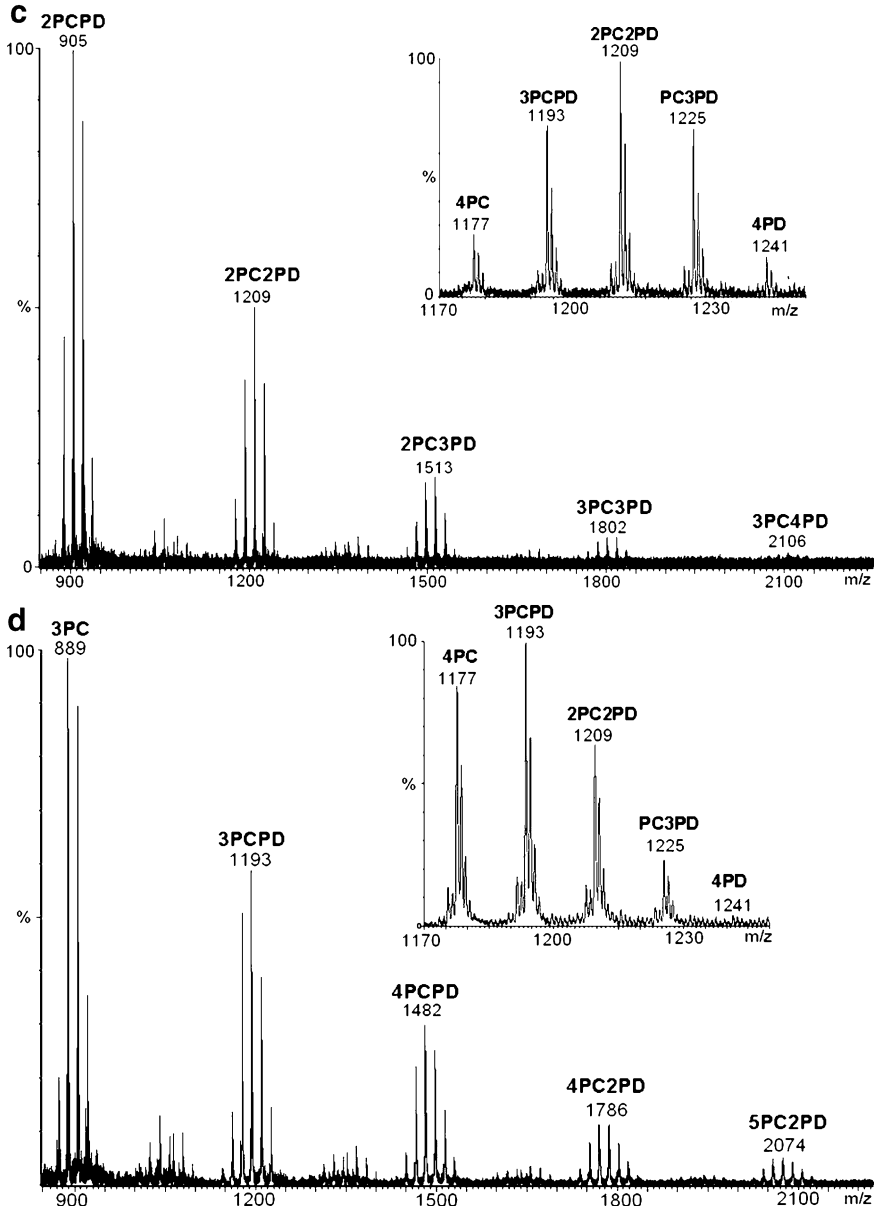


Fig. 2 (continued)

ronments (Steiner, 1999). *L. pedunculatus* and *L. americanus* PAs differ from other *Lotu* spp. examined here not only in having the highest PA concentrations, but also in polymer dispersion, containing higher mDP (>30) HMWPA polymer fractions, and in polymer composition, being dominated by PD-type extender units. An intermediate composition was observed for the “creeping” selection of *L. corniculatus* with a PD content of extender groups in the LMWPA fraction much higher than that for the standard *L. corniculatus*

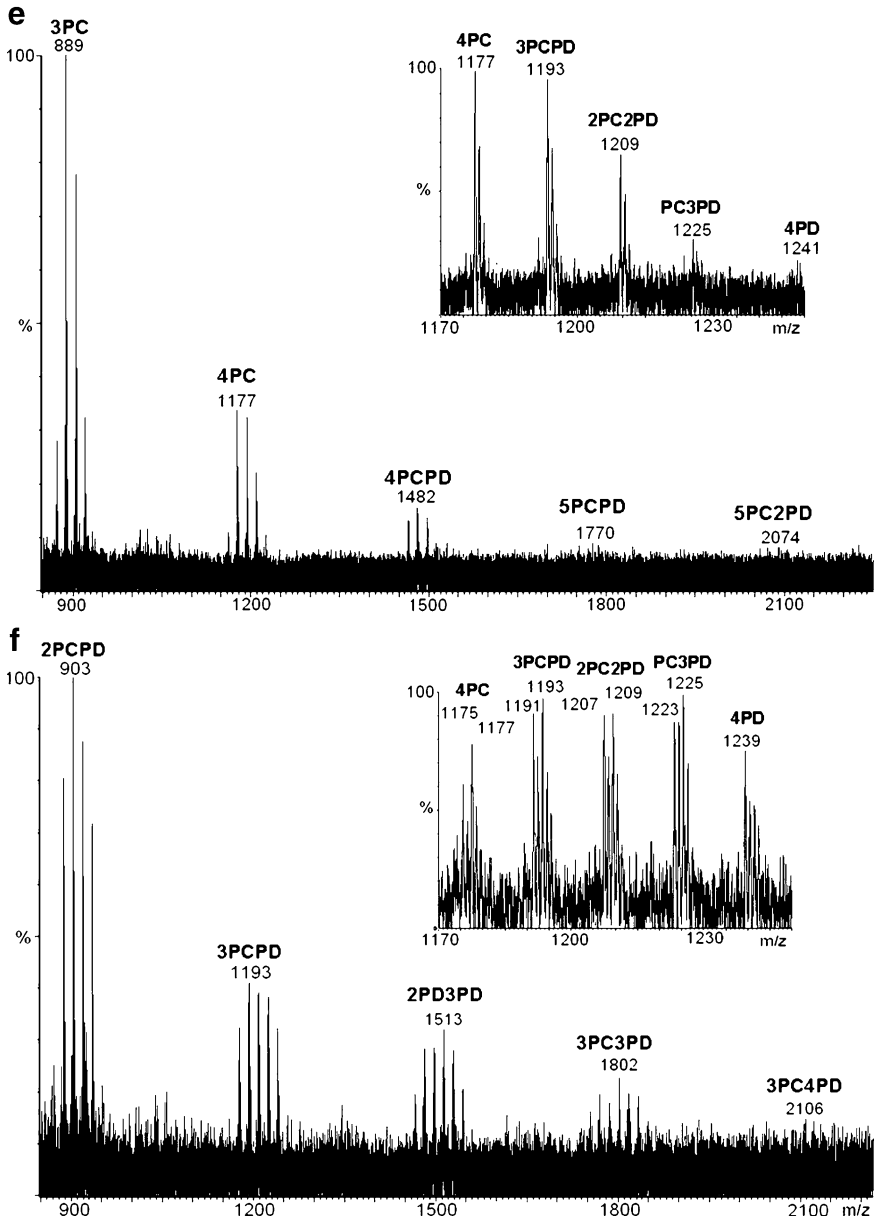


Fig. 2 (continued)

cultivar and comparable to that for *L. americanus*. However, no HMWPA fraction was separated in this case. The “creeping” selection is derived from material collected in Morocco, subsequently crossed and introgressed with standard agricultural cultivars of *L. corniculatus* (Rumball, unpublished observations). The observed structural differences in PAs suggest that there may be considerable variation between *L. corniculatus* populations in the wild. The sole “New World” species studied, *L. americanus*, contains PAs with doubly linked (A-type) units, and this is unique among reported PAs of not only these seven

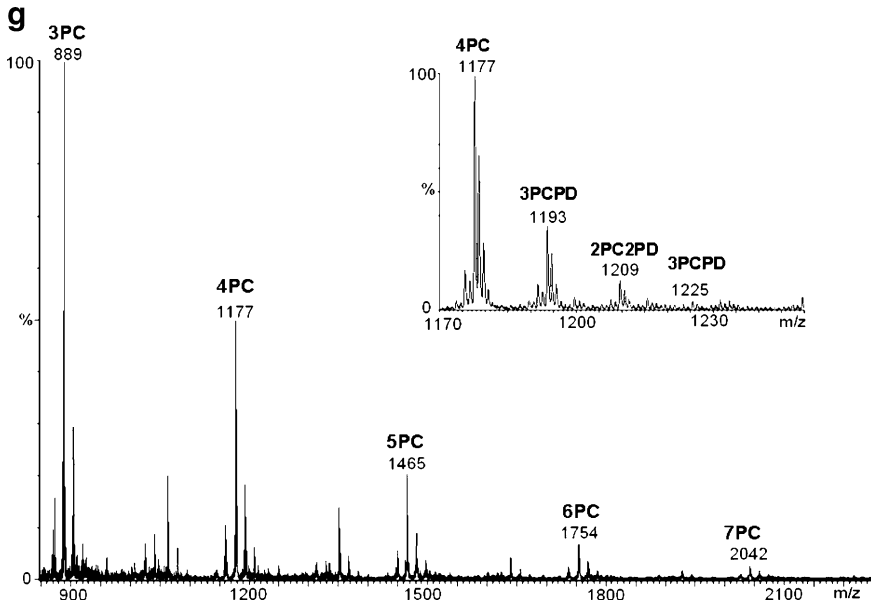


Fig. 2 (continued)

Lotus spp. but also legumes in general (Koupai-Abyazani et al., 1993; Foo et al., 1982, 1996, 1997, 2000b; Sivakumaran et al., 2004).

Functional relationships between the differences observed and between the structures and estimated concentrations of PAs in these *Lotus* spp. and the ecological niche the plants occupy remain to be elucidated. This would require study of the variations in chemical composition of *Lotus* PAs in terms of growth period, season, and other environmental factors, as well as a detailed examination of their interactions with the environment. However, these findings draw attention to the need to consider the complexities of PA structure as well as the concentration of PAs in considering the ecological role of *Lotus* PAs and their effects on herbivores both in nature and in agricultural systems.

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Introduction

Soil solution concentrations of phenolic acids are a product of the inputs of phenolic acids (e.g., leaching of plant materials, microbial activity, root secretions and exudations, and root cell autolysis), losses of phenolic acids (e.g., sorption by soil particles, microbial utilization, uptake by roots), and soil water content. Unfortunately, we know little about “real-time” inputs and losses of phenolic acids into and from soil solutions under field conditions. For a discussion of the potential processes by which phenolic acids may be added and lost from soil solutions, see reviews by Siqueira et al. (1991) and Blum et al. (1999). Soil water content, which is highly variable, is determined by precipitation and/or irrigation, soil water holding capacity, and evapotranspiration. As soil water content decreases, concentrations of phenolic acids in the soil solution should increase, and soil water potentials (i.e., solute and matric) should become more negative. The opposite should happen when soil water content increases. Changes in soil solution concentrations of phenolic acids, however, may not be linearly related to changes in soil water content because changes in soil water content may also modify microbial utilization, soil sorption, leaching, and root uptake of phenolic acids.

Although it has been demonstrated that the inhibitory effects of phenolic acids on transpiration and leaf area expansion are concentration-dependent and that mild moisture stress and pretreatment with phenolic acid or water stress can enhance and reduce, respectively, the inhibitory activity of phenolic acids (Einhellig, 1995; Lehman and Blum, 1999; Blum and Gerig, 2005), no attempt has been made to determine how soil water content may be influenced by phenolic acid inhibition of transpiration or how soil water content may influence the level and duration of the inhibition of phenolic acids. Based on previous studies (Blum and Gerig, 2005; unpublished data), we have chosen to use *p*-coumaric acid, a *p*-hydroxycinnamic acid, and a (cucumber seedling)–[Cecil A_p soil–sand mixture (or soil)] system to address the following questions:

1. How do *p*-coumaric acid treatments affect evaporation, evapotranspiration, soil water content, leaf area, and leaf area expansion of cucumber seedlings?
2. How does soil water content influence the effects of *p*-coumaric acid?
3. What are the response and recovery times of evapotranspiration, lowest daily soil water, leaf area, and absolute and relative rates of leaf expansion for cucumber seedlings growing in soil treated one to four times with a range of *p*-coumaric acid concentrations?

Methods and Materials

Experimental Designs

Experiment 1a (effects of *p*-coumaric acid on soil evaporation): no cucumber seedlings, soil only—three *p*-coumaric acid concentrations (0, 0.5, and 1 $\mu\text{mol/g}$ soil), four replicates, four treatment times (d 6, 8, 10, and 12), and termination of experiment on d 14. Experiment 1b (effects of *p*-coumaric acid on cucumber seedlings and soil processes): plus cucumber seedlings—six *p*-coumaric acid concentrations (0, 0.125, 0.25, 0.5, 0.75, and 1 $\mu\text{mol/g}$ soil), four replicates, four treatment times (d 6, 8, 10, and 12), and termination of experiment on d 14. Experiment 2 (influence of soil water on the effects of *p*-coumaric acid): *p*-coumaric acid treatment (0.5 $\mu\text{mol/g}$ soil on d 6 and 8 and 1 $\mu\text{mol/g}$ soil on d 10 and 12),

two water levels (20 and 25 g water/150 g soil), four or eight replicates, and termination of experiment on d 13. Exp. 3 (response and recovery times after *p*-coumaric acid treatment): four *p*-coumaric acid concentrations (0, 0.25, 0.5, 0.75 $\mu\text{mol/g}$ soil), four different *p*-coumaric acid treatment combinations (d 6; d 6 and 8; d 6, 8, and 10; d 6, 8, 10, and 12), four replicates, and termination of experiment on d 16.

Daily water levels in seedling–soil systems were brought to 20 g water/150 g soil for Exp. 1 (determined values 19.31 ± 0.15 g water/150 g soil for Exp. 1a and 18.84 ± 0.21 g water/150 g soil for Exp. 1b), 20 (determined value 18.96 ± 0.08 g water/150 g soil) or 25 g water/150 g soil (determined value 23.65 ± 0.04 g water/150 g soil) for Exp. 2, and 25 g water/150 g soil (determined value 23.77 ± 0.03 g water/150 g soil) for Exp. 3. Field capacity for the Cecil A_p soil–sand mixture was approximately 0.095 g water/g soil (Lehman and Blum, 1997), which is below the 0.13 g water/g soil (20 g water) and 0.16 g water/g soil (25 g water) treatments used.

General Procedures

Cucumber seeds (*Cucumis sativus* cv. Early Green Clusters; Wyatt Quarles Seed Company, Raleigh, NC, USA) were germinated in trays of vermiculite in the dark at 30°C for 2 d. The resulting seedlings were placed under cool white fluorescent light banks (12-hr photoperiod, 140 $\mu\text{E}/\text{m}^2/\text{sec}$ PAR) located on laboratory benches for 2 d before being transplanted into cups containing 150 g of a Cecil A_p -horizon soil–sand mixture (1 part soil to 2 parts distilled water rinsed sand; w/w). Cecil A_p -horizon soil materials [Typic Kanhapludults, clayey, kaolinitic, thermic; pH = 5.7 ± 0.03 (mean \pm standard error); total carbon $2.2 \pm 0.20\%$] were collected in the summer of 1999 from experimental field plots at the Lake Wheeler Field Research Site, approximately 5 km south of North Carolina State University, Raleigh, NC, air-dried, sieved (2.5-mm mesh), and stored at room temperature ranging from 21 to 30°C in the laboratory. For additional soil characteristics, see Dalton et al. (1987). Cecil A_p soil was chosen because it is a dominate top soil in the southeastern United States and because it has been utilized by a number of researchers to study allelopathic–phenolic acid interactions (see Blum et al., 1999). Cups containing the soil–sand mixture and/or cucumber seedlings were placed in a randomized-blocked design under light banks (see above) in wooden racks so that substrate in the cups, except for the soil surface, was protected from light. Soil and seedling–soil systems under the light banks were kept at room temperature ranging from 21 to 30°C. The systems were supplied with nutrient solution (7 ml double strength Hoagland’s solution, pH 5.0; Hoagland and Arnon, 1950) every other day starting with d 5 and alternated with *p*-coumaric acid solution (pH 5.0) treatments starting with d 6. Water was added each day so that final volume was constant across treatments. Soil water levels and the number and concentrations of *p*-coumaric acid treatments varied with experiment (see “Experimental Designs”). *p*-Coumaric acid was chosen as the model compound to represent simple phenolic acids, such as ferulic, vanillic, and *p*-hydroxybenzoic acids, because their action on cucumber seedlings was so similar (Blum and Gerig, 2005). Because *p*-coumaric acid was rapidly depleted from soil solutions by sorption, microbial utilization, and root uptake (Blum et al., 1999), *p*-coumaric acid was added every other day to maintain a consistent range of soil solution concentrations over the experimental intervals of each experiment. A broad range of *p*-coumaric acid concentrations (0–1 $\mu\text{mol/g}$ soil) was added to systems in this modeling effort to maximize the likelihood of observing clear-cut responses by the soil and seedling–soil systems to *p*-coumaric acid.

Leaf Area, Absolute and Relative Rates of Leaf Expansion, and Seedling Weights

Cucumber seedling leaf areas were determined from leaf length and width measurements and the following equation: leaf area in $\text{cm}^2 = -1.457 + 0.008(L \times W)$, $P < 0.001$, $r^2 = 0.98$, where L and W are in mm (Blum and Dalton, 1985). Surface areas for cotyledons were determined by measuring cotyledon length and width and the following equation: area of cotyledon in $\text{cm}^2 = 1.293 + 0.006(L \times W)$, $P < 0.001$, $r^2 = 0.76$, where L and W are in mm. The mean absolute rates of leaf expansion ($\text{cm}^2/\text{seedling}/\text{unit time}$) for the cotyledons and/or true leaves were determined as follows: leaf area at time_{x+1} – leaf area at time_x . The mean relative rates of leaf expansion ($\text{cm}^2/\text{cm}^2/\text{unit time}$) were determined as follows: $\ln(\text{leaf area at } \text{time}_{x+1}) - \ln(\text{leaf area at } \text{time}_x)$. For a discussion on calculating growth rates, see Radford (1967). Fresh and dry (100°C for 24 hr) weights of shoots and dry weights of roots were determined at termination of each experiment. However, because shoot weights were only impacted by *p*-coumaric acid treatments, root dry weights were not modified by any treatment, and weights were determined at only one point in time, the end of each experiment, the data are not presented.

Evaporation, Evapotranspiration, and Soil Water

Evaporation and evapotranspiration were based on soil water depletion over various time intervals for soil and seedling–soil systems. Soil water content of systems was determined gravimetrically (i.e., differences in weight of systems between two time intervals).

Depletion of Phenolic Acid from Soil Solutions

Twenty to 30 g of soil subsamples from the seedling–soil systems were added to 30 ml autoclaved-distilled water in Erlenmeyer flasks. The flask containing the water and soil sample was shaken for 10 min on a platform shaker. Subsamples of the resulting soil slurry were placed into centrifuge tubes and centrifuged at $10,000 \times g$ for 10 min. Resulting supernatants were filter sterilized with 0.2- μm filters (Supor-200, Gelman Sciences, Ann Arbor, MI, USA) before high-performance liquid chromatographic (HPLC) analysis (Blum et al., 1994). *p*-Coumaric acid was quantified with a Waters (Milford, MA, USA) fully automated HPLC equipped with a model 484 absorbance detector set at 254 nm. A 4- μm particle size Nova-pak C_{18} Radial Pak cartridge in an RCM-100 cartridge holder was eluted for 20 min with 22% methanol, 0.5% ethyl acetate, 0.9% acetic acid, and 76.6% water to isolate and quantify the *p*-coumaric acid in the subsamples. Identification and quantification were confirmed by comparing retention times and areas with those of standard amounts of *p*-coumaric acid obtained from Sigma (St. Louis, MO, USA). *p*-Coumaric acid recovered (i.e., total available based on HPLC analysis) was then used to calculate available $\mu\text{mol } p\text{-coumaric acid/g oven dried soil}$ and $\text{mM of } p\text{-coumaric acid in the soil solution of the seedling–soil systems at various time intervals}$. Soil dry weight (100°C at 24 hr) and water content of the soil (i.e., soil solution; weight of moist soil – weight of oven dry soil) were determined gravimetrically. Available *p*-coumaric acid in $\mu\text{mol/g soil}$ or mM represents only the free or active fraction of *p*-coumaric acid remaining in the soil and excluded *p*-coumaric acid sorbed to soil particles, utilized by microbes, or taken up by roots.

Soil Bacterial Population

Bacterial populations that could utilize *p*-coumaric acid as a sole carbon source were determined by the plate dilution frequency technique (Harris and Sommers, 1968) utilizing a basal medium containing 0.5 mM *p*-coumaric acid as the only carbon source (Blum and Shafer, 1988; Shafer and Blum, 1991; Blum et al., 2000).

Data Analysis

Data were analyzed using SAS[®] system (SAS Institute Inc., 1999) employing SAS/GLM and SAS/MIXED procedures. For each data set, models were developed with the joint goals of providing a parsimonious explanation of the data while maintaining maximum predictive power. Initial model screening and computation of the r^2 statistic were handled using SAS/GLM. However, for many of the analyses, the data contained observations on the same unit measured repeatedly over time. This created a violation of the assumptions for standard analysis of variance and the need to use methodology for handling linear models with repeated measures. The tool used in this case was the SAS/MIXED procedure (Littell et al., 1996).

Results

How Do *p*-Coumaric Acid Treatments Affect Soil Evaporation (Exp. 1a)?

Multiple *p*-coumaric acid treatments up to 1 $\mu\text{mol/g}$ soil did not modify evaporation from Cecil A_p soil–sand mixture (or soil). However, evaporation varied significantly for time of day (daytime vs. nighttime) and from day-to-day (24 hr, d 6–13). The mean evaporation of 5.15 ± 0.091 g water/12 hr (mean \pm standard error) for the daytime was 1.58 times higher than the 3.27 ± 0.073 g water/12 hr for the nighttime (Fig. 1). Daily evaporation from the

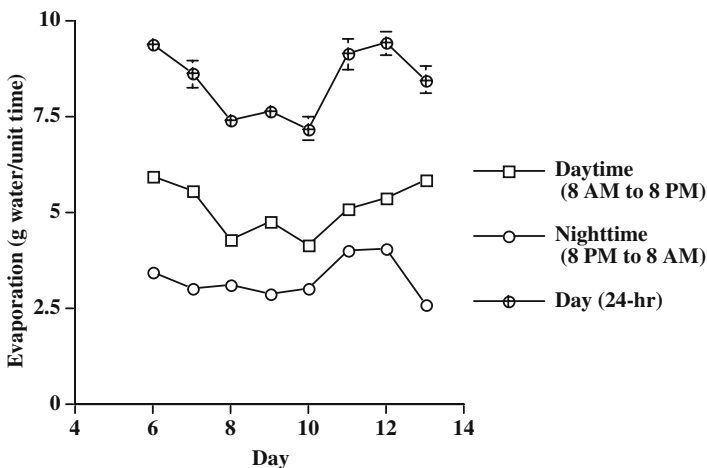


Fig. 1 Evaporation (g water/unit time) from Cecil A_p soil–sand mixture treated with 0–1 $\mu\text{mol/g}$ soil *p*-coumaric acid on d 6, 8, 10, and 12. Because effects of *p*-coumaric acid were not significant, only overall means \pm SE are presented. Absence of error bars indicates that error bars are smaller than the symbol representing the mean

soil systems ranged from 7.20 ± 0.30 g water/24 hr on d 10 to 9.44 ± 0.30 g water/24 hr on d 12. The mean daily evaporation was 8.42 ± 0.14 g water/24 hr.

How Do *p*-Coumaric Acid Treatments Affect Evapotranspiration, Soil Water Content, Leaf Area, and Leaf Area Expansion of Cucumber Seedlings (Exp. 1b)?

Increasing concentrations of multiple treatments of *p*-coumaric acid reduced evapotranspiration, leaf area, and absolute and relative rates of leaf expansion, slowed the decline of soil water in the (cucumber seedling)–soil system, and elevated the daily lowest soil water content (see Figs. 2 and 3; Tables 1, 2, and 3 in [Supplementary Material](#)). Based on the models in Table 1, reductions for mean daytime (6.64 ± 0.35 g water/12 hr for controls), nighttime (3.82 ± 0.30 g water/12 hr for controls), and 24 hr (10.47 ± 0.40 g water/24 hr for controls) evapotranspiration ranged from 3% for the $0.125 \mu\text{mol/g}$ soil treatment to 22% for the $1 \mu\text{mol/g}$ soil treatment. Because evapotranspiration was reduced by *p*-coumaric acid, soil water content declined at a slower rate (see Figs. 2 and 3; Table 2 in [Supplementary Material](#)). Based on the models in Table 2, a 25% loss of water from the systems required approximately 7.8, 8.8, 9.1, 9.8, 11.4, and 13.5 hr for the 0, 0.125, 0.25, 0.5, 0.75, and $1 \mu\text{mol}/150$ g soil treatments, respectively. The largest difference in soil water content occurred just before solutions were added each day (Fig. 2). Based on the models in Table 2, mean lowest daily soil water ranged from 8.31 ± 1.08 g water/150 g soil for the control to 11.03 ± 1.08 g water/150 g soil for the $1 \mu\text{mol/g}$ soil *p*-coumaric acid treatment.

Leaf area (cm^2 , areas of cotyledons not determined) and relative rates of leaf expansion ($\text{cm}^2/\text{cm}^2/\text{d}$) of control cucumber seedlings increased approximately in a linear manner from d 10 to 14, whereas absolute rates of leaf expansion (cm^2/d) of control seedlings varied significantly from day-to-day (see Table 3 in [Supplementary Material](#)). Based on the models in Table 3, reductions in mean leaf areas ($13.95 \pm 3.71 \text{ cm}^2$ for control) and mean absolute rates of leaf expansion ($5.20 \pm 0.43 \text{ cm}^2/\text{day}$ for controls) ranged from 4% for

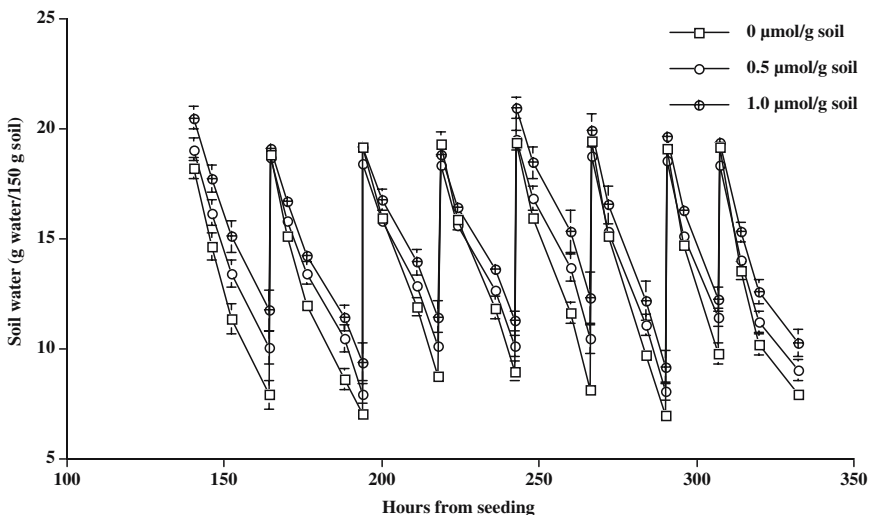


Fig. 2 Soil water content (g water/150 g soil) of (cucumber seedling)–(Cecil A_p soil–sand mixture) systems treated with 0–1 $\mu\text{mol/g}$ soil *p*-coumaric acid on d 6, 8, 10, and 12. Absence of error bars indicates that error bars are smaller than the symbol representing the mean

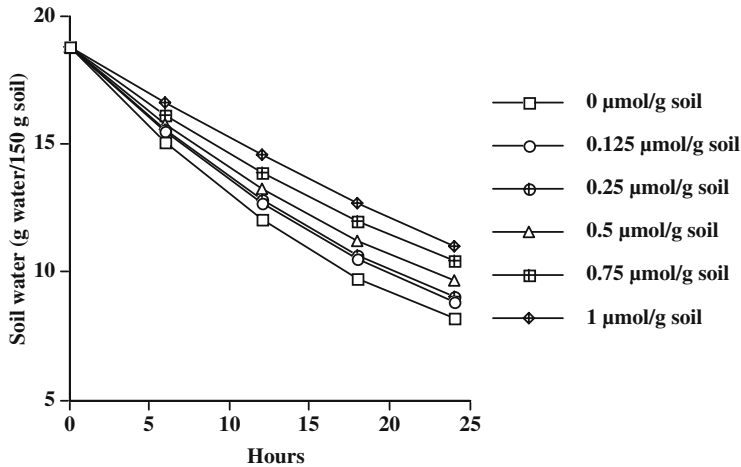


Fig. 3 Soil water content (g water/ 150 g soil) averaged over 24-hr periods for (cucumber seedling)–(Cecil A_p soil–sand mixture) systems treated with 0–1 $\mu\text{mol/g}$ soil *p*-coumaric acid on d 6, 8, 10, and 12. Data derived from models in Table 2 (see [Supplementary Material](#))

0.125 $\mu\text{mol/g}$ soil to 35% for 1 $\mu\text{mol/g}$ soil. Reductions of mean relative rates of leaf expansion ($1.14 \pm 0.90 \text{ cm}^2/\text{cm}^2/\text{d}$ for controls) ranged from 2% for 0.125 $\mu\text{mol/g}$ soil to 12% for 1 $\mu\text{mol/g}$ soil.

How Does Soil Water Content Influence the Effects of *p*-Coumaric Acid (Exp. 2)?

Effects of *p*-coumaric acid were not significantly different for the 20- and 25-g soil water treatments; that is, there were no significant interactions between *p*-coumaric acid and water treatments. (Cucumber seedling)–soil systems were treated with 0.5 $\mu\text{mol/g}$ soil on d 6 and 8 and 1 $\mu\text{mol/g}$ soil on d 10 and 12. Based on the models in Tables 4 and 5 (see tables in [Supplementary Material](#)), evapotranspiration (Fig. 4) and total leaf area (includes cotyledons) were inhibited by 10–19%, and lowest daily soil water was elevated by 8–21% by the 1 $\mu\text{mol/g}$ soil *p*-coumaric acid treatments given on d 10 and 12. Absolute rates of leaf expansion (Fig. 5) were inhibited 17–40% by 0.5 $\mu\text{mol/g}$ soil on d 6 and by 1 $\mu\text{mol/g}$ soil on d 10, 11, and 12. Relative rates of leaf expansion were inhibited 10–28% by both 0.5 and 1 $\mu\text{mol/g}$ soil *p*-coumaric acid (d 6–12).

The water treatments, however, did modify responses for the seedling–soil systems over the entire experimental period. Based on the models in Tables 4 and 5 (see tables in [Supplementary Material](#)), evapotranspiration was 8–15% (Fig. 4), lowest daily soil water was 26–42%, and absolute (Fig. 5) and relative rates of leaf expansion were 2–21% greater for the 25-g water treatments than the 20-g water treatments. Total leaf area, however, was 2–9% greater for the 20-g water treatments than the 25-g water treatments.

Starting with d 12, extra seedling–soil systems were destructively sampled at 0, 16, and 24 hr to determine soil moisture, water extractable $\mu\text{mol/g}$ soil *p*-coumaric acid, and mM of *p*-coumaric acid in soil solution (Fig. 6; see Table 6 in [Supplementary Material](#)). Based on models in Table 6, the 1 $\mu\text{mol/g}$ soil of *p*-coumaric acid added to the seedling–soil systems dropped immediately to 0.92 $\mu\text{mol/g}$ soil (time = 0) and then declined more gradually to 0.19 $\mu\text{mol/g}$ soil by 24 hr, a reduction of 79%. Over the same time period, soil water declined from 18 to 8 g/150 g soil for the 20-g water treatment and from 23 to 10 g/150 g

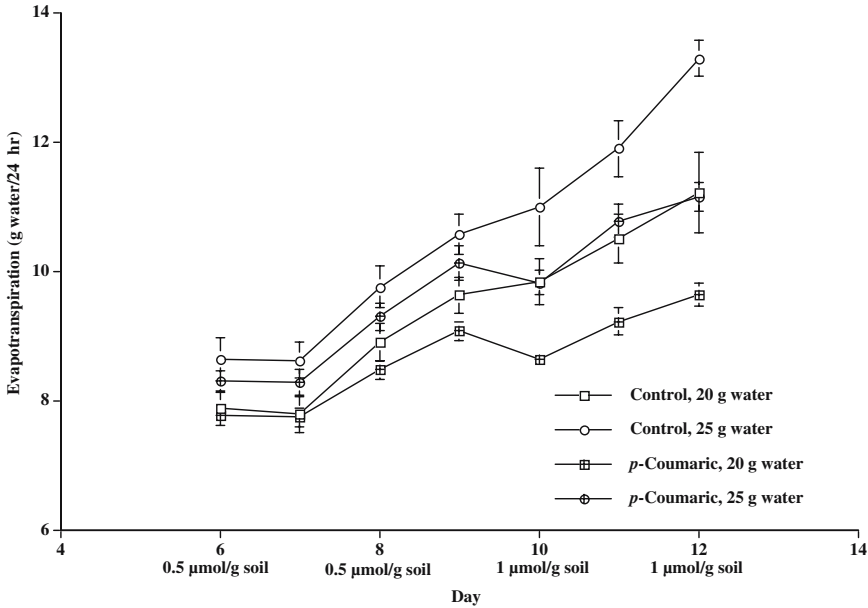


Fig. 4 Evapotranspiration (g water/24 hr) from (cucumber seedling)–(Cecil A_p soil–sand mixture) systems treated with two water levels (20 and 25 g water/150 g soil daily) and *p*-coumaric acid (0.5 $\mu\text{mol/g}$ soil was added on d 6 and 8, and 1 $\mu\text{mol/g}$ soil was added on d 10 and 12). Absence of error bars indicates that error bars are smaller than the symbol representing the mean

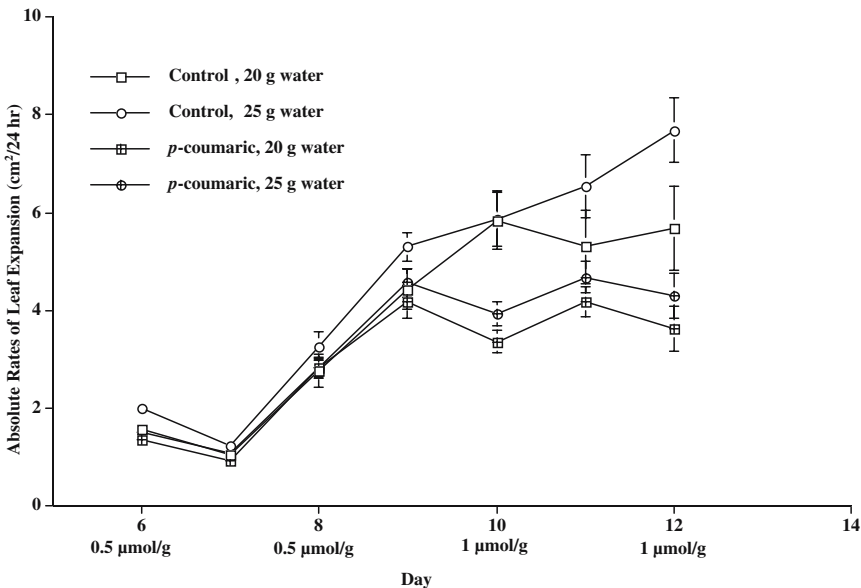


Fig. 5 Absolute rates of leaf expansion ($\text{cm}^2/24 \text{ hr}$) of cucumber seedling growing in (Cecil A_p soil–sand mixture) systems treated with two water levels (20 and 25 g water/150 g soil daily) and *p*-coumaric acid (0.5 $\mu\text{mol/g}$ soil was added on d 6 and 8, and 1 $\mu\text{mol/g}$ soil was added on d 10 and 12). Absence of error bars indicates that error bars are smaller than the symbol representing the mean

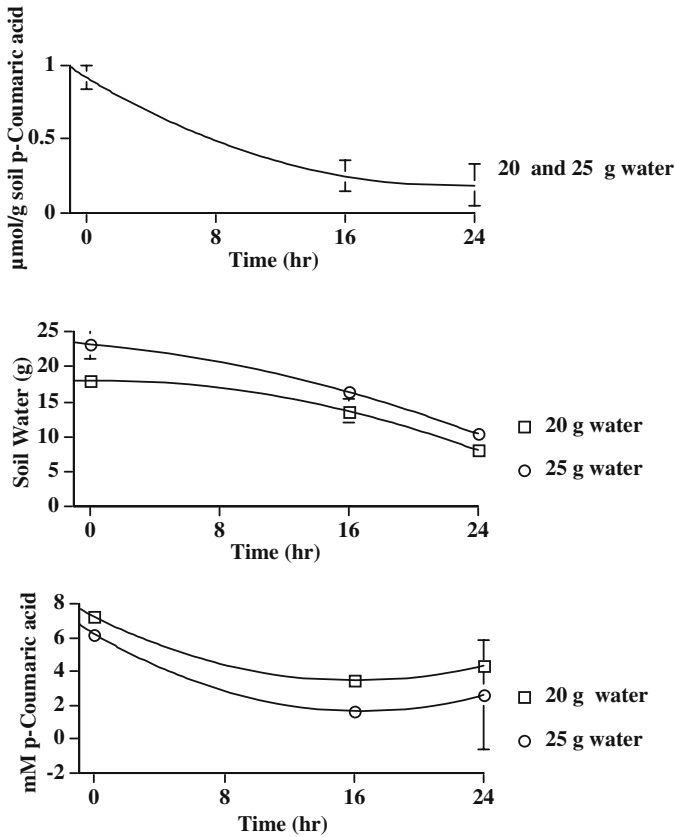


Fig. 6 $\mu\text{mol/g}$ soil *p*-coumaric acid, soil water (g/150 g soil), and mM of *p*-coumaric acid for 12- to 13-d-old (cucumber seedling)–(Cecil A_p soil–sand mixture) systems treated with 1 $\mu\text{mol/g}$ soil *p*-coumaric acid. Absence of error bars indicates that error bars are smaller than the symbol representing the mean

soil for the 25-g water treatment, approximately a 55% reduction for both water treatments. The concentration of *p*-coumaric acid in the soil solution based on the water-extractable *p*-coumaric acid and soil water content declined over the first 16 hr from 7.21 to 3.44 mM for the 20-g water treatment (a 52% reduction) and from 6.19 to 1.64 mM for the 25-g water treatment (a 74% reduction). Beyond 16 hr, the concentration increased for the next 8 hr from 3.44 to 4.34 mM for the 20-g water treatment (a 26% increase) and from 1.64 to 2.59 mM for the 25-g water treatment (a 58% increase). Finally, soil water and mM *p*-coumaric acid were higher and lower, respectively, for the 25-g water treatment than for the 20-g water treatment. Based on models in Table 6 (see [Supplementary Material](#)), soil water for the 25-g water treatment was 28, 20, and 29% greater than the 20-g water treatment at 0, 16, and 24 hr, respectively. The concentration of *p*-coumaric acid in the soil solution for the 25-g water treatment was 14, 52, and 40% lower than the 20-g water treatment at 0, 16, and 24 hr, respectively.

On d 13, additional sets of seedling–soil systems were also destructively sampled to determine the log colony forming units (CFU) of *p*-coumaric acid utilizing bacteria/g soil and soil pH. *p*-Coumaric acid utilizing bacteria were not modified by the 20- and 25-g water treatments, but were stimulated by the *p*-coumaric acid treatments. The control soil

contained 10.50 ± 0.31 log CFU/g soil (2.24×10^{11} CFU/g soil), and the *p*-coumaric-acid-treated soil contained 11.70 ± 0.17 log CFU/g soil (7.85×10^{11} CFU/g). Soil pH was not modified by the 20- and 25-g water treatments, but was increased by the *p*-coumaric acid treatments. The control soils had a pH value of 5.68 ± 0.03 , and the *p*-coumaric-acid-treated soil had a pH value of 6.10 ± 0.02 .

What Are the Response and Recovery Times of Evapotranspiration, Lowest Daily Soil Water, Leaf Area, and Absolute and Relative Rates of Leaf Expansion for Cucumber Seedlings Growing in Soil Treated One to Four Times with a Range of *p*-Coumaric Acid Concentrations (Exp. 3)?

Inhibition of evapotranspiration, total leaf area, absolute and relative rates of leaf expansion, and the elevation of daily lowest soil water were increased in a linear manner

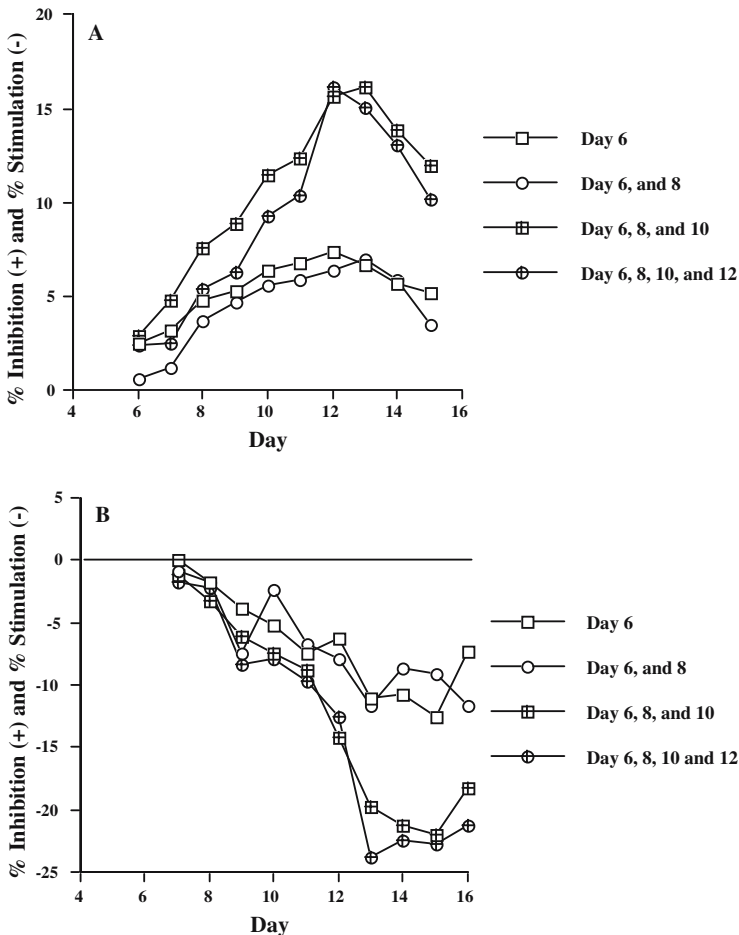


Fig. 7 Percent inhibition/stimulation of evapotranspiration (A) and daily lowest soil water (B) of (cucumber seedling)–(Cecil A_p soil–sand mixture) systems treated with $0.5 \mu\text{mol/g}$ soil *p*-coumaric acid one to four times. Percent inhibition presented as positive values and percent stimulation as negative values. Data derived from models in Table 7 (see [Supplementary Material](#))

with increasing concentrations of *p*-coumaric acid ranging from 0 to 0.75 $\mu\text{mol/g}$ soil (see Tables 7 and 8 in [Supplementary Material](#)). Significant effects of *p*-coumaric acid were first observed on the day of the first treatment (d 6) for absolute and relative rates of leaf expansion and on d 7–9 for evapotranspiration, lowest daily soil water, and total leaf area. Utilizing the models in Tables 7 and 8, maximum inhibition for all *p*-coumaric acid treatment combinations occurred for absolute and relative rates of leaf expansion on d 8 or 10 and for evapotranspiration and total leaf area on d 12 or 13, whereas maximum elevation of lowest daily soil water occurred on d 13 or 15.

Maximum inhibition for evapotranspiration and total leaf area and elevation of lowest daily soil water were not significantly different for the single (d 6) and double (d 6 and 8) *p*-coumaric acid treatments and averaged from 4 to 24% for the 0.25–0.75 μmol *p*-coumaric acid/g soil treatments (see Figs. 7 and 8, and Tables 7 and 8 in [Supplementary Material](#)).

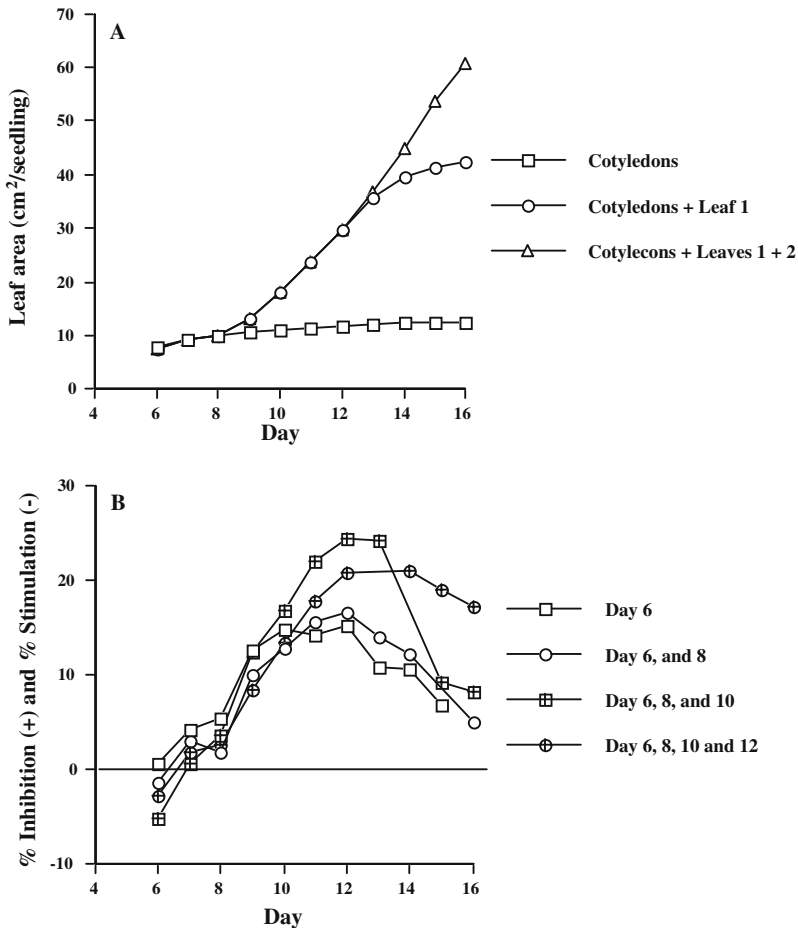


Fig. 8 Leaf area of control seedlings (cm²/seedling) (A) and inhibition/stimulation of total leaf area (B) of seedlings growing in (Cecil A_p soil–sand mixture) systems treated with 0.5 $\mu\text{mol/g}$ soil *p*-coumaric acid one to four times. Percent inhibition presented as positive values and percent stimulation as negative values. Data for percent inhibition/stimulation were derived from models in Table 8 (see [Supplementary Material](#))

Also, maximum effects were not significantly different for the triple (d 6, 8, and 10) and quadruple (d 6, 8, 10, and 12) *p*-coumaric acid/g soil treatments and averaged from 8 to 34% for the 0.25–0.75 μmol *p*-coumaric acid/g soil combinations. Recovery for evapotranspiration and total leaf area was evident for all treatment exposures after d 12 or 13. Recovery for lowest daily soil water was evident on d 13 or 15. Complete recovery (not significantly different from control), which occurred from d 14 to 16, was observed only for the single and/or double *p*-coumaric acid treatments.

Total leaf area and evapotranspiration, and total leaf area and lowest daily soil water for Exp. 3 were positively and negatively related, respectively [(evapotranspiration, g water/day = $8.79 + [0.11 \times \text{total leaf area}] - [0.002 \times \{\text{total leaf area} - 27.24\}^2] + [0.00004 \times \{\text{total leaf area} - 27.24\}^3]$], where total leaf area is in cm^2 and 27.24 is a constant; $P < 0.001$ and $r^2 = 0.88$, observations = 620) and (lowest daily soil water, g water/150 g soil = $14.38 - [0.09 \times \text{total leaf area}] + [0.002 \times \{\text{total leaf area} - 27.24\}^2] - [0.00005 \times \{\text{total leaf area} -$

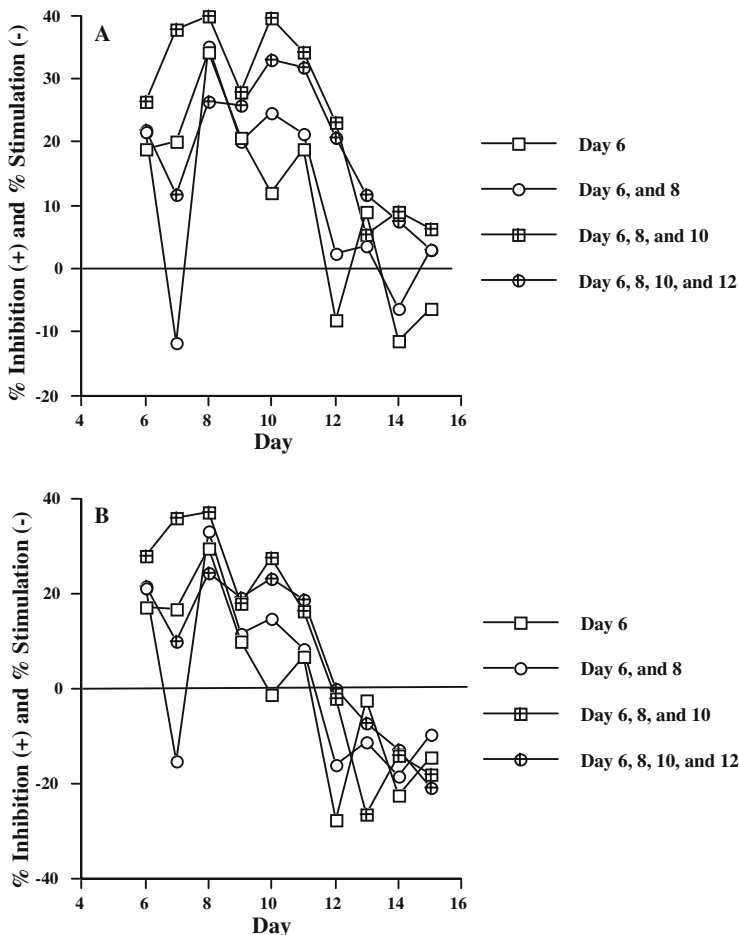


Fig. 9 Percent inhibition/stimulation of absolute (A) and relative rates (B) of leaf expansion of seedlings growing in (Cecil A_p soil–sand mixture) systems treated with 0.5 $\mu\text{mol/g}$ soil *p*-coumaric acid one to four times. Percent inhibition presented as positive values and percent stimulation as negative values. Data derived from models in Table 8 (see [Supplementary Material](#))

27.24 $\}^3]$, where total leaf area is in cm^2 and 27.24 is a constant; $P < 0.001$ and $r^2 = 0.69$, observations = 619)].

Maximum inhibition of absolute and relative rates of leaf expansion occurred on d 8 or 10, was not significantly different for all *p*-coumaric acid treatment combinations, and averaged from 16 to 53% for the 0.25–0.75 μmol *p*-coumaric acid/g soil treatments (see Fig. 9; Table 8 in [Supplementary Material](#)). Recovery was evident for all treatment combinations after d 8 or 10. Complete recovery (not significantly different from control), however, varied with treatment combination. Complete recovery for absolute rates of leaf expansion occurred on d 12 and 13 for the single and double treatments, and the triple and quadruple treatments, respectively. Complete recovery for relative rates of leaf expansion occurred on d 10, 11, and 12 for the single, double, and triple and quadruple treatments, respectively. Furthermore, relative rates of leaf expansion were subsequently stimulated. Maximum stimulations ranged from 12 to 35% for the 0.25–0.75 $\mu\text{mol/g}$ soil treatments for all treatment combinations.

Discussion

Phenolic acids, such as *p*-coumaric acid, inhibit transpiration and leaf area expansion and reduce leaf area of cucumber seedlings (Blum et al., 1985a,b; Blum and Gerig, 2005). What is new here is the demonstration that inhibition of phenolic acids, such as *p*-coumaric acid, on transpiration and leaf area expansion resulted in reduced rates of water depletion from soil and an elevation of mean lowest daily soil water (Exp. 1). Because the level of inhibition of transpiration and leaf area expansion is a function of concentrations of phenolic acids in soil solutions surrounding roots (Lehman and Blum, 1999; Blum and Gerig, 2005), one might anticipate that elevated soil water content associated with the presence of phenolic acids in soil solutions could decrease the level and duration of phenolic acid inhibition. In addition, such increases in water content and associated changes in soil solution concentrations could result in incorrectly identifying the actual dose in dose response studies. Furthermore, the increased soil moisture associated with phenolic acid treatments of sensitive species suggested a means by which the magnitude of some allelopathic interactions might be modified, and resource competition and allelopathy could interact in the presence of sensitive and insensitive species.

As a first step toward determining if such changes in soil water might influence the observed inhibition of phenolic acids, we attempted to maintain different water levels in the (cucumber seedling)–[Cecil A_p soil–sand mixture (or soil)] systems (Exp. 2). However, maintaining a specific narrow range of soil water for the seedling–soil systems was essentially impossible given the size of the cup system and the variations in seedling size and evapotranspiration. We therefore chose to supply different levels of water once a day, at the same time, to all systems, essentially a differential “dry-down” procedure. Bringing water levels to less than 20 g water/150 g soil each day required that water be added more than once during the day, particularly for control systems, to prevent wilting of cucumber seedlings. The first signs of wilting by cucumber leaves occurred whenever soil water in the seedling–soil system reached 7.24 ± 0.17 g water/150 g soil. Bringing water levels to more than 25 g water/150 g soil each day resulted in standing soil surface water for several hours during each 24-hr watering cycle, the time varying with seedling age and with *p*-coumaric acid treatment. Field capacity for the Cecil A_p soil–sand mixture was approximately 0.095 g water/g soil (Lehman and Blum, 1997), which is below the 0.13 g water/g soil (20 g water) and 0.16 g water/g soil (25 g water) water treatments used. Finally, the amount

of water actually added daily to the systems declined slightly over time as seedling weight increased. It also varied some with inhibition of seedlings by *p*-coumaric acid. Recall that water added at the beginning of each 24-hr water cycle was based on seedling–soil system weight. However, this error amounted to less than 1.5 g at the termination of all experiments and less than 0.5 g between controls and the *p*-coumaric acid treatments.

As expected, soil water was higher for the daily 25 g water/150 g soil treatment than the 20 g water/150 g soil treatment throughout each 24-hr watering cycle. However, leaf area expansion and evapotranspiration were also greater for the 25-g water treatment. Thus, watering the seedling–soil systems above field capacity apparently was not a problem for cucumber seedling development. Systems supplied with 25 g water/150 g soil (determined value 23.65 ± 0.045 g) had a range of 8–18% greater evapotranspiration, 26–42% higher lowest daily soil water, and 2–21% greater leaf area expansion than those supplied with 20 g water/150 g soil (determined value 18.96 ± 0.075 g). We suspect that this was primarily because of the more rapid decline (i.e., more negative) in soil solute and matric potentials each day for the 20-g water than for the 25-g water-treated (cucumber seedling)–soil systems. In other words, soil water became limiting earlier during each 24-hr watering cycle for the 20-g water than the 25-g water treatment. However, because the lowest daily soil water experienced by all seedlings was above the wilting point of cucumber seedlings and no visible wilting was observed, seedlings were not severely drought stressed at the end of each 24-hr water cycle.

Because the initial treatments of 0.5 $\mu\text{mol/g}$ soil of *p*-coumaric acid on d 6 and 8 in Exp. 2 did not result in a rapid and striking inhibition of evapotranspiration and leaf area expansion (only relative rates of leaf expansion were inhibited from d 6 to 10), the systems were treated with 1 $\mu\text{mol/g}$ soil on d 10 and 12. To determine the range and the differences in soil solution concentrations of *p*-coumaric acid for the two water treatments, we thus chose to extract soils toward the end of the experiment. Over a 24-hr period, seedling–soil systems containing 12-d-old cucumber seedlings treated with 1 $\mu\text{mol/g}$ soil *p*-coumaric acid had decreasing and then increasing soil solutions concentrations ranging from 7.12 to 3.44 mM for the 20-g water treatment and from 6.19 to 1.64 mM for the 25-g water treatment. The decreasing soil solution concentrations for the first 16 hr were a result of microbial utilization, soil sorption, and cucumber seedling root uptake (Blum and Shafer, 1988; Blum et al., 1994, 2000; Blum and Gerig, 2005). The increasing soil solution concentrations sometime after 16 hr were likely a result of a faster decline in the rates of microbial utilization, soil sorption, and/or root uptake of *p*-coumaric acid during the “dry down” than evapotranspiration; that is, water was declining proportionally faster than *p*-coumaric acid. The large error bars at 24 hr for mM of *p*-coumaric acid (Fig. 6) in soil solution (available *p*-coumaric acid) appeared to be caused by the variation in soil water content. Variation in soil water for the cup systems was small at time 0, when systems were watered, and became progressively larger during the “dry down” procedure, reaching a maximum at 24 hr, just before the next water addition. When the available *p*-coumaric acid was expressed in $\mu\text{mol/g}$ soil, soil being a constant, the increase in the error bars over the 24-hr water cycle was much smaller than when available *p*-coumaric acid was expressed in mM.

Given the significant differences in soil solution concentrations between the 20- and 25-g water treatments, it was surprising to find no significant differences in *p*-coumaric acid effects on cucumber seedlings for these two water treatments; that is, there were no significant interactions between *p*-coumaric acid and water treatment. In other words, the effects of water and *p*-coumaric acid treatments were independent of each other. The independence of *p*-coumaric acid and soil water treatments is counter to dose response studies and the observations in nutrient culture that effects of phenolic acids are primarily

determined by their active concentrations surrounding roots (Lehman and Blum, 1999; Blum and Gerig, 2005). Four possible reasons suggested themselves for this independence of response in soil systems: (1) that the mM of *p*-coumaric acid for both water treatments were too high; that is, range of concentrations experienced by seedling roots for both water treatments were at or close to maximum inhibition; (2) that the roots in both water treatments experienced identical doses despite different solution concentrations; (3) that the two water treatments lead to different cucumber seedlings responses to *p*-coumaric acid molecules, i.e., a difference in sensitivity; and/or (4) seedling responses in soil were too slow to determine these interactions within the time frame of this study.

Of the four, the first (1) appeared to be the least likely because inhibition of relative rates of leaf expansion (approximately 15%) by 0.5 $\mu\text{mol/g}$ soil *p*-coumaric acid treatments was also not significantly different for the two water treatments. In a subsequent experiment, *p*-coumaric acid soil solution concentrations ranging from 2.72 mM (1 hr after addition) to 0.92 mM (24 hr after addition) were observed for 12-d-old seedlings treated with 0.5 $\mu\text{mol/g}$ soil *p*-coumaric acid and 20 g water/150 g soil (unpublished data). Using a ratio method and data from the 1- $\mu\text{mol/g}$ soil treatment, we estimate that the range of soil solutions for a 25-g water treatment would have been approximately 2.36–0.44 mM.

The total amount of *p*-coumaric acid added to the seedling–soil systems every other day, the declining $\mu\text{mol/g}$ soil of available *p*-coumaric acid over a 24-hr watering cycle, soil pH, and the *p*-coumaric acid utilizing microbial populations were not significantly different between the two water treatments. The lack of difference in these variables indicated that the combined activities of root uptake and sorption by soil particles also were not different for the two water treatments. However, soil solution concentrations (mM) of *p*-coumaric acid were significantly lower and rates of evapotranspiration (primarily transpiration), lowest daily soil water, and absolute and relative rates of leaf expansion were significantly higher for the 25-g water treatment than for the 20-g water treatment. Thus, it is possible that either increased movement of *p*-coumaric acid molecules through the soil matrix to the roots, associated with higher transpiration rates, or a greater sensitivity of the faster-growing cucumber seedlings to *p*-coumaric acid molecules for the 25-g water treatment led to similar levels of *p*-coumaric acid inhibition for the two water treatments. Further research will be required to determine if either one or the other is true.

What about response times? To determine the time required for initial inhibition, maximum expression of inhibition, and recovery after *p*-coumaric acid treatment, (cucumber seedling)–soil systems were given single (d 6) to quadruple (d 6, 8, 10, and 12) *p*-coumaric acid treatment combinations (Exp. 3). The time required to initially detect inhibition of *p*-coumaric acid on evapotranspiration, lowest daily soil water, total leaf area, and absolute and relative rates of leaf expansion required 1–3 d after the first *p*-coumaric acid treatment. Maximum expression of inhibition of *p*-coumaric acid, however, required an additional 1 (leaf expansion) to 6 d (evapotranspiration and lowest daily soil water). Recovery occurred shortly after maximum expression, but full recovery for evapotranspiration, lowest daily soil water, and total leaf area only occurred for the single and/or double *p*-coumaric acid treatments. Full recovery of absolute and relative rates of leaf expansion for all treatment exposures occurred 2–5 d after maximum inhibition. Relative rates of leaf expansion not only recovered, but were also stimulated, maximum stimulation occurring 4–7 d after maximum inhibition.

The recovery after the single (d 6) *p*-coumaric acid treatment took longer than the recovery after the last *p*-coumaric acid treatment (d 12) of the quadruple treatment. This difference in recovery may have been related to the changing seedling leaf characteristics over the experimental period described below. Furthermore, given this range of times for

expression of inhibition, recovery, and/or stimulation, it was not surprising that the patterns and trends for the different *p*-coumaric acid treatment combinations were so similar and overlapping. However, the significant differences observed in *p*-coumaric acid effects between the single/double treatments and the triple/quadruple treatments were unexpected. The maximum effects of *p*-coumaric acid on evapotranspiration and lowest daily soil water for the triple/quadruple treatments were approximately twice that of the single/double treatments (Fig. 7). The difference for total leaf area was 1.4 (Fig. 8). One possible explanation for this outcome is as follows: On d 6, the start of *p*-coumaric acid treatments, cucumber seedling had no true leaves, only cotyledons, and these were almost fully developed (Fig. 8). The first and second true measurable leaves for the control seedlings did not occur until d 9 and 13, respectively. Thus, the single and double *p*-coumaric acid treatments primarily influenced the transpiration and growth of the cotyledons, whereas the triple and quadruple *p*-coumaric acid treatments influenced the transpiration and growth of both cotyledons and true leaves. The difference in leaf area of the developing seedlings (type and stage of leaf area expansion) and the ratio of succulent (cotyledons) and nonsucculent (true leaves) leaf tissue may help to explain the significant difference in the observed effects on evapotranspiration and lowest daily soil water for the single/double and triple/quadruple *p*-coumaric acid treatments.

Because *p*-coumaric acid treatments did not modify soil evaporation in the absence of cucumber seedlings (Exp. 1), the observed *p*-coumaric acid effects on evapotranspiration and lowest daily soil water were primarily related to effects on transpiration and/or leaf area. However, some effects on soil evaporation would be expected because of differences in soil surface shading by cucumber seedlings with different leaf areas. In a previous study, Blum and Gerig (2005) observed in nutrient culture that the effects of increasing concentrations of *p*-coumaric acid on water loss by cucumber seedlings were largely determined by leaf area. It was not surprising to find that total leaf area and evapotranspiration, and total leaf area and lowest daily soil water were positively and negatively related, respectively (see Exp. 3). Leaf areas of cucumber seedlings have been shown to be directly related to shoot and root dry weights, total and average root lengths of primary and secondary roots, and root numbers (Blum and Rebbeck, 1989). Thus, leaf area and the dynamics of leaf expansion (i.e., absolute and relative growth rates) provide an excellent means by which short- and longer-term responses of (cucumber seedling)–soil systems to phenolic acids, such a *p*-coumaric acid, may be monitored.

The rapid response times in nutrient culture led Blum and Gerig (2005) to suggest that negative feedback regulation of transpiration could be an important factor in determining the observed inhibitory activity of phenolic acids, such as *p*-coumaric acid. However, the slower initiation, maximum expression, and subsequent recovery after *p*-coumaric acid treatments in soil culture, particularly as related to transpiration, soil moisture, and leaf area, and the lack of significant responses to different water treatments suggested that negative feedback regulation by transpiration on seedling development (i.e., growth) is not a major concern to dose response studies in soil systems when time intervals are short. What role, if any, feedback inhibition may have over longer time intervals is still to be determined. We may also conclude that at least for this (cucumber seedling)–soil system and the time frame of this study that the initial input or treatment concentrations of *p*-coumaric acid represented a reasonable estimate of dose experienced by cucumber seedlings despite the dynamic nature of soil solution concentrations.

Allelopathic interactions can be altered by a variety of physical factors of the environment, among them, soil moisture (Einhellig, 1987; Lehman and Blum, 1997). Lehman and Blum (1997) observed that the inhibitory effects of soil-incorporated wheat

cover crop debris on pigweed seedling emergence were eliminated by increasing soil moisture. This suggested that increasing soil moisture associated with a reduction in water utilization by phenolic-acid-susceptible species could influence the magnitude of some allelopathic interactions. Furthermore, soil moisture will also influence a variety of other physical and chemical characteristics of soil, such as aeration, nutrient availability, and soil temperature, which also are known to influence the magnitude of allelopathic interactions (see Einhellig, 1987; Blum, 2006).

Finally, given two species with differing sensitivities to phenolic acids, such as *p*-coumaric acid, growing in proximity to each other in a water-limiting soil, water uptake would be inhibited more for the phenolic-acid-sensitive species than the less sensitive species, leaving more water for the less sensitive species. Similar observations can be made for mineral nutrition (Glass and Dunlop, 1974; Harper and Balke, 1981; Bergmark et al., 1991; Booker et al., 1992; Lehman and Blum, 1999). As the less sensitive species outgrows the sensitive species, its competitive effects on the sensitive species would intensify. Thus, resource competition by the less phenolic-acid-sensitive species could be intensified on the more phenolic acid sensitive species by the differential allelopathic effects of phenolic acids.

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An Evaluation of the Allelopathic Potential of Selected Perennial Groundcovers: Foliar Volatiles of Catmint (*Nepeta* × *faassenii*) Inhibit Seedling Growth

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Abstract Six perennial groundcovers including *Alchemilla mollis*, *Nepeta* × *faassenii*, *Phlox subulata*, *Sedum acre*, *Solidago cutleri*, and *Thymus praecox* were investigated for the allelopathic potential of their respective foliar tissues via evaluation of volatile constituents produced by foliage. These groundcovers were selected for further laboratory evaluation because of superior performance as weed-suppressive groundcovers in previous field experiments. Foliar volatile components of *N.* × *faassenii* exhibited the strongest inhibitory effects on seedling growth of curly cress (*Lepidium sativum*), but *S. cutleri* also showed allelopathic potential by reducing shoot growth of curly cress seedlings with extracted volatiles. Although *A. mollis* and *P. subulata* exhibited strong weed-suppressive traits in past field experiments, weed suppression is apparently associated with either competition for resources or other allelopathic mechanisms rather than an allelopathic effect caused by volatiles. Volatiles of *N.* × *faassenii* were further evaluated with gas chromatography coupled to mass spectrometry (GC-MS). A total of 21 chemical constituents were identified in the volatile cocktail; 17 components were identified from a direct crude leaf sample extraction, including sabinene, β -pinene, β -myrcene, 2-(2-ethoxyethoxy)-ethanol, 1,8-cineole, ocimene, neryl Acetate, 4 α ,7 α ,7 α -nepetalactone, α -copaene, *trans*-caryophyllene, alloaromadendrene, 4 α β ,7 α ,7 α β -nepetalactone, germacrene D, β -farnesene, χ -cadinene, germacrene B, and β -sesquiphellandrene. Five additional constituents were identified in a methanolic extract of dried of *N.* × *faassenii* foliage, but not the volatile cocktail collected from *N.* × *faassenii* foliage. These included methyl benzoate, 2,4-decadienal, neryl acetate, isodihydronepetalactone, and caryophyllene oxide. Three components, 2-(2-ethoxyethoxy)-ethanol, alloaromadendrene, and χ -cadinene, were not only

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detected in both the volatile mixture and the methanolic extract, but also in an aqueous foliar extract that exhibited potential allelopathic activity.

Keywords Allelochemical · Essential oils · Terpenes · *Nepeta* × *faassenii* · Ornamental groundcover · Herbaceous perennials · Volatiles

Introduction

Herbaceous perennial groundcovers have been popularly used in home gardens and landscapes because of their ability to rapidly cover the soil surface and prevent erosion, as well as for their aesthetic appeal. Certain herbaceous perennials evaluated in past field experiments have shown potential as weed-suppressive groundcovers for utilization in many urban landscapes including commercial properties, municipal settings, and even along roadsides (Eom et al., 2005).

Weed-suppressive groundcovers can be used in both private landscapes and roadside settings, and generally form dense foliar canopies, preventing light transmittance to the soil surface beneath the groundcover, thereby limiting weed seed germination and seedling establishment (Eom et al., 2005). The height and density of the groundcover canopy contribute to its ability to suppress weeds, and allelochemicals from vegetative plant parts may also contribute to weed suppression (Eom 2004). Establishment of groundcovers is limited by various environmental factors that influence plant growth, including physical soil condition, salinity, drought, previously established plant species, and temperature extremes (Usón and Poch 2000). Furthermore, relatively large-scale landscape settings are often affected by uncontrollable and undesirable environmental factors. Although we selected groundcovers that exhibited a strong ability to suppress weeds over time because of their competitive interference with weeds, the growth and establishment of groundcovers in difficult urban landscape settings is dependent upon many factors. Therefore, groundcovers that exhibit inherent allelopathic as well as competitive traits may be more successful in suppressing surrounding vegetation, especially on a long-term basis.

Allelochemicals are released from plants into the environment in several ways, including volatilization from leaf tissues, leaching of nonvolatiles from foliage by rainfall, exudation from living roots, or decomposition of residues by soil microorganisms (Shiraishi et al., 2002; Rai et al., 2003; Kobayashi, 2004). Persistence and activity of allelochemicals released under natural conditions or field settings are influenced by the environment, specifically meteorological and rhizospheric conditions, as well as physical distance from the source of production (Jose and Gillespie, 1998; Romero-Romero et al., 2002). Unlike other mechanisms of allelopathy, studies on volatile effects in plant–plant interactions have been less often investigated, although some significant effects have been associated with volatiles produced by mugwort (*Artemisia vulgaris*) and other species as well (Bradow and Connick, 1990; Barney et al., 2005). The evaluation of certain herbaceous ornamentals for their ability to suppress weeds through allelopathic interference has been reported (Shiraishi et al., 2002). Several groundcovers, including *Oxalis brasiliensis*, *Phlox subulata*, and *Lycoris radiata* were observed to

be strongly weed-suppressive when foliar tissues were bioassayed in the laboratory under controlled conditions.

As little information is available regarding the allelopathic potential of such groundcovers, we selected six of our top-performing weed-suppressive species for further evaluation in the laboratory. In our field evaluation, certain groundcovers, including *Alchemilla mollis*, *Nepeta* × *faassenii*, *P. subulata*, and *Thymus praecox*, were superior performers in terms of establishment and/or weed suppression (Eom et al., 2005). In addition, *Solidago cutleri* and *Sedum acre* were included for further investigation of allelopathic traits because these two groundcovers and related species are relatively stress-tolerant and establish well in field settings (Eom et al., 2005). We specifically sought to determine if these groundcovers exhibited allelopathic potential in *in vitro* bioassays by examining the activity of the foliar volatiles they produced. Certain herbaceous perennials, such as mints and common potherbs, emit large quantities of volatiles, likely associated with plant defense against pests or insect attraction. Because *N. × faassenii* exhibited strong potential allelopathic activity in these assays, we focused on isolation and evaluation of its volatile constituents.

Methods and Materials

Groundcover Growth

Six groundcovers and a commercial tomato plant (*Solanum lycopersicum* L.) were propagated for use in our volatile assays. The tomato plant was used as a positive control for production of foliar volatiles. *S. acre* L. (*Sedum* “Acre”) was propagated from 5-cm stem cuttings. Seeds of other perennial groundcovers—*A. mollis* L. (lady’s mantle), *N. × faassenii* L. (catmint “Walker’s Low”), *P. subulata* L. (creeping phlox “Emerald blue”), *S. cutleri* L. (ornamental goldenrod), and *T. praecox* L. (creeping thyme) were purchased from Jelitto Staudensamen GmbH (Schwarmstedt, Germany). Selected groundcovers had previously exhibited strong weed suppression (*A. mollis*, *N. × faassenii*, and *P. subulata*) in field and roadside experiments (Eom et al., 2005) or excellent establishment (*S. cutleri*, *T. praecox*, and *S. acre*) in landscape settings. Stratification of seed was required for uniform germination; therefore, seed was initially placed in moist, filter-paper-lined Petri dishes (4 ml of distilled H₂O/Petri dish) for 6 d at a 4°C dark condition, and then moved to a lighted growth chamber [150 μmol m⁻² s⁻¹ photosynthetic photon flux (PPF) overhead light intensity] at 25°C for 7 d according to instructions available in the Jelitto catalogue, 2000. Groundcovers were sown into Metro-mix[®] 510 growth media (Scotts Co., Marysville, OH, USA) and later transplanted into a soil mixture comprised of 50% local soil (Hudson silt clay loam), 25% sand, and 25% Metro-mix[®] 510 growth media based on volume. All groundcovers in 25 pots of each species for this experiment were grown in a propagation house for 12 mo. Plants were maintained under a 14-hr photoperiod with a light intensity of 200 μmol m⁻² s⁻¹ PPF from supplemental overhead lighting. Daytime temperatures in the greenhouse were maintained within 24–28°C, whereas nighttime temperatures were maintained at 18–22°C. Plants were fertilized biweekly with full-strength Hoagland’s solution (200–300 ml/pot).

Volatile Bioassay

In an assay similar to that developed in our laboratory to assess volatile activity in mugwort tissue (Barney et al., 2005), mature leaves of each groundcover species were excised and directly placed in a plastic ziplock bag submerged in ice water. In the laboratory shortly after collection, leaf samples were folded and wrapped in cheesecloth at amounts of 0, 5, 10, and 20 g of tissue (fresh weight). Each wrapped sample was suspended in a capped 500-ml glass flask in which curly cress (*Lepidium sativum* L.) seeds (20/bottle) were placed on moistened filter paper (Whatman #4) wetted with 5 ml distilled water (Figure 1). Selection of seedling indicators for use in the bioassay requires that the selected species exhibit a high level of germination and uniform growth over time (Bertin et al., 2003). Curly cress meets these requirements (Bertin et al., 2003) and does not require seed pretreatment or stratification; seeds rapidly germinated after storage in a dark, cold room (5°C) upon transfer to a moistened filter paper (4 ml of distilled H₂O/Petri dish) in parafilm-sealed Petri dishes and a light regime of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF overhead light intensity. As a control treatment, foliar tissues of tomato (*S. lycopersicum* L.), commonly known to produce volatile constituents from its foliage (Smith et al., 1996; Farag and Paré, 2002; Feng et al., 2004), were utilized for comparative purposes. Fully expanded leaves of tomato grown for 30 d were selected, and treatments were arranged in a completely randomized design maintained in a growth chamber at 28°C daily temperature and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF overhead light intensity. After 72 hr of growth in enclosed environments, length of curly cress seedling roots and shoots were measured. All treatments were replicated four times.

Sample Preparations for Analyzing *Nepeta Foliar* Constituents

A direct crude leaf volatile extract was made to analyze the volatile constituents from fresh leaves of *N. × faassenii* grown in a greenhouse. A potted *N. × faassenii*

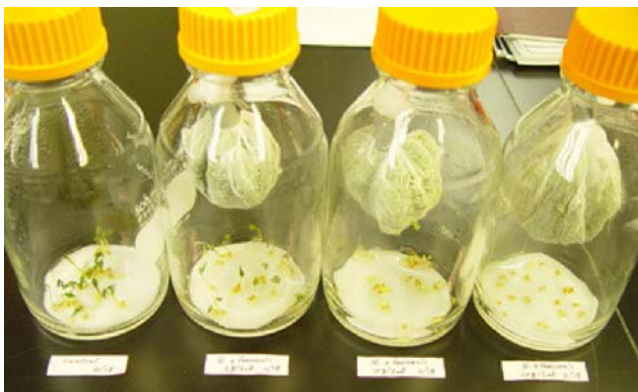


Fig. 1 Volatile bioassay showing suspended foliar tissue of *Nepeta × faassenii* above germinating seedling indicators. From left to right, control, and increasing levels of catmint tissue at 5, 10, and 20 g/cheesecloth.

plant was brought into the laboratory where volatile analysis was performed with a gas chromatograph coupled to a mass spectrometer at room temperature and ambient conditions. Detached fully expanded young leaves were immediately submitted for volatile analyses as described below. In addition, both aqueous and methanolic extracts were formulated by using dried leaves. Ground foliage (100 g) was extracted with 1500 ml distilled water or HPLC (high-performance liquid chromatography)-grade methanol with a rotating shaker maintained in the dark at 5°C for 48 hr. Both extracts were filtered through eight layers of cheesecloth to remove coarse particulates, and centrifuged at 25,000 rpm for 20 min (RC5C[®], Sorvall Instruments, Du Pont, Wilmington, MA, USA) to remove the fine particulates. Final filtration was performed with #4 and #42 filter papers (Whatman) placed in a Büchner funnel. The methanol solvent was vacuum-evaporated at 38°C, and the remaining constituents were analyzed by gas chromatography-mass spectrometry (GC-MS). The aqueous water extract was reduced to dryness with a vacuum rotary evaporator at 38°C, and the residue was resolved in 250 ml distilled deionized water. The aqueous extract was partitioned sequentially $\times 6$ with 250 ml HPLC-grade solvents that included *n*-hexane, diethyl ether, and ethyl acetate by using a separatory funnel. After evaporation of solvents, activity of those residues was assessed by measuring the growth of 72 hr cress seedlings in comparison to an aqueous control with a standard Petri dish germination assay. Each concentrated fraction dissolved in its initial solvent was applied to filter paper in Petri dish. The Petri dish was placed into a 40°C oven for 48 hr to allow solvent evaporation. Ten cress seeds were arranged in glass Petri dishes containing 1 ml distilled water and #1 Whatman filter paper. After parafilm sealing, dishes were maintained in the dark at high humidity at 25–26°C for 72 hr, and root and shoot elongation were measured. Upon evaluation of the seedling bioassay, ether and ethyl acetate fractions were combined and subjected to flash column chromatography. Silica gel flash column chromatography was performed by using a chloroform/methanol solvent gradient, starting with 100% chloroform and terminating with 100% methanol, to further separate the active extracts. Fraction number 4 out of 25 total fractions collected was analyzed by GC-MS, based on activity-guided fractionation with the same cress seedling bioassay.

Identification of Volatiles by SPME Headspace GC-MS

Clear vials (2 ml, Supelco) with PTFE rubber seals (11 mm, Supelco) were used to obtain equilibrium of sample solids (groundcover foliage) with their respective gaseous phase (volatile constituents). After weighing fresh foliar tissues (1 g) of *N. \times faassenii* for analysis, volatile adsorption to SPME fiber (polyacrylate, 85 μ m) was conducted at room temperature for 30 min. In the injection port of the GC, temperatures of 260°C resulted in desorption of volatiles, which were continuously injected into the GC for 5 min. GC-MS (Agilent 6890 + Agilent 5973) was used to analyze all volatile constituents present in each sample. A DB-5MS capillary column (30 m \times 0.25 mm, film thickness 0.25 μ m) was used for the separation of analytes. The column was temperature-programmed from 50°C (held for 3 min) to 200°C (held for 2 min) at a rate of 10°C/min, and then to 300°C (held for 5 min) at a rate of 15°C/min. Helium was used as a carrier gas with a constant flow rate of 1.0 ml/min. For the mass selective detector (MSD), the electron energy was 70 eV, ion source

temperature was 230°C, quadruple temperature was 150°C, and interface temperature was 280°C. MSD was used in SCAN mode over a mass scan range at m/z 30–400, with every analysis verified with air as a blank control for injection between samples to reduce contamination.

Retention indices for all compounds on the DB-5MS capillary column were determined according to the Van Den Dool approach, with *n*-alkanes (especially *n*-hexane) as standards (Kovats 1958). Identification of the components was based on

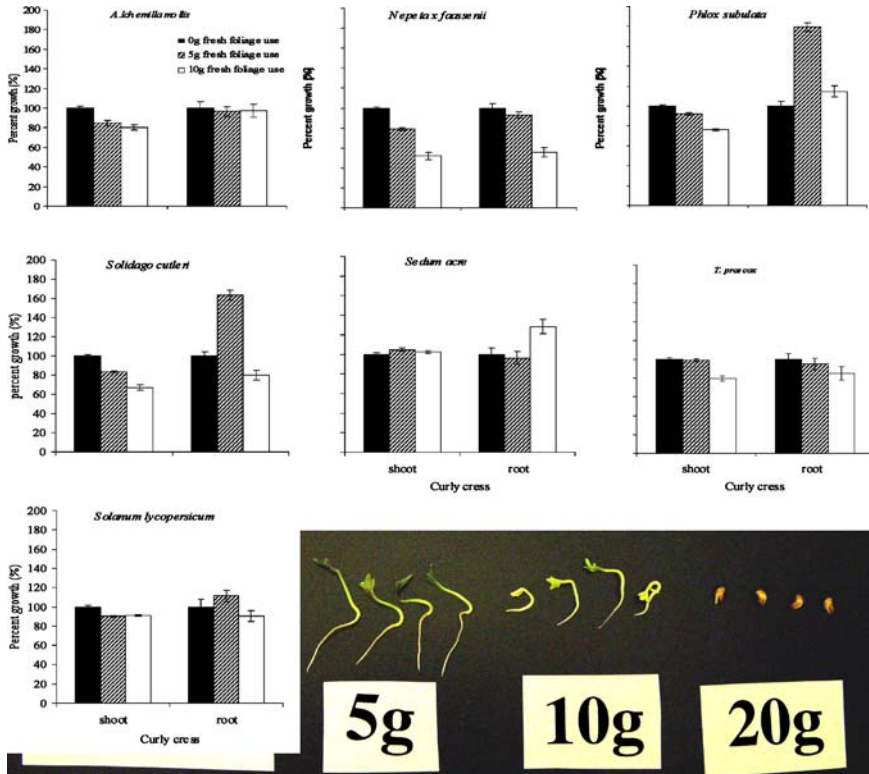


Fig. 2 Top: Inhibition of seedling growth of curly cress (*Lepidium sativum* L.) by volatiles produced by groundcover and tomato foliage (5 and 10 g fresh weight) as compared to an untreated control. Values presented are means of curly cress seedlings ($N = 80$) with standard errors. *Nepeta x faassenii* volatile effect: overall $Pr > F$ of curly cress shoot is <0.001 and $LSD = 1.336$, and overall $Pr > F$ of curly cress root is <0.001 and $LSD = 1.831$. *Solidago cutleri* volatile effect: overall $Pr > F$ of curly cress shoot is <0.001 and $LSD = 0.784$, and overall $Pr > F$ of curly cress root is 0.083 and $LSD = 2.0389$. *Phlox subulata* volatile effect: overall $Pr > F$ of curly cress shoot is >0.001 and $LSD = 1.042$, and overall $Pr > F$ of curly cress root is 0.001 and $LSD = 1.837$. *Alchemilla mollis* volatile effect: overall $Pr > F$ of curly cress shoot is >0.001 and $LSD = 1.183$, and overall $Pr > F$ of curly cress root is 0.795 and $LSD = 2.046$. *Thymus praecox* volatile effect: overall $Pr > F$ of curly cress shoot is >0.001 and $LSD = 1.078$, and overall $Pr > F$ of root is 0.023 and $LSD = 2.044$. *Sedum acre* volatile effect: overall $Pr > F$ of curly cress shoot is 0.009 and $LSD = 1.117$, and overall $Pr > F$ of curly cress root is 0.006 and $LSD = 2.476$. *Solanum lycopersicum* volatile effect: overall $Pr > F$ of curly cress shoot is >0.001 and $LSD = 1.012$, and overall $Pr > F$ of curly cress root is 0.423 , $LSD = 2.118$. Bottom: Seedling growth of curly cress after exposure to *Nepeta x faassenii* foliage. Activity of fresh foliage of *Nepeta x faassenii* was assessed at 0, 5, 10, and 20 g fresh weight in enclosed volatile bioassays.

comparison of their mass spectra with those of Wiley Libraries and those described by R. Adams, as well as by comparison of their retention indices with published values (Van Den Dool and Kratz, 1963).

Results

Effects of Groundcover Foliar Volatiles on the Growth of Curly Cress Seedlings

Of the groundcovers evaluated for volatile production, foliar tissue of most, with the exception of *S. acre*, inhibited the shoot growth of curly cress seedlings, with increasing inhibition observed with increasing rates of fresh foliage placed in the enclosed environment (Figure 1). In contrast, the volatiles of tomato leaves did not inhibit the growth of curly cress seedlings (Figure 2). Overall, *N. × faassenii* volatiles produced the greatest inhibitory effects by consistently reducing both shoot and root elongation. *N. × faassenii* volatiles inhibited shoot growth by 21% and 48% relative to the untreated controls at 5 and 10 g of foliage, respectively. Root elongation was inhibited by 7% and 44%, respectively

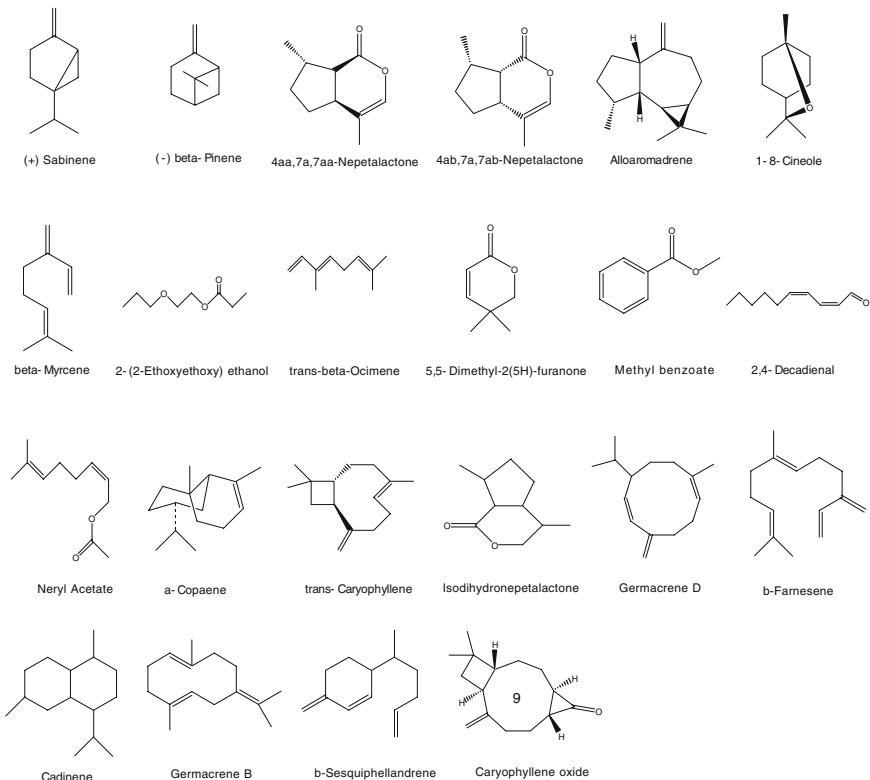


Fig. 3 Chemical structures of volatile components identified in leaf extracts of *Nepeta × faassenii*.

(Figure 2), in comparison to controls. Furthermore, radicle elongation and shoot growth were completely inhibited at the 20 g foliage treatment (Figure 2). Foliar volatiles of *A. mollis* also significantly inhibited shoot growth, but volatiles had little impact on root elongation. Interestingly, radical elongation of curly cress exposed to 5 g of *P. subulata* and *S. cutleri* foliage was increased by 179% and 164%, respectively, indicating some stimulatory activity of certain collected volatile constituents. In comparison, root elongation, when exposed to 5 g of *S. acre* foliage, was not significantly different from the control, but increased with exposure to 10 g of foliage, indicating that *S. acre* foliage also possessed stimulatory potential (Figure 2).

Identification of Volatiles in *N. × faassenii* by SPME-Headspace GC-MS

A total of 22 compounds were identified in *N. × faassenii* volatile headspace by GC-MS (Figure 3 and Table 1). Seventeen compounds were detected directly within the volatile cocktail collected from *N. × faassenii* leaf tissue (Table 1). The components included sabinene, β -pinene, β -myrcene, 2-(2-ethoxyethoxy)-ethanol, 1,8-cineole, ocimene, neryl acetate, 4 α ,7 α ,7 α -nepetalactone, α -copaene, trans-caryophyllene, alloaromadendrene, 4 α β ,7 α ,7 α β -nepetalactone, germacrene D, β -farnesene, χ -cadinene, germacrene B, and β -sesquiphellandrene. Many of the volatiles we observed were similar to those reported previously in the essential oils of *N. crassifolia*, *N. cataria* L., and *N. macrosiphon* Boiss. (Baranauskiene et al., 2003; Dabiri and Sefidkon, 2003; Javidnia et al., 2004).

As root elongation of curly cress seedlings were most affected by aqueous extracts of crude *N. × faassenii* foliar tissues, polyacrylate fibers (85 m) were also utilized to detect polar or semivolatile materials within one fraction (number 4) that was collected and further purified by column chromatography after aqueous extraction. Three components were detected, including 2-(2-ethoxyethoxy)-ethanol, alloaromadendrene, and γ -cadinene, at the detection limit ($\leq 0.04\%$ of GC peak

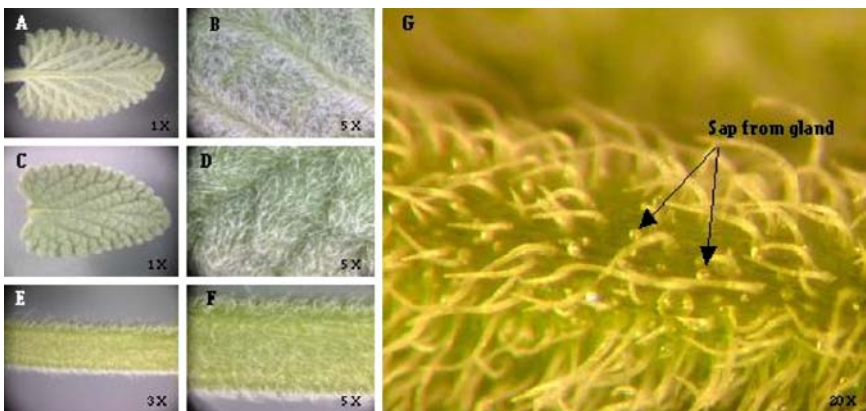


Fig. 4 Leaf and stem morphology of *Nepeta × faassenii*, showing trichomes and glands on leaf surfaces. (A, B) Leaf abaxial surface. (C, D) Leaf adaxial surface. (E, F) Stem. (G) Trichomes and glands.

area). These constituents were detected within the volatile cocktail collected from *N. × faassenii* foliage.

Methanolic extraction of *N. × faassenii* foliage produced similar results, with 10 similar constituents observed at similar detectable levels as above. Five of the 10 components, 5,5-dimethyl-2(5*H*)-furanone, methyl benzoate, 2,4-decadienal, isodihydronepetalactone, and caryophyllene oxide were only detected in the methanolic extract.

Leaf and Stem Structures of *N. × faassenii*

N. × faassenii, commonly known as catmint, is an attractive ornamental ground-cover used in the landscape for mass plantings. It forms a dense canopy of pubescent foliage, and produces attractive blue inflorescences for most of the summer season (Armitage 1997). Trichomes of *N. × faassenii* were observed in great density on the surface of both leaf and stem tissues (Figure 4). Trichomes were denser on the underside of the leaf in comparison to the upper surface, and were less numerous on the stem surface in comparison to leaf. Glandular exudates were also observed on both catmint leaf surfaces and stems.

Seedling Growth of Curly Cress on Either Aqueous or Methanolic Extracts

Seedling growth of curly cress was measured to compare the influence of extraction method, with water or methanol upon extract phytotoxicity (Figure 5). Inhibitory effects upon the seedling growth were more significant with water than with methanolic extracts, when similar dosages of extracts were evaluated (Figure 5). However, total yield of extracts was 26-fold higher in methanolic extracts (17.20 g/100 g DW foliar tissues) than in water extracts (0.77 g/100 g DW). Further fractionation with ether provided greater yield in comparison to other organic solvents (14.09 g).

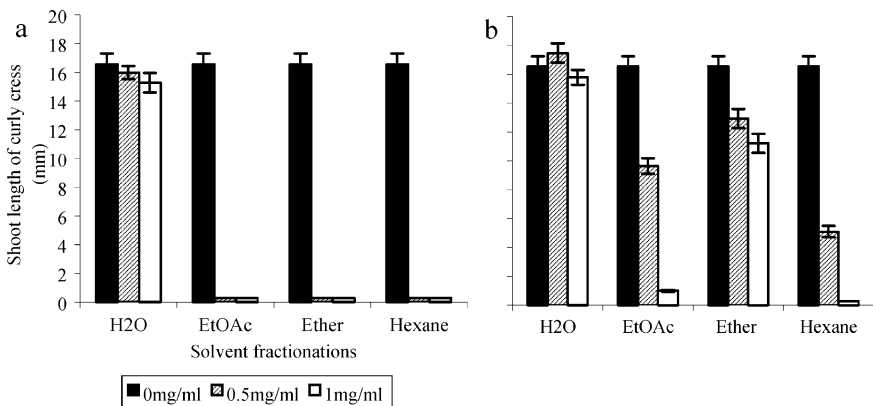


Fig. 5 A comparison of activity observed from aqueous (a) or methanolic (b) extraction of dried *N. × faassenii* shoot tissue using a curly cress seedling elongation bioassay. Each extract was fractionated with organic solvents using stepwise fractionation. Each fraction was dissolved in distilled water and bioassayed with solutions of 0, 5, and 10 mg/ml. Shoot length of curly cress was measured at 4 d after germination initiation. Values presented are means ($N = 80$) with standard errors.

Table 1 Identification of constituents in collected volatiles from *Nepeta × faassenii* foliage as well as from aqueous and methanolic extraction of foliage by GC-MS

Identified constituents	RI ^a	GC peak area (%)			
		Crude sample	H ₂ O ^b	MeOH	MI ^c
Sabinene	972	0.44	nd ^d	1.84	RI, MS
β-Pinene	981	0.86	nd	nd	RI, MS
β-Myrcene	992	0.10	nd	nd	RI, MS
2-(2-Ethoxyethoxy)-ethanol	1011	0.57	25.73	3.52	RI, MS
1,8-Cineole	1030	1.71	nd	2.54	RI, MS
Ocimene	1052	0.68	nd	nd	RI, MS
5,5-Dimethyl-2(5H)-furanone	1060	nd	nd	4.31	RI, MS
Methyl benzoate	1103	nd	nd	1.78	RI, MS
2,4-Decadienal	1284	nd	nd	69.17	RI, MS
Neryl Acetate	1362	0.39	nd	nd	RI, MS
4α,7α,7α-Nepetalactone	1370	73.05	nd	nd	RI, MS
α-Copaene	1377	0.36	nd	nd	RI, MS
trans-Caryophyllene	1467	1.53	nd	nd	RI, MS
Alloaromadendrene	1477	1.99	60.32	13.38	RI, MS
4aβ,7α,7β-Nepetalactone	1485	5.79	nd	nd	RI, MS
Isodihydronepetalactone	1490	nd	nd	1.65	RI, MS
Germacrene D	1496	0.30	nd	nd	RI, MS
β-Farnesene	1500	1.43	nd	nd	RI, MS
χ-Cadinene	1543	0.36	13.96	1.35	RI, MS
Germacrene B	1562	10.25	nd	nd	RI, MS
β-Sesquiphellandrene	1560	0.21	nd	nd	RI, MS
Caryophyllene oxide	1573	nd	nd	3.75	RI, MS

^a Retention indices (RI) indicating elution order from DB-5 column.

^b Aqueous extract was further separated after partitioning using liquid column chromatography and subjected to GC analysis.

^c MI = method of identification; RI = retention indices; MS = mass spectroscopy.

^d Not detected or ≤ 0.04%.

0.5 mg/ml aqueous extracts or above. When methanolic extracts were further partitioned, the hexane fraction at 1.0 mg/ml dosage showed the strongest inhibitory activity among all fractions evaluated, indicating that less polar constituents were associated with inhibitory activity; curly cress seed germination was completely inhibited at 1.0 mg/ml concentrations.

Discussion

The same volatile bioassays we designed to assess *in vitro* inhibitory activity could be considered a potential means to assess the impact of leaf detachment upon volatile production from living plants. Leaf detachment may trigger the production of abscisic acid and ethylene that is caused by change in water status or availability in the leaf (Taiz and Zeiger, 2002). The presence of these hormones may further impact volatile production by inducing metabolic changes in higher plant systems (Taiz and Zeiger, 2002; Feng et al., 2004; LeNoble et al., 2004). We utilized foliar

tissues from tomato (*S. lycopersicum* L.) for comparison with groundcover tissues in this volatile bioassay as a positive control. It is well known that excised tomato leaves can produce significant levels of ethylene over time, besides other scented volatiles (Smith et al., 1996; Farag and Paré, 2002; Feng et al., 2004). In these studies, foliar volatiles of tomato did not greatly inhibit seedling growth of curly cress when compared to groundcover foliage (Figure 2), and neither groundcovers nor tomato produced significant levels of detectable ethylene in this closed assay system.

Given the large numbers of surface glands or trichomes observed on the abaxial surface of *N. × faassenii* leaves, it is likely that significant quantities of volatiles may be emitted by foliage under laboratory or field conditions. In past studies, we have shown that volatiles produced by foliage of mugwort (*A. vulgaris*) successfully bind to soil particles, and render soil in an enclosed environment inhibitory to subsequent seedling germination and growth (Barney 2004). Although we did not perform groundcover volatile assays in the presence of field soil in the laboratory or conduct field-based collections of volatiles under actual field conditions, few—if any—weed seedlings emerged directly around or beneath established *N. × faassenii* plants over a 3-yr period in two separate locations across New York state (Eom et al., 2005). Our findings indicate that foliarly produced allelochemicals may play a role in reduction of weed seedling growth, besides that of resource competition in plant interference. The volatiles of *N. × faassenii* could act as allelochemicals in field settings, if significant concentrations occur under field conditions. Our laboratory assays indicate that in an enclosed environment with detached tissues, foliar volatiles emitted from small quantities of *N. × faassenii* foliage are quite active as seedling growth inhibitors.

Plants of the genus *Nepeta*, commonly called catmints, synthesize and emit various volatiles through foliar trichomes and glands (Plepys et al., 2002). Trichomes were highly abundant in catmint foliage, especially along the abaxial surface of *N. × faassenii* leaves (Figure 4). Circular glands containing volatiles produced by *N. × faassenii* were also observed to be numerous and were located on the leaf surface itself. Of the volatiles detected, isomers of 7S-nepetalactone, which are monoterpenoids, were the principal constituents of the essential oil accumulated by the genus *Nepeta* (Hallahan et al., 1998). In our experiment, three forms of nepetalactone were detected in *N. × faassenii* foliar volatiles. Of the three forms, 4 α ,7,7 α -nepetalactone was the most abundant component, representing 73% of all detected components in a direct crude sample analysis using GC-MS (Table 1). Nepetalactones are believed to attract insects for insect plant pollination (Plepys et al., 2002). However, they have not yet been associated with inhibition of plant growth. Because of their complex structure and our inability to purchase or synthesize these compounds, we have yet to assess their biological activity. Many of the other volatile components detected are also reported allelochemicals, influencing either plant–plant or plant–microbial interactions, including β -myrcene (Ward et al., 1997), ocimene, β -farnesene (McAuslane and Alborn, 1998), and caryophyllene (Stipanovic et al., 1990). Subjected to volatile emission, curly cress was completely inhibited when 20 g or more of fresh foliar tissues of *N. × faassenii* were suspended in closed bioassay system in 500 ml glass bottles (Figure 2).

To investigate another possibility for the release of allelopathic constituents, we evaluated both the aqueous and methanolic extracts from foliar tissues of ground-

covers for inhibitory potential in order to assess possibilities for production of non-volatile inhibitors. By using a standard seed germination/radical elongation bioassay, both aqueous and methanolic extracts of dried foliar tissues of *N. × faassenii* (Figure 5) and *S. cutleri* inhibited seedling growth (data not shown). Although the use of solvent extraction for increasing yield of allelochemicals from extracts of plant tissues is common in the literature, certain extracted chemicals may not be typically released into a natural environment under average field conditions (Lovett and Jessor, 1982). Instead of the relatively harsh solvent extraction technique with methanol, we also decided to use an aqueous method to extract volatile and non-volatile constituents from the foliar tissues of *N. × faassenii*. In an activity comparison between aqueous and methanol extracts, the aqueous extracts of foliage exhibited greater specific activity in terms of seedling growth reduction on a per-weight basis (Figure 5). Not surprisingly, the actual amount of dried residue obtained after aqueous extraction was 26-fold less than that obtained by methanolic extraction. Volatile components were evaluated in both aqueous and methanolic extracts, even though nonvolatile allelopathic components may also be present in considerable quantities in catmint foliage.

From the GC-MS results, fractionation of the aqueous extracts by column chromatography resulted in only three chemicals being detected in purified active fraction number 4 including 2-(2-ethoxyethoxy)-ethanol, alloaromadendrene, and γ -cadinene, at the detected level ($\leq 0.04\%$ of GC peak area). Methanolic extraction yielded 10 chemical constituents including the key components also present in aqueous extracts (Table 1). Volatile analysis yielded 17 chemical constituents present in the crude sample of *N. × faassenii* foliage. As we were not able to evaluate all of these components individually or in mixtures in separate plant growth bioassays, it is unknown which constituents are associated with plant growth inhibition. Further analysis and separation of aqueous *N. × faassenii* extracts by HPLC would likely result in isolation of other active non-volatile constituents.

In summary, previous field experiments showed that *A. mollis* and *N. × faassenii* were highly suppressive of weed growth, as were several other groundcovers that were moderately suppressive. Laboratory experiments indicate that only one of these, *N. × faassenii*, may show strong potential to suppress curly cress growth by volatile allelopathic interference. It evidently produces allelochemicals that are active both as volatile and non-volatile forms from its foliar tissues. Although we examined the effects of allelopathic traits in an *in vitro* volatile assay, our fieldwork mainly focused on examination of weed suppression and competition for resources. Although we have no proof of allelopathy under field conditions, we have evidence to support the concept that catmint tissues could potentially interfere with weed seedling growth due to allelochemical production.

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tosterone) male temporal gland secretions (TGS) is a multifaceted pheromone (Greenwood et al., 2005). Both enantiomers of frontalin are present in TGS, and behavioral responses by conspecifics depend on precise enantiomeric ratios. Bioassays strongly support the presence of a preovulatory pheromone in female African elephant urine (*Loxodonta africana*; Bagley et al., 2006). This communication describes our search for this African elephant pheromone using solid-phase dynamic extraction (SPDE) and gas chromatography–mass spectrometry (GC-MS), which has resulted in the discovery of several compounds (among hundreds) that are known insect pheromones and their precursors (Fig. 1).

Methods and Materials

Volatile organic components of African elephant urine, thawed after having been frozen (-70°C) since collection, were sampled by the powerful technique of automated SPDE/GC-MS (Lipinski, 2001), as well as by solid-phase microextraction (SPME) coupled with chiral column GC-FID and GC-MS (Goodwin et al., 2005; Greenwood et al., 2005). For automated SPDE analysis, the necessary accessories (Chromsys) were mounted on a CTC Analytics CombiPAL multipurpose sampler (Gerstel, MPS2). Urine samples (10 ml) in 20-ml screw-top vials (Viton septa) with a stir bar were analyzed under three conditions: unaltered, with addition of NaCl (3 g), or a protease (1 mg, Invitrogen proteinase K, fungal; #25530-015). Elephant albumin binds urinary and TGS pheromones in the Asian elephant; therefore, protease and NaCl may denature putative protein carriers in African elephant urine and force possible pheromones into the headspace (Lazar et al., 2004). After incubation at 37°C (15 min) and extraction (200 up-and-down syringe strokes over 13 min) by SPDE, desorption was at 250°C in the GC inlet. GC-MS analyses were conducted as published (Goodwin et al., 2005). For GC-MS references, racemic frontalin (**2**), *exo*- (**3**) and *endo*-brevicomins (**4**), enriched samples of (+) and (–) *exo*- and *endo*-brevicomins, ketones **7** (*E* and *Z*) and **9**, and (*E,E*)- α -farnesene (**6**) were obtained

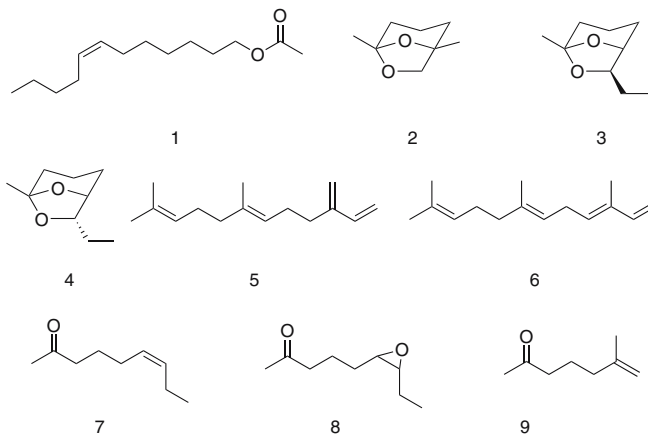


Fig. 1 Chemical structures for insect pheromones and related compounds found in female African elephant urine. **1** = (*Z*)-7-dodecenyl acetate, **2** = frontalin, **3** = *exo*-brevicomins, **4** = *endo*-brevicomins, **5** = (*E*)- β -farnesene, **6** = (*E,E*)- α -farnesene, **7** = (*Z*)-6-nonen-2-one, **8** = 6,7-epoxy-2-nonanone, **9** = 6-methyl-6-hepten-2-one

Table 1 Insect pheromones in African elephant urine (Average Areas^a, *N* Samples)

	Frontalin	<i>N</i>	Precursor 9	<i>N</i>	<i>exo</i> -Brev.	<i>N</i>	<i>endo</i> -Brev.	<i>N</i>	Precursor 7 ^b	<i>N</i>	β -Farn.	<i>N</i>	α -Farn.	<i>N</i>
Natural—day 1	19,868	1	1,697,528	3	315,372	2	303,914	1			82,754	1	16,300	1
Natural—day 3	23,462	2	2,337,089	3	948,664	3	637,825	2			1,948,720	3	1,145,830	3
Natural—day 5	222,659	2	61,416	2	294,704	2	178,004	1	734,301	1	1,291,553	3	1,223,010	3
NaCl—day 1	542,920	2	162,580	3	2,593,626	3	900,098	3			213,791	1	47,040	1
NaCl—day 3	566,862	2	293,201	2	3,019,347	3	1,228,742	3			101,267	1	31,163	1
NaCl—day 5	6,386	1	59,176	1									277,506	1
ProK—day 1	37,174	2	299,443	3	428,326	2			689,491	1	879,681	1	292,602	1
ProK—day 3	36,819	2	2,413,629	3	955,355	2	615,004	1	1,017,276	2	1,564,024	3	1,023,991	3
ProK—day 5											1,168,456	3	1,411,340	3

^a Average peak areas from the GC-MS total ion chromatograms.

^b *E/Z* ratio unknown.

from Pherotech. (*E*)- β -Farnesene (**5**) was purchased from Bedoukian. For enantiomerically enriched frontalin, ratios were established as published (Greenwood et al., 2005).

Results and Discussion

Representative data are shown in Table 1, for which aliquots of ovulatory urine samples from three African elephants were either untreated or treated with NaCl or protease. SPME/GC-MS was run daily for 7 d (stored at 24°C). Data such as those in the table lead to tentative conclusions for urine headspace analysis: (1) addition of NaCl increases the release of frontalin, maximizing around day 3; (2) frontalin precursor **9** is higher in untreated urine; (3) *exo*-brevicommin exceeds the *endo*- isomer, and both increase daily through day 7 in NaCl-treated urine; (4) brevicomin precursor **7** appears on day 3 after protease treatment and increases through day 7; (5) the farnesenes are present almost equally and generally require no treatment for detection after 2 or 3 days. Compounds **2**, **3**, and **4** are bark beetle aggregation pheromones (Francke and Schröder, 1999), whereas farnesenes **5** and **6** are aphid alarm pheromones and components of the mouse puberty accelerating pheromone mixture (Wyatt, 2003).

The importance of frontalin enantiomeric ratios in Asian elephant TGS chemical signaling (Greenwood et al., 2005) led us to investigate these ratios for frontalin and *endo*- and *exo*-brevicommins in female African elephant urine via SPME/chiral column GC-MS. Frontalin, difficult to detect by SPME/chiral column in urine samples, was consistently (-). For unaltered samples on the day of thawing (day 1), no brevicommins were detected. Day 3 revealed (-)-*exo* only, and on day 5, the ratio of (-)-*exo*/(+)-*exo*/(+)-*endo*/(+)-*endo* was 16.7/1/3.3/0. For NaCl-treated samples up to day 5, we saw (-)-*exo* only, but larger amounts. For protease-treated samples, we observed the following: day 1 (14.3/1/2.1/0), day 3 (12.1/1/1.8/0), and day 5 (10/1/0.8/0). Thus, (-)-*exo*-brevicommin was generally the dominant stereoisomer.

The discovery of elephant-derived beetle pheromones and their precursors is particularly intriguing. Various bark beetles can convert (*E*)- and (*Z*)-6-nonen-2-one to *endo*- and *exo*-brevicommin, respectively. (*Z*)-6-Nonen-2-one (**7**) was identified in the volatiles from western balsam bark beetles and male mountain pine beetles, suggesting it as a natural biosynthetic precursor. Furthermore, the latter have been shown to convert (*Z*)-6-nonen-2-one to (+)-*exo*-brevicommin through a keto epoxide intermediate (**8**), followed by an intramolecular carbonyl epoxide rearrangement (Vanderwel and Oehlschlager, 1992). Several beetle species synthesize frontalin when exposed to exogenous 6-methyl-6-hepten-2-one (**9**), although the ketone could not be detected naturally in the beetles (Francke and Schröder, 1999). Ketone **9** has been detected in hindgut extracts of frontalin-producing eastern larch beetles (Francke and Schröder, 1999) and Asian elephant TGS. Evidence exists that some *Dendroctonus* beetles synthesize frontalin via mevalonate in the isoprenoid pathway (Seybold and Tittiger, 2003). Our findings suggest that biosynthesis of brevicommins and frontalin via precursors **7** and **9** proceeds similarly in African elephants and beetles.¹

¹ The presence of both *exo*- and *endo*-brevicommin in the urine suggests the presence of both (*E*)- and (*Z*)-6-nonen-2-one precursors. Nonetheless, these are not yet separable via GC, and their mass spectra are similar; thus, the presence of both cannot be verified.

Questions yet to be answered include the following. (1) Are any of the insect pheromones functioning as pheromones in African elephants? (2) If so, is the chemical signal a single compound, or a blend? (3) Are these putative chemical signals from females to males, from females to females, or both? (4) Do enantiomeric ratios play a role in the proposed chemical signaling? (5) In nature, are these putative pheromones released slowly over time from carrier proteins, and if so, does the chemical signal change? (6) How do concentrations and ratios of the insect pheromones and precursors vary throughout the African elephant estrous cycle? In our laboratories, these questions and others are under active investigation involving both chemical and behavioral studies with a large number of samples.

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et al., 2005; Theis and Raguso, 2005), and circadian rhythms (e.g., Dötterl et al., 2005; Negre et al., 2003).

Of particular interest is the variation in scent emission before and after pollination. Postpollination changes in scent production reduce metabolic costs and decrease the attractiveness of the flower, thus directing the pollinator to other flowers of the plant and increasing overall reproductive success (Schiestl and Ayasse, 2001). In *Silene latifolia* Poir (Caryophyllaceae), a perennial native to Europe, postpollination changes may have the additional function of reducing the attraction of seed predators (Dötterl et al., 2005). *S. latifolia* has a close relationship with *Hadena bicruris* Hufnagel (Lepidoptera, Noctuidae), with *H. bicruris* designated as a parasitic pollinator. The female moths pollinate the female flowers. After nectar ingestion, they lay their eggs in the flower. The larvae subsequently feed on the developing seeds (Bopp and Gottsberger, 2004). Usually, there is one larva hatching per vessel. Once the larva has eaten the whole seed set, it moves to another intact vessel (personal observation). Adult female moths are attracted to flowers mainly by scent, and lilac aldehydes A–D and veratrole were found to be key compounds in their attraction (Dötterl et al., 2006). These compounds make up about 80% of the total blend (Table 1).

If postpollination changes are adaptive in terms of reducing the attraction of seed predators, a specific downregulation of the behaviorally active compounds should be observed. The present study aimed at analyzing compositional and quantitative changes in the floral scent emission of *S. latifolia* after pollination. Furthermore, we analyzed whether pollinator-attracting and nonattracting compounds changed differently in the pollinated flowers.

Methods and Materials

Plant Material and Sample Size

Sixteen potted female plants were grown from seeds collected in 2003 from a *S. latifolia* population located in Leuk (Switzerland). The inflorescence of each potted plant was separated into two equal parts of 3–10 flowers. One part of each inflorescence was hand-pollinated, whereas the other remained unpollinated. Pollination was achieved at 7 p.m. by dabbing a female flower with a male flower. We used only female flowers that underwent anthesis 24 hr before treatment. Older flowers and buds were removed.

Scent Collection

Floral scent was collected during the first and second night after pollination, from 8 p.m. to 7 a.m. Each treated inflorescence was separately packed in an oven-baking bag (PET, Toppits®). A filter was placed inside the bag and connected to a vacuum pump (Personal Air Sampler, SKC Inc.) drawing the air at a rate of about 140 ml/min. Air in the bag was drawn over the filter consisting of 5 mg of Porapak Q as an adsorbent material, sealed in a small glass tube. Nine filters were connected by silicone tubes to one vacuum pump, one of them sampling surrounding air to identify background contamination. Before scent collection, filters were cleaned with 100 µl dichloromethane and 100 µl hexane. After sampling, desorption was achieved by slowly running 50 µl of a hexane/acetone mixture (9/1) through the filter. Desorption was carried out immediately after sampling. Eluates were stored at –20°C in sealed glass vials for later analysis.

Quantitative Gas Chromatographic Analysis and Compound Identification

Before analysis, 100 ng of n-octadecane (Fluka, Buchs) were added to each sample as an internal standard. One microliter of the eluate was injected splitless at a start temperature of 50°C into a gas chromatograph (Agilent 6890N) equipped with an HP5 column (30 m × 0.32 mm × 0.25 μm) and a flame ionization detector. Hydrogen was used as the carrier gas and nitrogen as make-up gas. The injector temperature was kept at 300°C. The oven was kept at 50°C for 1 min, then heated to 150°C at a rate of 5°C/min, and subsequently to 300°C at a rate of 10°C/min, and kept at 300°C for 10 min. Chromatograms were analyzed with the program ChemStation (Agilent Technologies). Absolute amounts of compounds were quantified by using the internal standard (IS) method, i.e., by integrating the peak of every substance and dividing every peak area with the integrated IS peak area and multiplying with the IS amount. None of the samples of surrounding air contained considerable amounts of one of the compounds used in our analyses. Thus, we did not subtract these samples. For a few samples, gas chromatographic analyses with mass selective detection (Hewlett Packard G1800 A) were conducted by using identical column and oven parameters. Compounds were identified by comparison of retention times and gas chromatography–mass spectrometry (GC-MS) spectra with those of known standard compounds.

Data were first checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene test) and then analyzed with *t* test and ANOVA.

Results and Discussion

We found 28 odor compounds in our samples, 2 of which belong to the class of fatty acid derivatives (nonanal and octanal), 8 to the class of benzoids (benzyl acetate, benzaldehyde, benzyl benzoate, methyl benzoate, 2-methoxyphenol, methyl salicylate, phenylacetaldehyde, phenylethyl alcohol, and veratrole), and 12 to the class of terpenoids (camphene, eucalyptol, lilac aldehyde A, lilac aldehyde B, lilac aldehyde C, limonene, linalool, 6-methyl-5-hepten-2-one, trans-β-ocimene, α-pinene, β-pinene, and β-farnesene) (see also Dötterl et al., 2005). Five of the reported compounds remained unidentified (Table 1).

Pollinated plants emitted significantly lower total scent amounts over the two nights than unpollinated plants (unpollinated: mean ± SE, 408.90 ± 105.33 ng·h⁻¹·l⁻¹·flower⁻¹; pollinated: mean ± SE, 95.68 ± 29.55 ng·h⁻¹·l⁻¹·flower⁻¹; *t* test: *t* = 2.86, *df* = 60, *P* < 0.01). Earlier studies on postpollination changes have also found a decrease in total scent emission. Tollsten and Bergström (1989) and Tollsten (1993), for example, detected a decrease in scent production of *Platanthera bifolia*. In *Ophrys sphegodes*, the scent emission also decreased after pollination (Schiestl et al., 1997). In two thistle species, scent decreased strongly after pollination leading to reduced attractiveness of the flowers to honey bees (Theis and Raguso, 2005). Dötterl et al. (2005) showed that *S. latifolia* almost completely stopped emitting scent only 24 hr after pollination. In our study, there was an approximately fourfold decrease in total scent emission (see above). Lilac aldehydes A and B as well as veratrole, which made up about 80% of total scent emission before pollination, contributed most to this decrease (Fig. 1). Most of the other compounds remained unchanged after pollination (Fig. 1). In terms of relative amounts, pollinated plants sampled on the first and second night emitted significantly lower proportions of lilac aldehydes A and B than unpollinated plants sampled in the first night [lilac aldehyde A: ANOVA, *F*(3,58) = 6.21,

Table 1 Mean (\pm SE) relative amounts (%) of odor compounds in pollinated and nonpollinated plants of *S. latifolia* over two nights

Compound	Nonpollinated		Pollinated	
	First night	Second night	First night	Second night
<i>Fatty acid derivates</i>				
Octanal	0.56 \pm 0.14 bc	0.59 \pm 0.13 bc	1.77 \pm 0.29 a	1.30 \pm 0.23 ab
Nonanal ^a	0.29 \pm 0.04 bc	0.77 \pm 0.15 bc	1.46 \pm 0.30 a	1.96 \pm 0.33 ab
Octanal	0.56 \pm 0.14 bc	0.59 \pm 0.13 bc	1.77 \pm 0.29 a	1.30 \pm 0.23 ab
<i>Benzoids</i>				
Benzaldehyde ^a	2.19 \pm 0.29 bc	3.12 \pm 0.61 bc	7.70 \pm 0.99 a	6.92 \pm 1.11 ab
Phenylacetaldehyde ^a	0.95 \pm 0.19 bd	1.41 \pm 0.36 abd	2.85 \pm 0.81 acd	3.33 \pm 0.83 ac
2-Methoxyphenol ^a	0.14 \pm 0.03 ab	0.39 \pm 0.10 bc	0.29 \pm 0.10 abc	0.54 \pm 0.20 ac
Methyl benzoate ^a	0.09 \pm 0.03 a	0.21 \pm 0.08 a	0.09 \pm 0.05 a	0.50 \pm 0.14 c
Phenylethyl alcohol ^a	0.16 \pm 0.04 bc	0.18 \pm 0.09 bc	0.67 \pm 0.15 a	0.62 \pm 0.15 ab
Veratrole ^a	15.66 \pm 4.48 b	11.08 \pm 3.08 ab	4.21 \pm 0.99 a	7.92 \pm 3.68 ab
Methyl salicylate ^a	1.05 \pm 0.14 bd	2.39 \pm 0.60 abd	4.19 \pm 0.77 acd	5.37 \pm 0.88 ac
Benzyl benzoate	1.44 \pm 0.62 a	1.14 \pm 0.59 a	0.68 \pm 0.14 a	1.23 \pm 0.15 a
<i>Terpenoids</i>				
α -Pinene	0.16 \pm 0.05 ab	0.66 \pm 0.15 ad	0.53 \pm 0.13 abd	1.53 \pm 0.26 c
Camphene	0.74 \pm 0.09 b	2.52 \pm 0.59 a	2.94 \pm 0.42 a	5.71 \pm 0.89 c
β -Pinene	0.31 \pm 0.05 bc	0.51 \pm 0.12 bc	1.30 \pm 0.20 a	1.17 \pm 0.19 ab
6-Methyl-5-hepten-2-one	0.25 \pm 0.04 bc	0.37 \pm 0.11 bc	1.06 \pm 0.23 a	1.13 \pm 0.22 ab
Limonene	0.40 \pm 0.14 ab	1.13 \pm 0.29 abc	1.50 \pm 0.27 abc	2.50 \pm 0.97 ac
Eucalyptol	2.50 \pm 0.45 b	1.41 \pm 0.41 b	9.25 \pm 1.48 a	3.06 \pm 0.58 b
<i>trans</i> - β -Ocimene ^a	1.46 \pm 1.22 a	2.18 \pm 1.09 a	0.85 \pm 0.21 a	2.64 \pm 0.56 a
Linalool ^a	0.20 \pm 0.04 bc	0.43 \pm 0.09 bc	1.07 \pm 0.20 a	0.84 \pm 0.17 ab
Lilac aldehyde A ^a	22.69 \pm 1.93 b	16.77 \pm 2.52 ab	13.80 \pm 2.42 ac	9.24 \pm 2.09 ac
Lilac aldehyde B ^a	41.45 \pm 2.85 b	32.99 \pm 4.71 ab	23.98 \pm 4.04 ac	18.78 \pm 4.50 ac
Lilac aldehyde C	2.68 \pm 0.36 bc	3.54 \pm 0.40 bc	6.27 \pm 0.70 a	5.90 \pm 0.70 ab
and benzyl acetate ^{a,b}				
β -Farnesene	0.11 \pm 0.04 abd	0.43 \pm 0.17 abcd	0.09 \pm 0.05 abd	0.74 \pm 0.22 cd
<i>Unknowns with Kovats retention index, R_i</i>				
U1 978	1.08 \pm 0.16 bc	2.16 \pm 0.42 bc	4.11 \pm 0.52 a	4.60 \pm 0.68 ab
U2 992	1.98 \pm 0.89 ab	12.21 \pm 3.59 ad	6.15 \pm 1.77 abd	10.09 \pm 2.47 c
U3 1009	0.15 \pm 0.04 a	0.43 \pm 0.10 a	0.52 \pm 0.15 a	0.93 \pm 0.14 a
U4 1112	0.11 \pm 0.05 a	0.12 \pm 0.05 a	0.18 \pm 0.07 a	0.04 \pm 0.03 a
U5 1191	1.22 \pm 0.27 b	0.85 \pm 0.26 b	2.51 \pm 0.40 a	1.41 \pm 0.21 b

Different letters indicate significant differences between groups as calculated by one-way ANOVA with LSD post hoc test, $P < 0.05$.

^aCompounds elicit electrophysiological signals (Dötterl et al., 2006).

^bAs lilac aldehyde C and benzyl acetate coelute and the amounts of benzyl acetate are much lower than the amounts of lilac aldehyde C, they are listed together in the class of terpenoids.

$P = 0.001$; Lilac aldehyde B: ANOVA, $F(3,58) = 5.84$, $P = 0.001$]. Unpollinated plants sampled on the second night, however, emitted the same relative amounts of lilac aldehydes A and B as pollinated plants sampled on the first and second night [lilac aldehyde A: ANOVA, $F(3,58) = 6.21$, $P = 0.001$; lilac aldehyde B: ANOVA, $F(3,58) = 5.84$, $P = 0.001$]. This could be due to senescence in flowers. For most of the other compounds, pollinated plants emitted the same or greater relative amounts than unpollinated ones (Table 1).

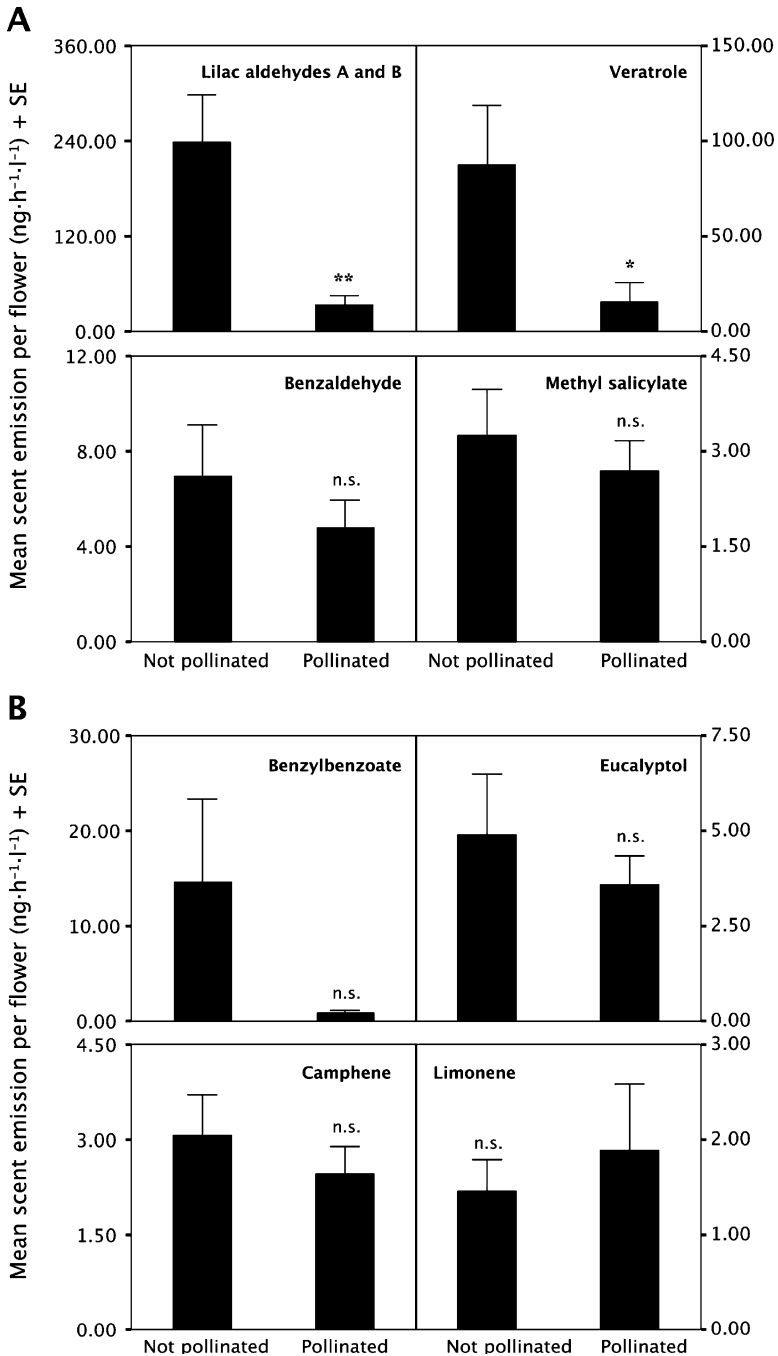


Fig. 1 Mean (+ SE) absolute amounts of (a) the four most abundant electrophysiologically active scent compounds and (b) the four most abundant electrophysiologically nonactive compounds (Dötterl et al., 2006) sampled over the 2 days. Lilac aldehydes A and B and veratrole significantly decreased after pollination (**t* test: $t = 2.20$, $df = 60$, $P < 0.05$; ***t* test: $t = 3.33$, $df = 60$, $P < 0.01$), whereas the other six analyzed compounds did not change significantly after pollination (*t* test, $df = 60$, $P > 0.05$, n.s.)

There are contradictory results on compositional changes in scent emission reported in the literature. In *P. bifolia*, the relative amounts of scent compounds remained almost constant after pollination (Tollsten and Bergström, 1989; Tollsten, 1993). By contrast, pollination changed the bouquet composition of *Cirsium repandum* (Theis and Raguso, 2005) and *O. sphegodes* (Schiestl et al., 1997). Interestingly, in the latter species, the emission of farnesyl hexanoate, which functions as a repellent compound for the pollinator (Schiestl and Ayasse, 2001), increases after pollination. This mechanism is thought to direct pollinators to other, as yet unpollinated flowers, thereby maximizing the overall reproductive success of the plant. The strong compositional changes in the floral odor of *S. latifolia* are mostly due to a decrease in lilac aldehydes A and B as well as in veratrole. Since these compounds have been demonstrated to be of key importance for host finding in *S. latifolia* (Dötterl et al., 2006), we interpret their drastic reduction after pollination as a mechanism for avoiding or reducing further parasitism by the nursery pollinator *H. bicruris*.

Taken together, our results show how adaptations in the chemical communication between plants and their pollinators can be fine-tuned to the regulation of specific compounds in the odor bouquet. Furthermore, our findings underline the key functions of the lilac aldehydes and veratrole for plants' reproductive success, which is largely determined by the attraction of pollinators and avoidance of seed predators.

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(Thompson and Pellmyr, 1991). Plant compounds often serve as cues for phytophagous herbivores to identify suitable hosts for feeding and oviposition.

Many plant defenses are based on active compounds that are enzymatically released from glycosylated precursors accumulated in vacuoles. The general question arises whether the deterrent function is exclusively provided by the aglycones or whether the precursors also have a protective effect in themselves, as has been reported for bitter-tasting cyanogenic glycosides (Nahrstedt, 1985).

Cyanogenesis is a two-step mechanism in which cyanide-containing precursors are cleaved by specific β -glucosidases. The resulting α -hydroxynitriles are unstable and decompose spontaneously, or enzymatically, in the presence of α -hydroxynitrile lyase to yield hydrogen cyanide (HCN) and an aldehyde or a ketone. It is, thus, necessary to consider the effect on oviposition choices of both the release of hydrogen cyanide and the content of cyanogenic precursors to understand the role of host plant cyanogenic features in this ecologically important interaction. In lima bean, both factors underlie high quantitative variability (Baudoin et al., 1991; Ballhorn et al., 2005).

We used Mexican bean beetle, which represents an herbivore specialized on members of the Fabaceae. However, the insects show strong preference for cyanogenic lima bean [*Phaseolus lunatus* (Dover et al., 1988)], and cyanogenic glycosides act as a feeding stimulant for the beetles (Lapidus et al., 1963) indicating some specialization to this host plant. In choice experiments with intact lima bean plants characterized by cyanogenic properties, we addressed effects of cyanide release and cyanogenic precursor concentration on oviposition preference of female beetles.

Methods and Materials

Plants

Lima beans and snap beans were cultivated under greenhouse conditions adjusted to 25:20°C in a 16-hr light/8-hr dark period and a relative humidity of 60–70%. Lima beans were grown from seeds obtained from one mother plant.

Cyanogenic Features of Plants

Cyanogenic capacity (HCN_c) was measured in an airflow apparatus according to Ballhorn et al. (2005) using chloroform as an inductive agent for release of gaseous hydrogen cyanide from leaves. By this treatment, the plant's general capacity for cyanogenesis can be quantified. Cyanogenic potential (HCN_p) was measured by complete enzymatic degradation of cyanogenic precursors and quantitative measurement of released HCN (Ballhorn et al., 2005).

Beetles

Beetles were reared on noncyanogenic snap bean (*Phaseolus vulgaris* var. Saxa) under the same ambient conditions as plants. Snap bean represents another important host plant species of the beetle (Dover et al., 1988). Any possible adaptations to particular lima bean accessions were excluded by rearing beetles on snap beans. Beetles that emerged within 3 d were separated from the maintenance culture. Four days after they had started mating, females were removed. In parallel experimental settings, eight female beetles were placed

in cages (fabric tubes, 70 cm diam) containing four 30-d-old HC, LC⁽⁺⁾, or LC plants of different accessions (replications per combination of plants, $N = 4$). Order of placement of the four plants in each cage was random to eliminate any possible positional effects for oviposition. Plants were exchanged every 3 d, and the egg clutches on the leaves of the removed plants were counted. Oviposition of beetles was observed over a total period of 30 d.

Statistics

Effects of cyanogenic capacity on beetle oviposition choice and assignment of lima beans to homogeneous groups based on different cyanogenic capacity were carried out by application of a *post hoc* test [Tukey's honestly significant difference (HSD) test] after one-way analysis of variance (ANOVA) to the data set (Statistica 7.0).

Results

We found significant differences in HCNc and HCNp among the plants. Based on the differences in HCNc, plants were assigned to a high HCN-releasing group (HC) with also high HCNp and to a low HCN-releasing group (LC) with low HCNp. Accessions 2116 showed low HCNc under high HCNp (LC⁽⁺⁾). *P. vulgaris* cv. Saxa showed no cyanogenic precursors, and thus no ability to release gaseous HCN (Table 1). Screening of *P. lunatus* leaf material for nutritive factors, such as protein content and total phenolics, revealed, in contrast to cyanogenic variability, no significant differences among the accessions (unpublished data).

Each female beetle usually produced three egg clutches. Clutches were positioned on the undersurface of leaves in intervals of 8–9 d. The oviposition behavior of beetles was

Table 1 Cyanogenic features of lima beans (*Phaseolus lunatus*)

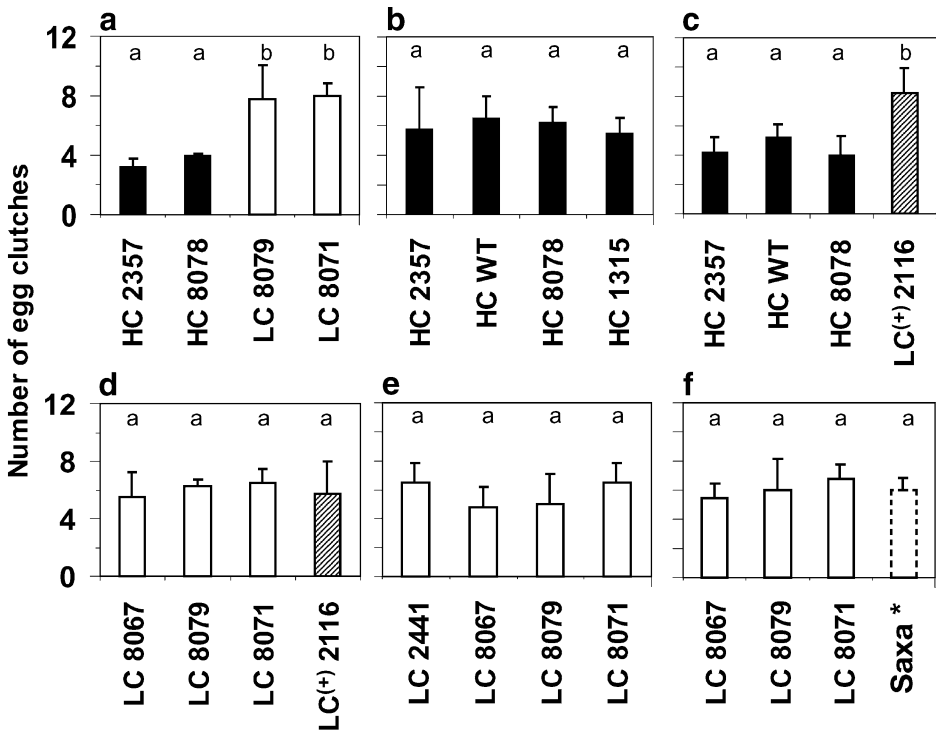
Accessions of <i>P. lunatus</i>	Cyanogenic status: high (HC) or low (LC)	Cyanogenic capacity (HCNc; $\mu\text{mol HCN g fw}^{-1} \text{ min}^{-1}$)	Cyanogenic potential (HCNp; $\mu\text{mol HCN g fw}^{-1}$)
2357	HC	0.58 ± 0.07^a	68.36 ± 14.82^{ab}
2233 (WT) ¹	HC	0.52 ± 0.07^a	64.72 ± 11.38^{ab}
8078	HC	0.56 ± 0.05^a	61.81 ± 5.99^{ab}
1315	HC	0.49 ± 0.08^a	55.92 ± 11.44^b
8067	LC	0.17 ± 0.08^b	18.54 ± 4.23^c
8079	LC	0.16 ± 0.04^{bc}	9.29 ± 3.20^{cd}
8071	LC	0.16 ± 0.03^{bc}	7.31 ± 2.09^{cd}
2116 ²	LC ⁽⁺⁾	0.13 ± 0.03^{bc}	76.53 ± 15.30^a
2441	LC	0.08 ± 0.01^{cd}	19.80 ± 4.84^c
Saxa ³	Noncyanogenic	0.00 ± 0.00^d	0.00 ± 0.00^d

Young leaves of the lima bean accessions were screened for their cyanogenic characteristics. Values shown for HCNc and HCNp are means \pm SD ($N = 12$ individual plants per accession). Significant differences were calculated by a *post hoc* test (Tukey's HSD; $P < 0.001$) after one-way ANOVA and are indicated by different letters.

¹ Accession 2233 represents lima bean, *P. lunatus*, wild type (WT) with small seeds, and small, relatively hard textured leaves.

² Accession 2116 showed low release of gaseous HCN under high concentrations of cyanogenic precursors.

³ Saxa represents *P. vulgaris* cv. Saxa.



Lima bean accessions with different cyanogenic capacity

Fig. 1 Oviposition choice of beetles. Shown is the number of egg clutches (mean \pm SD, $N = 4$ per choice experiment) deposited by eight females on leaves of lima beans from accessions with different cyanogenic features (HC \blacksquare : high HCN-releasing plants; LC \square : low HCN-releasing plants; LC⁽⁺⁾ \square : low HCN-releasing plants caused by low activity of endogenous β -glucosidase under high concentration of cyanogenic precursors). Plants were assigned to groups based on significant differences in cyanogenic capacity (HCN; see Table 1). Oviposition preference of beetles was analyzed in parallel experimental settings comprising different combinations of plants (a–f). Saxa* represents noncyanogenic *Phaseolus vulgaris* cv. Saxa. Different small-typed letters on top of the figure represent significant differences at $P < 0.001$ (*post hoc* test after one-way ANOVA; Tukey's HSD)

affected by the cyanogenic capacity of the lima beans. Beetles showed significant preference for LC plants compared to HC plants (Fig. 1a). In addition, LC⁽⁺⁾ plants were preferred for oviposition over HC plants (Fig. 1c), but they were not significantly refused compared to the LC plants (Fig. 1d). Female beetles that were kept exclusively on HC or LC plants showed no preference for single accessions (Fig. 1b, e). Choice experiments including noncyanogenic *P. vulgaris* plants revealed similar preference of beetles for LC plants compared to *P. vulgaris* (Fig. 1f).

Discussion

The results show that the Mexican bean beetle, although preferring to feed on lima bean (Dover et al., 1988), avoids high HCN-releasing varieties of its host plant. It was the

cyanogenic capacity that affected female oviposition choice. Beetles were repelled by high cyanogenic capacity, whereas high concentrations of cyanogenic precursors did not result in reduced oviposition.

Release of HCN is the result of cell damage. Thus, insect feeding on leaves is required as a precondition for repellent activity. In contrast to other defenses, which can be triggered by oviposition (Hilker et al., 2002), deposition of eggs on plant surfaces *per se* has no ability to initiate cyanogenesis.

Considering quantitative aspects, beetles did not differentiate between lima beans showing low capacity for release of HCN and snap beans, which did not contain any cyanogenic precursors at all. This finding indicates that cyanogenic capacity at lower concentrations provides no repellent activity against oviposition by female beetles. Both a role of cyanogenic capacity in female choice behavior as well as a homogeneous food quality of lima bean are supported by the finding that female beetles that were kept exclusively on HC or LC plants showed no oviposition preference for single accessions.

In general, nutritive traits have a strong impact on host plant quality and affect female oviposition preference (Raubenheimer and Simpson, 1994). However, substantial differences in host plant quality can be excluded here because we found contents of protein and total phenolics in leaves among the accessions to be homogenous (data not shown).

Taken together, the results demonstrate that oviposition choice of Mexican bean beetles depends on the cyanogenic capacity of host plants at high thresholds.

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Introduction

Volatile organic compounds (VOCs) are commonly found to function as infochemicals in terrestrial ecosystems (e.g., Metcalf, 1987; Kessler and Baldwin, 2001). However, in aquatic ecosystems, they have only received attention in the limited context of water quality issues: VOCs produced by algae and cyanobacteria frequently cause unpleasant odors in source water and are a nuisance in water treatment, especially for drinking water. Important for most consumer complaints are the earthy–musty odor compounds geosmin and 2-methylisoborneol (Watson and Ridal, 2004). Planktonic (Watson, 2003) and benthic cyanobacteria (Izaguirre and Taylor, 1995) are the major sources of both compounds. Numerous other VOCs belonging to lipoxygenase, carotene oxygenase, and fermentation products have been detected in lake and river water (Jüttner, 1995). The large number of compounds described for fresh water contrasts with the sparse studies on their ecological functions.

Some possible biological functions of VOCs in aquatic ecosystems are becoming clear (Watson, 2003). Volatile aldehydes might play a role in an activated defense mechanism of marine diatoms (Miralto, et al., 1999). Wound-activated diatom cells release α -, β -, γ -, δ -unsaturated aldehydes (Pohnert, 2000; Pohnert et al., 2002) that drastically lower the hatching rate of copepod eggs and might serve as a defense at the population level (Ianora et al., 2004). An alternative role of polyunsaturated aldehydes is serving as repellents for crustacean grazers (Jüttner, 2005).

Aquatic systems are ideally suited for communication via chemical cues because infochemicals can be easily distributed in concentrations sufficient for a response (Wisenden 2000). In highly structured benthic ecosystems, VOCs remain more localized than in planktonic ecosystems and may form robust microzones (Blackburn et al., 1998). Infochemicals related to predation (von Elert and Loose, 1996), reproduction (Müller et al., 1971), and foraging (Thomas et al., 1980) are perceived by many groups of aquatic organisms, which use this information to optimize their fitness. Indeed, VOCs function as habitat-finding cues for both aquatic insects (Evans, 1982) and nematodes (Höckelmann et al., 2004).

In littoral marine and freshwater ecosystems, herbivorous gastropods are of major importance because they structure the algal community by strong top–down predation pressure; they also form an important link to higher trophic levels because they are preyed upon by both vertebrate (fish, birds) and invertebrate predators, e.g., crayfish (Turner et al., 2000). Gastropods generally lack good vision, and cannot easily locate food patches visually (Gal et al., 2004). Their locomotion is energetically demanding, primarily owing to the production of pedal mucus (Denny, 1980). Therefore, it should be adaptive for snails to rely on chemical cues to minimize these costs by directed chemotaxis toward potential food sources.

Chemical stimuli affect the behavior of gastropods, and aquatic snails use their sensitivity to chemical cues as a principal modality to detect distant objects in the environment (Croll, 1983). The osphradium, located in the mantle cavity, is considered to be the major chemosensory organ of freshwater gastropods, although it might also be involved in other sensory processes such as the detection of ambient CO₂ pressure (Wedemeyer and Schild, 1995). However, to date, it has not been investigated whether long-range chemical cues are involved in observed food preferences, which have been quantified only either as residence time on a particular food patch or as the amount of food consumed (e.g., Madsen, 1992; Brendelberger, 1995; Wakefield and Murray, 1998). Snail chemotaxis toward dissolved sugars, amino acids, and carboxylic acids has been determined only in the context of control mechanisms for snails that are intermediate hosts

for parasites (Thomas et al., 1980; Thomas, 1986). Therefore, it remains unclear how these dissolved chemical cues are related to the process of finding food.

Volatile organic compounds released from benthic algae are suitable for dispersion over distance, and are good predictors of the presence of algal food. We hypothesize that freshwater gastropods utilize algal VOCs as foraging kairomones (sensu Ruther et al., 2002) for finding food. To investigate this hypothesis, we first identified the VOCs released upon cell disintegration from the filamentous benthic green alga *Ulothrix fimbriata* (Bold). *Ulothrix* is a cosmopolitan genus commonly occurring in periphyton communities of marine and fresh waters (John, 2002). In a newly developed standardized assay, we then investigated the behavioral response of the common pulmonate snail *Radix ovata* (Draparnaud) to these VOCs to see if they could be utilized as food-finding infochemicals by the snails. We deliberately used snails naïve to *Ulothrix* odors to distinguish between genetically fixed food-locating abilities and preferences acquired by olfactory learning.

Methods and Materials

Cultures

An axenic culture of the filamentous benthic green alga *U. fimbriata* (Bold) SAG 36.86 (Chlorophyceae) was obtained from the Göttingen Algal Culture Collection, Germany. The alga was cultivated semicontinuously as a suspension at a dilution rate of 0.25 d^{-1} in WC medium (Guillard and Lorenzen, 1972) at a constant temperature of 20°C and a light intensity of 1×10^{16} quanta $\text{s}^{-1}\text{ cm}^{-2}$. The culture was aerated by heavy bubbling with sterile, compressed air to prevent sinking and attachment of the benthic algae to the bottom of the culture flasks. Algae were harvested, concentrated by centrifugation at $4000 \times g$, and resuspended in filtered ($0.45\text{ }\mu\text{m}$) Lake Constance water. In the resulting food suspensions, carbon concentrations were adjusted to 0.5 mg particulate organic carbon per milliliter by using photometric light extinction at 800 nm and carbon extinction regressions previously determined (P. Fink, unpublished data). Juvenile *R. ovata* (Draparnaud) snails with shell lengths of $5\text{--}10\text{ mm}$ were collected during the summer in the littoral zone of Lake Constance, and acclimatized in the laboratory (at 20°C under constant dim light) before use in the food choice experiments. During the acclimation period, snails were fed Tetra PlecoMin[®] fish food pellets (Tetra, Melle, Germany). Before the experiments, animals were moderately starved for 24 hr by placing them in filtered ($0.45\text{ }\mu\text{m}$) Lake Constance water without food to increase their food-searching activity.

VOC Analyses

To standardize VOC extraction, algal biomass equivalent to 10 mg particulate organic carbon was disintegrated by a freeze–thaw cycle in 40 ml ultrapure water. After addition of 25% NaCl, VOCs were extracted by closed-loop stripping for 45 min and sorbed onto Tenax TA (Supelco) as described by Jüttner (1988b). VOCs were then thermally desorbed from Tenax TA and directly transferred onto a capillary column (DB 1301, 30-m length, 0.32 mm i.d., J&W Scientific, Folsom, CA, USA) of a combined gas chromatograph–mass spectrometer (Thermo/Finnigan GCQ). Helium was used as the transfer and carrier gas. VOCs produced by *U. fimbriata* were separated with the temperature program 4 min at 0°C , $5^{\circ}\text{C min}^{-1}$ to 250°C , and 10 min at 250°C , and identified by comparing the retention times and mass spectra (EI at 70 eV) with those of reference compounds (Aldrich). The

major VOCs identified were quantified as described by Jüttner (1988b) by using the peak areas of characteristic mass fragments and a calibration curve for each compound. 3-Hexanone was chosen as the internal standard because it did not occur in *U. fimbriata* and exhibited intermediate volatility with respect to the VOCs of *U. fimbriata*.

Extraction of VOCs for Food Choice Assays

Volatile organic compounds adsorbed onto Tenax TA (see previous section) were eluted with 5 ml diethyl ether. The ether was gently evaporated to dryness with nitrogen gas, and the residue was immediately dissolved in 100 μ l of ethanol. Samples were stored in gas-tight vials at -20°C for <48 hr to avoid loss. Before the food choice assay, 7 ml filtered (0.45 μ m) Lake Constance water was added to each sample, and this solution was added to the containers placed in the aquarium (see below).

As a control, 40 ml of ultrapure water were stripped with 25% NaCl onto Tenax TA, eluted with diethyl ether, dried, and dissolved in ethanol. In another control, WC medium aerated for several days instead of ultrapure water was analyzed to exclude possible effects of contaminants introduced during cultivation.

Synthetic VOC Mixtures

Reference compounds (Aldrich) identified as the major VOCs of *U. fimbriata* were mixed to approximately match the concentrations in the algal VOC extract (Table 1). However, the (*Z*)-isomer of 2-pentenal and the (*E,Z*)-isomer of 2,4-heptadienal were not available. Thus, only the (*E*)- and (*E,E*)-isomers were used in the complete VOC mixture (Table 1). To resolve whether the attractant activity of *U. fimbriata* VOCs was dependent on specific substances or rather on multiple compounds, 2(*E*),4(*E*)-heptadienal and a mixture of the three C_5 compounds 1-penten-3-one, 1-penten-3-ol, and 2(*E*)-pentenal (C_5 mixture; Table 1) were also tested in the assay.

Food Choice Assays

The food choice assay was specifically designed to separate chemotaxis, mediated through foraging kairomones, from food preference resulting from taste-receptor-mediated effects on patch residence time. Therefore, it was important to offer the food source in a way that allowed for the release and detection of foraging kairomones, but prevented the experimental animals from accessing the food itself. The food choice assays were performed in an aquarium (320 \times 170 mm, 180 mm deep, total volume 10 l) in a climate-controlled room at 20°C . The aquarium was filled with 1 l of filtered (0.45 μ m)

Table 1 Composition of the synthetic VOC mixtures offered to *R. ovata* in the food choice assays

VOC	Supplier and product no.	Complete mixture ^a (μ g per container)	C_5 mixture (μ g per container)	C_7 compound (μ g per container)
1-Penten-3-one	Aldrich E5,130-9	85	85	–
1-Penten-3-ol	Aldrich P860-2	84	84	–
2(<i>E</i>)-Pentenal	Aldrich 26,925-5	86	86	–
2(<i>E</i>),4(<i>E</i>)-Heptadienal	Aldrich 18,054-8	0.9	–	0.9

^a The complete VOC mixture was designed to mimic the VOCs of *U. fimbriata*.

Lake Constance water. Special containers were designed to allow the introduction of algae and samples of extracted VOCs in water without disturbing the water body (Fig. 1a,b). The containers were modifications of the olfactometers described by Thomas et al. (1980) and consisted of two cylindrical Plexiglas® rings with radial bores (5 mm diam.) near the bottom side of the ring. The inner ring (40 mm diam) was closed at the bottom by gluing a circular Plexiglas® plate to the opening. The inner ring fitted exactly into the outer ring (Fig. 1a,b). Hence, the container could be opened by rotating the outer ring, thereby matching up the bores of the outer and inner rings (Fig. 1a). Likewise, the container could be closed by rotating the outer ring so that the bores did not match up; closing the container stopped the exchange of substances between the inside and the outside of the container (Fig. 1b). One container for the test extract was placed into the aquarium 10.5 cm from the center, and another container for the control extract was placed at the opposite end of the aquarium 10.5 cm from the center. Each container received 7 ml of sample in filtered lake water at the same temperature as the surroundings; the inner and outer water levels were the same. The water level was higher than the radial bores of the containers and lower than the opening at the top of the containers. The top of each container was covered with a circular glass plate. The assay was initiated by rotating the outer rings to open the containers, and introducing five juvenile *R. ovata* at the center of the aquarium. The relative distance (± 1 cm) of each individual snail to both containers was recorded every minute for a total time period of 40 min. The initial scoring value for each snail at the beginning of the experiment (equal distance to both containers) was set at 0; scoring values ranged from -21 (closest to the control) to $+21$ (closest to the test sample). After opening the containers containing samples, the dissolved substances could leave the containers through the bores. This was verified by analyzing the aquarium water 30 min after opening a container containing 1-penten-3-one: 16.6% of the compound ($1.7 \mu\text{g l}^{-1}$) were released into the surrounding water body of the aquarium within half an hour.

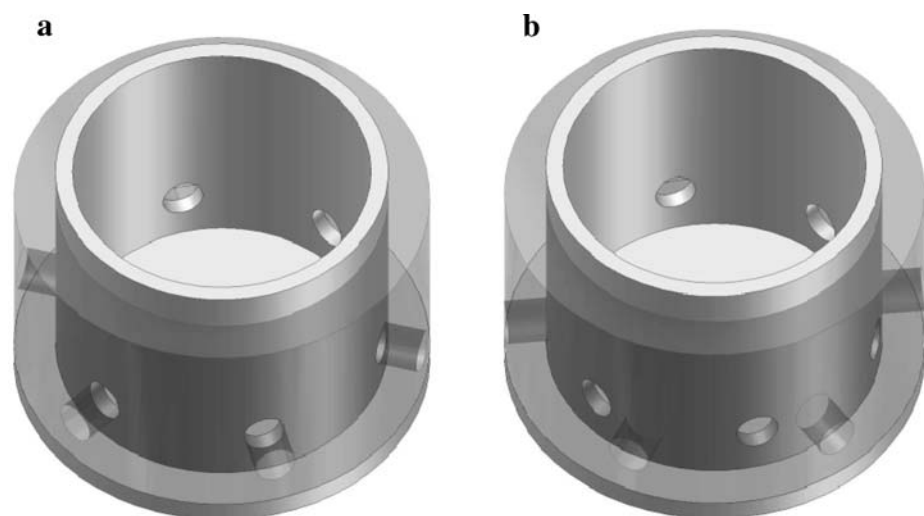


Fig. 1 Schematic drawing of the containers used in the food choice assays. (a) Open position. (b) Closed position

Statistical Analyses

Because the five individual snails in each experiment were not independent from each other, the mean of their distribution was calculated for every reading at intervals of 1 min. These mean values were plotted against the reading time and treated as one replicate experiment. All experiments were repeated at least five times, resulting in experimental series with $N = 5$ –10 for each treatment. Between replicate experiments, the sides of the test and control containers were exchanged to exclude directional effects introduced by the experimental setup. In a series of control experiments ($N = 14$), both containers were filled with filtered lake water. The distribution of the snails in this control series was tested against the distribution of the snails in the test series, in which one container contained the test sample and the other contained a control sample. Data were tested to meet assumptions for analysis of variance (ANOVA; normal distribution, homogeneity of variance, Levene's test) and, subsequently, results were compared by using repeated-measurement ANOVA and the GLM module of Statistica[®] v.6 software package (StatSoft, Inc., 2004) and a significance level of $\alpha = 0.05$.

Results

Analysis of VOCs Released by *U. fimbriata*

Although intact *U. fimbriata* cells did not release any detectable VOCs into the surrounding water, freeze-thawed *U. fimbriata* cells released a variety of VOCs (Table 2). The most dominant group detected was lipoxygenase products released upon cleavage of polyunsaturated fatty acids, e.g., 1-penten-3-one, 1-penten-3-ol, (*Z*)-2-pentenal, (*E*)-2-pentenal, (*E,Z*)-2,4-heptadienal, and (*E,E*)-2,4-heptadienal. Minor compounds were the nor-carotenoids 6-methyl-5-hepten-2-one, α -ionone, β -ionone, and β -cyclocitral (Table 2). These minor compounds resulted in peak areas of <10% of the peak area of the major compounds and were not quantified in detail. We determined the concentrations of the major VOCs released by *U. fimbriata* upon cell disintegration (Table 3). The C_5 compounds 1-penten-3-one, 1-penten-3-

Table 2 VOCS liberated from *U. fimbriata* upon freeze-thawing

Compound group	VOC	M (g mol ⁻¹)	Rt (min)
Fatty acid pathway	1-Penten-3-one	84	10.03
	1-Penten-3-ol	86	10.71
	2(<i>Z</i>)-Pentenal	84	12.44
	2(<i>E</i>)-Pentenal	84	12.83
	2(<i>E</i>),4(<i>Z</i>) -Heptadienal	110	21.62
	2(<i>E</i>),4(<i>E</i>) -Heptadienal	110	22.06
	Nonanal	142	24.35
	Pentadecane	212	34.12
Isoprenoid pathway	6-Methyl-5-hepten-2-one	126	21.15
	β -Cyclocitral	152	27.35
	Geranylacetone	194	33.59
	α -Ionone	192	34.41
	β -Ionone	192	35.08

^a Compounds are given with their molecular mass (M), grouped by their biosynthetic pathways, and sorted by retention time (Rt).

ol, and (*Z*)-2-pentenal were the dominant compounds, e.g., at concentrations of up to 0.9 µg per mg algal carbon (1-penten-3-ol). (*E*)-2-pentenal, (*E,Z*)-2,4-heptadienal, and (*E,E*)-2,4-heptadienal were released at concentrations 10.4–18.7% of that of 1-penten-3-ol (Table 3).

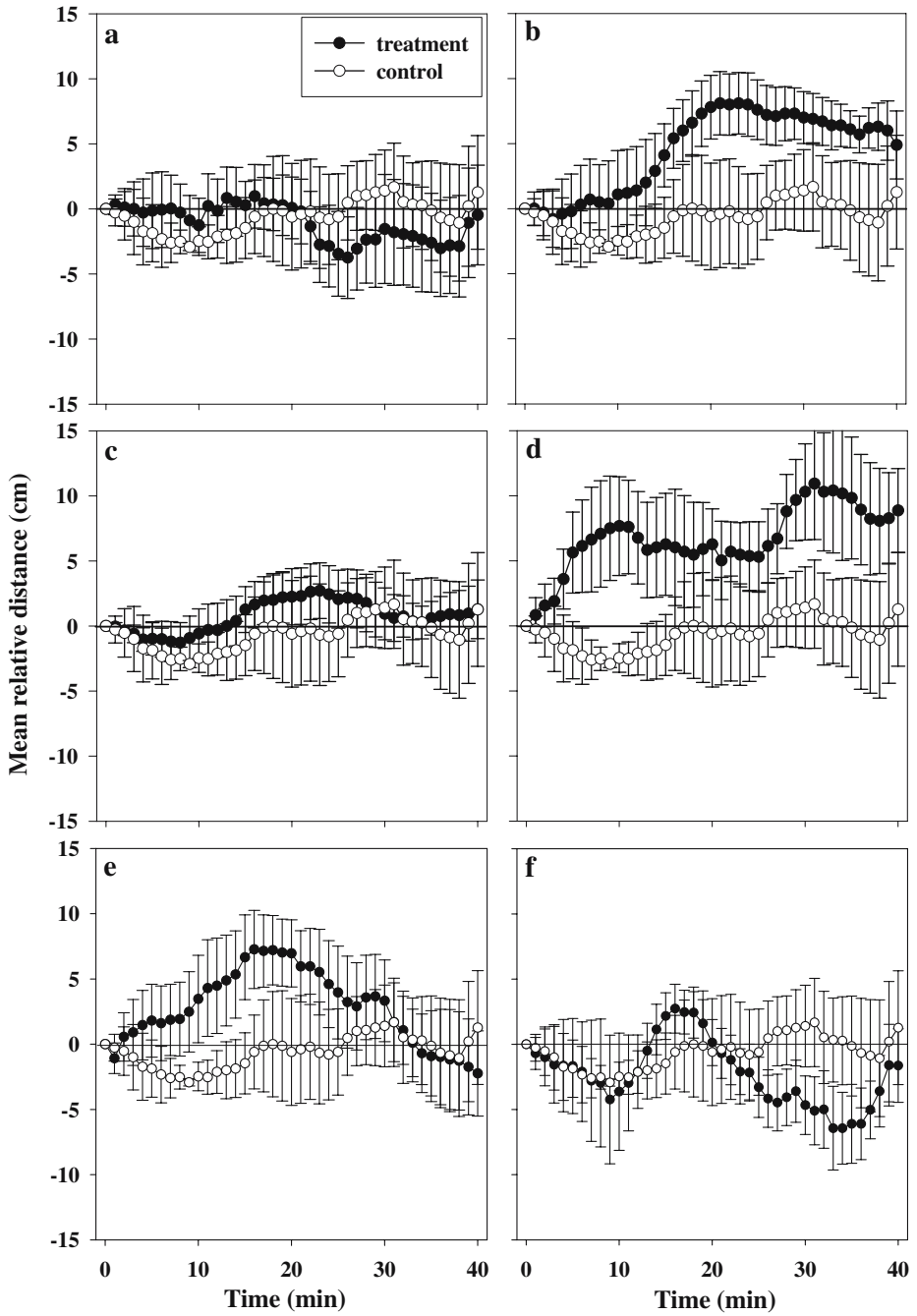
Food Choice Experiments with *R. ovata*

The results obtained in food choice experiments with intact and damaged *U. fimbriata* cells offered to *R. ovata* corroborated the hypothesized VOC release mechanism and the results of the VOC analysis: When a suspension of intact *U. fimbriata* cells was added to the test container and filtered lake water was added to the other container, juvenile *R. ovata* did not show any positive or negative chemotactic response to the algae (Fig. 2a; Table 4). Because intact algal cells did not release VOCs, the results indicate that the snails did not respond chemotactically to any other potential chemical gradient formed by the algae. Similarly, no response occurred when the supernatant of an exponentially growing culture of *U. fimbriata* was placed in the test container (Table 4), which indicated that exudates of actively growing cells do not evoke a chemotactic response. However, a positive chemotactic response was observed when VOCs released from the same amount of biomass of disintegrated *U. fimbriata* cells (trapped and eluted from Tenax TA), were placed in the test container; that is, the snails preferred the VOCs over the control (Fig. 2b; Table 4). This attraction was not caused by possible contaminants introduced during cultivation of the algae or by the VOC extraction process, because an equivalent VOC extract of aerated, sterile culture medium without algae was not preferred over the control (Fig. 2c; Table 4). Hence, the attraction of the snails clearly depended on the VOCs released from *U. fimbriata*. As expected from the results of VOC analysis, *R. ovata* preferred a synthetic mixture of pure C₅ and C₇ compounds designed to mimic the bouquet of released algal VOCs (Table 1) over the solvent control (Fig. 2d; Table 4). To determine whether *R. ovata* was attracted to the C₅ compounds or the C₇ compound or all, either a mixture of the three C₅ compounds or the C₇ compound 2(*E*),4(*E*)-heptadienal was offered (Table 1). Neither the mixture of C₅ compounds nor 2(*E*),4(*E*)-heptadienal alone significantly attracted *R. ovata* (Fig. 2e,f; Table 4). The results of the assays indicated that a mixture of C₅ and C₇ VOCs was needed to attract *R. ovata*, rather than a mixture of the C₅ compounds or 2(*E*),4(*E*)-heptadienal alone.

Table 3 Quantities of the six major compounds in the VOCS bouquet of *U. fimbriata*

VOC	Rt (min)	Release (ng mg POC ⁻¹)	Food choice assay (µg per container)
1-Penten-3-one	10.03	536	5.4
1-Penten-3-ol	10.71	924	9.2
2(<i>Z</i>)-Pentenal	12.44	488	4.9
2(<i>E</i>)-Pentenal	12.83	173	1.7
2(<i>E</i>),4(<i>Z</i>)-Heptadienal	21.62	170	1.7
2(<i>E</i>),4(<i>E</i>)-Heptadienal	22.06	97	1.0

^a Compounds are sorted by retention time (Rt). The amount released is given as mean of *N* = 3 replicate measurements as nanograms of compound per milligram of algal particulate organic carbon (POC). In the food choice assays, the indicated amount of VOCs extracted from 10 mg algal carbon was added to the container. As the (*Z*)-isomer of 2-pentenal and the (*E,Z*)-isomer of 2,4-heptadienal were not available, the calibration for these compounds was done using the signal strengths of the respective (*E*)- and (*E,E*)-isomers.



Discussion

Although diatoms (e.g., Pohnert and Boland, 1996; Wendel and Jüttner, 1996; Jüttner and Dürst, 1997) and especially cyanobacteria (Jüttner et al., 1983; Jüttner 1987; Watson and Ridal, 2004) have been the subjects of numerous studies of VOC production, the ecologically important group of green algae has been widely neglected in such studies. Green algae are phylogenetically related to higher plants, and therefore might be expected to produce similar VOCs. The so-called green leaf volatiles (GLVs) of terrestrial plants consist mainly of C₆ compounds (Croft et al., 1993) and play an important role in herbivory-activated defense mechanisms (e.g., Kessler and Baldwin, 2001; Halitschke et al., 2004). The VOCs released from the benthic green alga *U. fimbriata* upon cell disintegration differed from the GLVs released by terrestrial plants (Table 2). As in higher plants, lipoxygenase products were the major reaction products. Such products arise from the degradation of polyunsaturated fatty acids (Pohnert, 2002), but C₆ compounds, such as (*E*)-2-hexenal and (*Z*)-3-hexenal, which are primarily found in GLV mixtures from leaves, could not be unambiguously identified and, therefore, are not included in Table 2. The peaks assigned to the C₆ compounds in our study were small compared with the peaks of the C₅ and C₇ compounds; therefore, these C₆ compounds are not major VOCs in *U. fimbriata*. The position specificity of lipoxygenase/hydroperoxide lyases in *U. fimbriata* differed markedly from that of higher plants. In addition to VOCs resulting from fatty acid degradation, a variety of volatile nor-carotenoids were detected that are degradation products of carotenoids (Jüttner, 1988a; Simkin et al., 2004). Among others, β -ionone was found, which is an important component of flower odors (e.g., *Viola* sp.) and was shown to be a repellent for the freshwater nematode *Bursilla monohystera* (Höckelmann et al., 2004).

Using our newly developed assay system, we showed that *R. ovata* significantly preferred VOCs liberated from damaged *U. fimbriata* cells over control extracts. Cell damage was necessary for the liberation of the infochemicals perceived by the snails because neither undamaged algae nor a culture supernatant of exponentially growing *U. fimbriata* led to a chemotactic response. These results are in keeping with current ideas on the liberation of VOCs from algal phospholipids via rapid enzymatic degradation (Jüttner, 2001). The enzyme cascade is thought to start with a wound-activated lipase that cleaves algal lipids and releases polyunsaturated free fatty acids. These free fatty acids, which can be potent toxins for benthic herbivores (Jüttner, 2001), are in part rapidly oxygenated by a lipoxygenase that introduces dioxygen into the fatty acid molecule (Gardner, 1991). The hydroperoxides obtained are cleaved by specific lyases into a volatile compound and a nonvolatile short-chain oxo-fatty acid (Pohnert, 2002). Thus, cell damage seems to be a prerequisite for the formation of volatile oxylipins in filamentous green algae.

We unequivocally demonstrated that VOCs and not other compounds released upon cell lysis were responsible for the observed attractant activity, as the eluate of the Tenax adsorbent loaded with *U. fimbriata* volatiles was clearly preferred over a control eluate.

◀**Fig. 2** Mean relative distance (\pm S.E.) of the snails from the two containers in the food choice assays. One container with the test extract was placed into the aquarium 10.5 cm from the center (scoring value +21) and another container with a control extract was placed at the opposite end of the aquarium 10.5 cm from the center (scoring value -21). Test extracts (●): (A) undamaged *U. fimbriata* cells ($N = 7$). (B) VOC extract from disintegrated *U. fimbriata* cells ($N = 5$). (C) VOCs extracted from aerated algal medium ($N = 10$). (D) Synthetic complete VOC mixture containing three C₅ compounds and 2(*E*),4(*E*)-heptadienal ($N = 6$). (E) Synthetic VOC mixture containing the three C₅ compounds ($N = 10$). (F) Synthetic 2(*E*),4(*E*)-heptadienal ($N = 6$). Results from a series of control experiments (both containers with filtered lake water) are plotted for comparison (○).

Table 4 Results of repeated-measurement analyses of variances on the mean positions of five juvenile *R. ovata* in the food choice experiments

	SS	df	F	P	
Intact cells^a					
Treatment	359.69	1	0.381	0.545	n.s.
Error	16,972.49	19			
Time	395.20	39	0.418	0.999	n.s.
Time × treatment	1106.98	39	1.172	0.221	n.s.
Error	16,998.87	702			
VOC extract^b					
Treatment	3959.27	1	4.575	0.047	*
Error	14,712.65	17			
Time	2019.12	39	3.205	<0.001	***
Time × treatment	1234.34	39	1.959	<0.001	***
Error	10,710.56	663			
Aer. medium^c					
Treatment	507.53	1	0.622	0.438	n.s.
Error	18,752.72	23			
Time	843.75	39	0.992	0.486	n.s.
Time × treatment	491.19	39	0.577	0.983	n.s.
Error	19,565.31	897			
Compl. mix.^d					
Treatment	9239.39	1	8.501	0.009	**
Error	19,564.60	18			
Time	1449.77	39	1.501	0.027	*
Time × treatment	956.60	39	0.991	0.488	n.s.
Error	17,381.63	702			
C₅ mix.^e					
Treatment	2715.27	1	2.185	0.154	n.s.
Error	27,344.71	22			
Time	1876.39	39	1.434	0.043	*
Time × treatment	2608.89	39	1.994	<0.001	***
Error	28,768.75	858			
C₇ compound^f					
Treatment	498.39	1	0.623	0.440	n.s.
Error	14,397.08	18			
Time	753.09	39	0.804	0.799	n.s.
Time × treatment	1908.12	39	2.037	<0.001	***
Error	16,863.48	702			
Supernatant^g					
Treatment	338.56	1	0.411	0.530	n.s.
Error	14,007.82	17			
Time	842.80	39	1.125	0.280	n.s.
Time × treatment	2341.34	39	3.125	<0.001	***
Error	12,738.88	663			

^a Choice between control and intact cells of *U. fimbriata* ($N = 7$).

^b Choice between control and VOC extract of *U. fimbriata* ($N = 5$).

^c Choice between control and VOC extract of aerated WC medium ($N = 11$).

^d Choice between control and the complete VOC mixture ($N = 6$).

^e Choice between control and the synthetic C₅ mixture ($N = 10$).

^f Choice between control and the synthetic C₇ compound ($N = 6$).

^g Choice between control and the supernatant of an *U. fimbriata* culture ($N = 5$).

^h All analyses compared the treatment (choice between a given test treatment and a control) with a series of control experiments ($N = 14$) in which snails had to choose between two containers with filtered lake water. Asterisks indicate significant differences at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***); n.s.: not significant.

Only volatile organic compounds adsorb to Tenax TA, so these compounds must have been responsible for the attractant activity of the eluate. This was further supported by the finding that a synthetic mixture of pure reference compounds designed to mimic the VOC bouquet of *U. fimbriata* was also highly attractive to *R. ovata*. Because a mixture of the three major C₅ VOCs and the major C₇ compound alone did not attract *R. ovata*, a multicomponent odor is required for the response of the snails. This type of response is remarkably similar to that of the benthic freshwater nematode *B. monohystera* (Höckelmann et al., 2004), which responds to a bouquet of cyanobacterial VOCs, but not to single compounds. In terrestrial systems, multi-component odors are also frequently more effective in eliciting responses in insects than single compounds (Metcalf, 1987). The sesquiterpene β -ionone was a minor constituent of the VOC bouquet released by *U. fimbriata*. It was not tested whether it has an effect on the behavior of *R. ovata* as has been observed for nematodes (Höckelmann et al., 2004). However, as the complete VOC bouquet extracted from *U. fimbriata* and the synthetic mixture of the four most abundant compounds tested showed similar attractant activity, we believe that β -ionone and other minor compounds contributed neither significant attractant nor repellent activity to the observed effect.

In some of the repeated measurement analyses, there was a significant effect of the factor time and/or significant time \times treatment interactions (Table 4). This is due to the fact that the response of the snails is neither immediate nor persistent. When the animals are introduced into the aquarium, they need a while to detect and respond to odor cues in their environment. Then, the behavioral response develops gradually, causing the effect of and the interaction with the factor time. In the assays without an attractant odor source, the snails start a so-called “random search” behavior (Streit, 1981), which leads to high fluctuations in the distribution of the animals, and causes the significant interaction terms with the factor time.

For the behavioral assays, we deliberately used the filamentous green alga *U. fimbriata*, as members of the genus *Ulothrix* are commonly found in lake periphyton assemblages (John, 2002), but do not occur in the summer periphyton of the lake where the snails were sampled (M. Kahlert, pers. comm.). Therefore, snails had probably not encountered the odor bouquet of *U. fimbriata* before. These bouquets seem to be species specific (Wendel and Jüttner, 1996; Höckelmann and Jüttner, 2004). This choice allowed us to focus exclusively on genetically fixed abilities for food location, as snails are known to be capable of learning to respond to stimuli they have previously encountered (Croll, 1983). For the terrestrial gastropod *Helix pomatia*, preconditioning to odors even seems to be essential for attraction responses (Teyke, 1995). Thus, snail food preferences are influenced by both innate factors and olfactory learning (Croll and Chase, 1980). Additionally, freshwater gastropods are able to adapt their digestive enzymes to optimally suit the digestion of the most abundant food source (Calow and Calow, 1975; Brendelberger, 1997). Hence, the response in natural systems could be much higher if olfactory learning and conditioning of the array of digestive enzymes play a role. The response observed might be a rather conservative estimate of the potential of VOCs to induce food-finding behavior.

Diatoms are known to produce polyunsaturated straight chain and cyclic hydrocarbons, aldehydes, and alcohols (Pohnert and Boland, 1996; Wendel and Jüttner, 1996), some of which function as pheromones in brown algae (Müller et al., 1971). This interaction can possibly be explained by the phylogenetic relationship between brown algae and diatoms (Pohnert and Boland, 1996). Recently, polyunsaturated aldehydes from diatoms have been shown to act as repellents for crustacean grazers (Jüttner, 2005). To our knowledge, no results have been published either on the possible role of eukaryotic VOCs in interspecific communication or on the VOCs released by (benthic) green algae. Especially green algae

have been largely neglected in the context of biogenic VOCs despite their considerable importance in the field (Stevenson et al., 1996).

The results of our study provide the first indications that VOCs from damaged green algae serve as a food-finding cue for freshwater benthic herbivores. Certainly, VOCs are not the only group of potential infochemicals liberated upon cell damage. Various other organic compounds, e.g., sugars, amino acids, and other short-chain carboxylic acids (e.g., propionic acid and butyric acid), have been described as attractants (Thomas et al., 1980; Thomas, 1986) and feeding stimulants (Thomas et al., 1986, 1989) for (tropical) freshwater snails. Some of these might also play a role in the chemical orientation of gastropod species in temperate latitudes. However, *R. ovata* was not attracted to butyric acid and (chironomid) carrion (P. Fink, unpublished results), probably because *R. ovata* feeds almost exclusively on periphyton and to a lesser degree on detritus (Calow, 1970; Lodge, 1986), and usually not on dead animal tissue that could release significant amounts of carboxylic acids and amino acids. Furthermore, in the heterogeneous benthic environment, many organisms and processes probably release such substances that do not necessarily indicate a food source to *R. ovata*. Algal VOCs are distinguishable from such diverse sources, and might, therefore, be the more appropriate food-finding signal for *R. ovata*. The effectiveness of VOCs as foraging kairomones is further supported by our observation that the concentrations sufficient for a chemotactic response of the snails are about an order of magnitude lower than the effective minimal concentrations of dissolved amino acids (Thomas et al., 1983). This difference is not surprising because, from terrestrial systems, it is known that the detection limit of invertebrates for volatile infochemicals is remarkably low (Harborne, 1995).

In terrestrial plant–herbivore interactions, it has repeatedly been shown that the liberation of volatile lipoxygenase products requires the damage of cells to trigger the enzyme cascade responsible for the release of VOCs. Apart from herbivory (Kessler and Baldwin, 2001), pathogens and senescence (Batten et al., 1995) are known to lead to the liberation of volatile lipoxygenase products. The release of volatile infochemicals from benthic algae under natural conditions can be caused by a variety of mechanisms. In laboratory experiments, intact algal cells do not release any lipoxygenase products (Pohnert, 2000; Jüttner, 2001; this study). However, this is a rather artificial situation, as natural periphyton communities are subject to a constant and high turnover of energy, nutrient, and biomass (Lamberti et al., 1995; Steinman et al., 1995), which contributes to their high productivity and ecological importance for littoral food webs (Klumpp et al., 1992; Pinckney and Zingmark, 1993). This turnover unavoidably involves damage and lysis of benthic algal cells. Thus, senescence and mechanical damage by hydrodynamic forces (Cattaneo, 1990; Watson and Ridal, 2004) and resultant release of VOCs into the water (Watson and Ridal, 2004) are likely to occur in natural environments. Furthermore, algal cells can become infected with parasitic fungi (Van Donk, 1989) or viruses (Reisser, 1993), resulting in increased cell lysis. Thus, a constant release of algal degradation products is expected in any natural biofilm community.

Another potentially important release factor is the grazing of herbivores. Similar to findings from terrestrial plant–herbivore interactions, Durrer et al. (1999) have shown that grazing by herbivorous cladocera on planktonic cyanobacteria leads to the release of significant amounts of VOCs in both the field and the laboratory. Likewise, when snails or other herbivorous invertebrates graze on an algal biofilm in the benthos, algal cells will rupture and release VOCs. Other snails would then be able to detect this food patch. A similar mechanism has been proposed for the mangrove snail *Terebralia palustris*, where active feeding of individual snails on leaf litter leads to the release of odor compounds and,

subsequently, to the attraction of conspecifics (Fratini et al., 2001). In this study, we found such an attraction using a widely distributed (ITIS, 2004) and often highly abundant snail species and a benthic green alga commonly found in lake littoral zones as model organisms. Probably, such a VOC-mediated attraction is not restricted to these species but rather represents a new, so far not investigated means of chemical communication that generally applies to gastropod–algae interactions. Such behavioral response of gastropods to volatile lipoxigenase products (or oxylipins; Pohnert et al., 2002) might also explain the frequently observed patchy occurrence of snails in littoral zones (Lodge, 1986; Klumpp et al., 1992; P. Fink, pers. obs.). The results reported here suggest that VOCs are not only important information-transmitting cues in terrestrial ecosystems (Metcalf, 1987), but also in (benthic) freshwater habitats.

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Introduction

Marine algae are defended from herbivory by a variety of secondary metabolites (Paul et al., 2001), including polyphenolics, acetogenins, terpenes, amino-acid-based and halogenated compounds, which can influence palatability. These metabolites deter feeding by marine herbivores in the field and in laboratory assays (Hay et al., 1987a, b; Van Alstyne et al., 2001; Van Alstyne and Houser, 2003). Because algal chemical defenses influence herbivore preference, and herbivores, such as sea urchins, can structure marine algal communities (Carpenter, 1986; Wright et al., 2005), chemical defenses indirectly structure algal populations and communities in coral reefs and other nearshore habitats.

Numerous green algae (Chlorophyta) are especially deterrent to coral reef herbivores. Rhizophytic algae in the order Bryopsidales, including *Caulerpa*, *Halimeda*, *Udotea*, *Penicillus*, and *Rhypocephalus* spp., are less palatable than other species commonly found in reef habitats (Paul and Hay, 1986; Wylie and Paul, 1988; Meyer et al., 1994). Their extracts deter feeding in artificial assays (Paul and Van Alstyne, 1992). Algae also produce higher concentrations or additional defensive compounds in areas subject to high herbivore pressure (Paul and Fenical, 1986). In addition, algae in the order Ulvales and Cladophorales, such as *Ulva*, *Enteromorpha*, and *Cladophora* spp., are less palatable than other algae in the field and in live algal and artificial feeding trials toward some temperate herbivores (Paul and Hay, 1986; Van Alstyne et al., 2001; Van Alstyne and Houser, 2003), although less is known about their secondary chemistry.

Some green algae use an activated defense system whereby damage, which could result from feeding, results in the conversion of a stored secondary metabolite, which may have biological activity, into a product with greater bioactivity (Paul and Van Alstyne, 1992). Two notable examples of activated defenses occur in the Bryopsidales, which commonly produce bioactive terpenoids (mainly sesquiterpenes and diterpenes). These are caulerpenyne from *Caulerpa* spp. and halimeda-tetraacetate from *Halimeda* spp., which, upon wounding, are transformed into the more toxic and deterrent oxytoxins and halimeda-trial, respectively (Paul and Van Alstyne, 1992; Cimino et al., 1990; Gavagnin et al., 1994; Jung and Pohnert, 2001). Another activated system used by some macroalgae involves the conversion of dimethylsulfoniopropionate (DMSP) into acrylic acid and dimethyl sulfide (DMS). Many genera of green macroalgae have high DMSP concentrations that deter feeding by marine herbivores (Van Alstyne et al., 2001; Van Alstyne and Houser, 2003). For instance, both conversion products deter feeding by temperate sea urchins at relatively low concentrations (Van Alstyne et al., 2001; Van Alstyne and Houser, 2003; Lyons et al., unpublished data).

This study tested the relative effectiveness of different types of defenses that are commonly found in green algae on feeding by a common tropical and subtropical reef herbivore, the rock-boring sea urchin *Echinometra lucunter*. Live algal feeding assays were conducted to assess palatability of green algae relative to co-occurring red and brown macroalgae, found in the Indian River Lagoon, and at offshore sites near Fort Pierce, Florida. In addition, relative palatability was tested among unpalatable green algae that may use different defense systems (caulerpenyne-related terpenoids vs. products from the activation of DMSP). Finally, artificial feeding assays were conducted, where algal extracts or isolated metabolites were incorporated into agar-based foods, to assess if trends in palatability were related to algal chemistry. This study is the first to examine how DMSP-related defenses influence feeding by a subtidal, subtropical herbivore, and to assess the relative susceptibility of this herbivore to components of these different systems.

Methods and Materials

Collection of Organisms

Algae used in live algal feeding assays were collected from Fort Pierce, Florida, USA, in the Indian River Lagoon (27°27.769'N, 80°19.291'W), offshore at Pepper Park (27°29.566'N, 80°17.796'W), and from the Smithsonian Marine Ecosystems Exhibit from August to October, 2003. Green algae included *Caulerpa prolifera*, *Caulerpa racemosa* var. *laetevirins*, *Caulerpa sertularioides*, *Cladophora* sp., *Codium taylorii*, *Halimeda discoidea*, and *Ulva lactuca*. Red algae included *Amphiroa fragilissima*, *Gracilaria caudata*, *Gracilaria cervicornis*, and *Gracilaria tikvahiae*. Brown algae included *Dictyopteris deliculata* and *Lobophora variegata*. The cyanobacterium *Lyngbya confervoides* was obtained from a bloom off Fort Lauderdale, Florida, in the fall of 2003 (26°16.408'N, 80°03.833'W). *G. tikvahiae* for artificial feeding assays was obtained from cultures maintained at Harbor Branch Oceanographic Institution by D. Hanisak. Sea urchins (*E. lucunter*) were obtained from a rocky sea wall in the Indian River Lagoon (27°27.769'N, 80°19.291'W). This species is found in reef communities and rocky shores throughout the Caribbean, from North Carolina south to Brazil, and on the Atlantic coast of Africa (Hendler et al., 1995).

Live Algal Feeding Assays

Multiple-choice feeding assays were conducted with live algae commonly found in reef and rocky habitats to gain perspective on how algae rank in palatability. Individual sea urchins ($N = 15\text{--}20$) were offered pieces of similar volumes of four to six algal species (see figures for species used in each experiment). *G. tikvahiae*, an abundant red drift alga readily consumed in preliminary live algal feeding assays, served as a positive control. Algae were weighed before and after feeding, and assays were stopped once an alga was completely consumed or after 70 hr. Paired sea urchin exclusion controls were run at the same time in each aquarium to account for changes in algal mass unrelated to feeding (Peterson and Renaud, 1989). Consumption of each species by each sea urchin was determined by using the formula $[T_i \times (C_f / C_i)] - T_f$, where T_i is the initial algal mass, T_f is the final algal mass, C_i is the initial control algal mass, and C_f is the final control algal mass. The amount of each algal species consumed was expressed as the percentage of the total algae consumed by an individual sea urchin (Lockwood, 1998). Sea urchins that consumed <10% or >90% of the total algal mass per aquarium⁻¹ were excluded from statistical analysis. Friedman's repeated-measures ANOVA and Student–Newman–Keuls multiple comparison test were used to identify significant differences in the percentage of total consumption among algae (Lockwood, 1998). Based on the results from the live algal feeding assays, additional assays were performed, comparing palatability among the green algae *U. lactuca*, *Cladophora* sp., and *C. prolifera* as described above.

Preparation of Algal Extracts

Freshly collected *U. lactuca* and *C. prolifera* were individually homogenized in solvent and extracted $\times 3$ in 1:1 ethyl acetate/methanol to yield a nonpolar extract and then $\times 2$ in 1:1 ethanol/distilled H₂O to yield a polar extract. It is possible that nonpolar extracts might contain some polar compounds that are partially soluble in methanol, and that some polar compounds were excluded from the polar extract due to the presence of 50% ethanol.

Extracts were filtered, dried by rotary evaporator, and stored at 4°C until used in feeding assays. There was not enough *Cladophora* sp. to perform extractions and use in feeding assays.

Artificial Feeding Assays with Algal Extracts

Methods were similar to those used by Hay et al. (1998). To make the artificial diet, 1 g agar was dissolved in 30 ml distilled H₂O and heated in a microwave. Two grams of dried, ground *G. tikvahiae* were added. Algal extracts were dissolved in 2 ml ethanol and incorporated into the food at natural concentrations on a dry weight basis. Ethanol (2 ml) also was added to the control foods. Artificial food with and without extracts was spread into a mold with parallel, rectangular wells over window screen, cooled, and cut into replicate strips containing one piece of each food type. Sea urchins were fed extract-free artificial food before feeding trials. For artificial assays, sea urchins ($N = 15\text{--}20$) were offered a strip of screen containing three pieces of food of equal size, one with nonpolar extract, one with polar extract, and a control piece without extract (placement of extract-containing and control food was randomized on strips among assays). Sea urchins were allowed to feed until half of the artificial food of one food type was consumed or until 48 hr passed. Preference was quantified as the number of window screen squares revealed after food was consumed. Sea urchins that did not eat or consumed all food were excluded from statistical analysis. Friedman's repeated-measures ANOVA and Student–Newman–Keuls multiple comparison test were used to identify significant differences in the number of squares consumed.

Measurement of DMSP in Green Algae

Tissue concentrations of DMSP in *U. lactuca*, *C. prolifera*, and *Cladophora* sp. were measured with methods similar to those described in Van Alstyne et al. (2001). Dried algae ($N = 10$) were weighed and placed in 4 N NaOH in 30 ml gas-tight vials that were stored at 4°C in darkness overnight. The next day, DMSP was measured as DMS from the headspaces of the vials by direct injection into an SRI gas chromatograph (Chromosil 330 column, flame photometric detector; detection limit: 5 µg DMS). Known concentrations of commercially obtained DMSP were used as standards. A Kruskal–Wallis ANOVA and Tukey's multiple comparison test were used to compare concentrations among species.

Artificial Feeding Assays with Algal Compounds

Dimethyl sulfide (Acros Organics) and acrylic acid (Sigma-Aldrich) were incorporated at natural concentrations, and multiples thereof, into artificial foods composed of 2:1 dried *G. tikvahiae*/agar. Because DMS is volatile, it was mixed into artificial food after food cooled to a temperature below 40°C. Equal amounts of distilled H₂O were added to control foods. Evaporative losses of DMS during food preparation were determined through gas chromatography. These numbers were used to adjust the concentration of DMS added to the foods to achieve the desired concentration for the start of the assay. The diet was presented on strips of window screen, as above, for acrylic acid assays. For DMS assays, artificial food was created by spreading food evenly into a thin layer over clean sand (to weight food down), allowing it to cool, and cutting replicate pieces by using different shapes of known size for DMS and controls. This was performed to reduce the time from

incorporation of volatile DMS into food and the start of the assay. Sea urchins were not starved before feeding assays, because starvation reduced feeding rates (A. Erickson, personal observations). Feeding assays ($N = 15\text{--}20$) consisted of pairs of food, with and without a compound, that were offered simultaneously. Sea urchins were allowed to feed until half of one food type was consumed or until 2 hr passed. This time period was based on established diffusion rates of DMS, where 50% was lost from experimental diets after 1 hr and 75% after 4 hr (Van Alstyne and Houser, 2003). Consumption was quantified for acrylic acid as the number of window screen squares revealed when food was consumed and for DMS assays as the number of squares eaten when food pieces were held against window screen. Sea urchins that did not eat or consumed all food were excluded from statistical analysis. Paired t -tests were used to identify significant differences in the number of squares consumed.

Caulerpenyne was semipurified from *C. sertularioides* crude extract with flash column chromatography (Paul and Fenical, 1986). It was found in the 95:5 hexane/ethyl acetate fraction of a florisil column, and nuclear magnetic resonance indicated ~90% purity. The caulerpenyne fraction was dissolved in ethanol and incorporated into artificial food (2:1 dried *G. tikvahiae*/agar) at concentrations that approximate the natural concentration in *Caulerpa* spp. Foods were presented on strips of window screen, as above, along with a control containing ethanol without caulerpenyne. Sea urchins were allowed to feed until half of one food type was consumed or until 48 hr passed. Consumption was quantified as

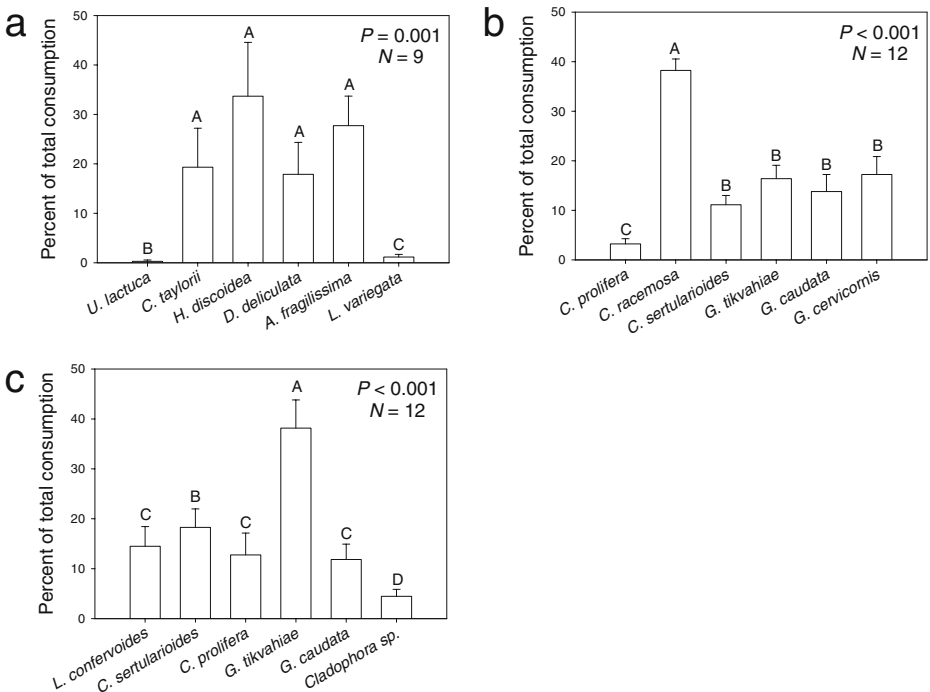
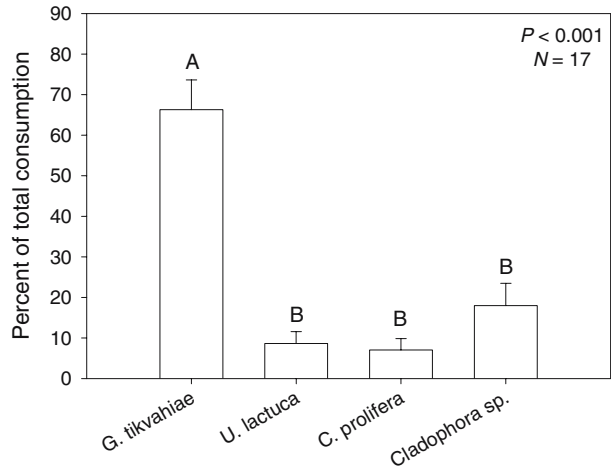


Fig. 1 Percentage of total consumption for a variety of algae offered to *E. lucunter* in live algal multiple-choice feeding assays. Treatments were compared by Friedman’s repeated-measures ANOVA followed by the Student–Newman–Keuls *post hoc* test. Error bars represent standard error and letters above bars denote significant differences

Fig. 2 Percentage of total consumption for select algae offered to *E. lucunter* in a live algal multiple-choice feeding assay. Algae include those deemed highly palatable (*G. tikvahiae*) and unpalatable (*U. lactuca*, *C. prolifera*, and *Cladophora* sp.) from assays in Fig. 1. Treatments were compared by Friedman's repeated-measures ANOVA followed by the Student–Newman–Keuls *post hoc* test. Error bars represent standard error and letters above bars denote significant differences



the number of window screen squares revealed when food was eaten. Sea urchins that did not eat or consumed all food were excluded from statistical analysis. Friedman's repeated-measures ANOVA and Student–Newman–Keuls multiple comparison test were used to identify significant differences in the number of squares consumed.

Results

Live algal multiple-choice feeding assays revealed that certain species of algae were of low preference to *E. lucunter*. *U. lactuca* and *L. variegata* were preferred less compared with other green, brown, and red algae (Friedman's $\chi_r^2 = 19.835$, $P = 0.001$; Fig. 1a). *C. prolifera* was the least preferred species of *Caulerpa* and was eaten less than *Gracilaria* spp. (Friedman's $\chi_r^2 = 32.446$, $P < 0.001$; Fig. 1b). *Cladophora* sp. was fed upon less than *Caulerpa* spp., *Gracilaria* spp., and the cyanobacterium *L. confervoides* (Friedman's $\chi_r^2 = 24.326$, $P < 0.001$; Fig. 1c). Three of the four least preferred algal species were green, suggesting that *E. lucunter* may be more sensitive to secondary metabolites of green algae than those found in other types. Hence, this report concentrates on the influence of

Fig. 3 The number of squares consumed by *E. lucunter* in an artificial feeding assay. Nonpolar (NP) and polar (P) extracts of *U. lactuca* (UI) were incorporated into artificial food and offered, in conjunction with extract-free controls, simultaneously to sea urchins. Treatments were compared by the Friedman's repeated-measures ANOVA followed by the Student–Newman–Keuls *post hoc* test. Error bars represent standard error and letters above bars denote significant differences

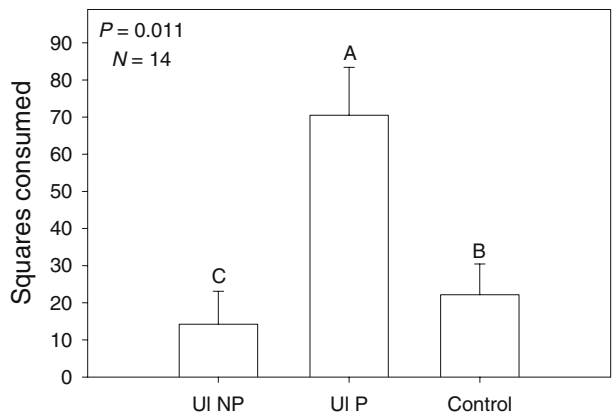
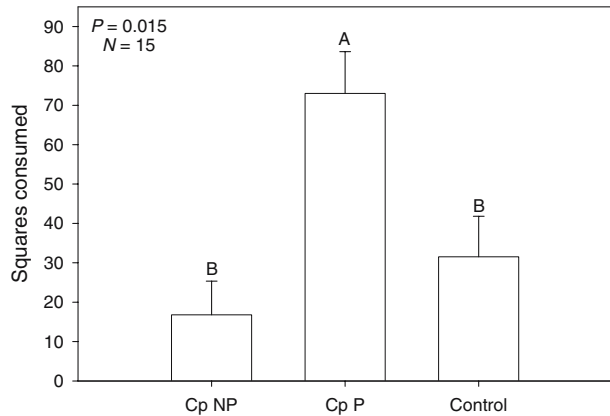


Fig. 4 The number of squares consumed by *E. lucunter* in an artificial feeding assay. Nonpolar (NP) and polar (P) extracts of *C. prolifera* (Cp) were incorporated into artificial food and offered, in conjunction with extract-free controls, simultaneously to sea urchins. Treatments were compared by the Friedman's repeated-measures ANOVA followed by the Student–Newman–Keuls *post hoc* test. Error bars represent standard error and letters above bars denote significant differences



green algal chemistry on feeding by *E. lucunter*. In each case, the low-preference algae composed <5% of the average total consumption. When the three green algae that were the least preferred in previous experiments were offered to sea urchins simultaneously, there was no significant difference in consumption among them. However, they were all consumed less than *G. tikvahiae* (Friedman's $\chi_r^2 = 21.663$, $P < 0.001$; Fig. 2). *U. lactuca* and *C. prolifera* each composed <10% of the average total consumption, whereas *Cladophora* sp. was just below 20% of the average total consumption.

Consumption of artificial food with polar *U. lactuca* extracts was about $\times 3$ greater than for food with nonpolar extract or the control (Friedman's $\chi_r^2 = 9.000$, $P = 0.011$; Fig. 3). Food with nonpolar *U. lactuca* extract also was consumed significantly less than the control. Similarly, artificial food with polar *C. prolifera* extract was consumed two to three times more than food with nonpolar extract or the control (Friedman's $\chi_r^2 = 8.423$, $P = 0.015$; Fig. 4); however, there was no difference in consumption between food with nonpolar extract and the control.

Dimethylsulfoniopropionate concentrations were $\sim 2.76 \pm 0.15\%$ of the dry mass (DM) or $0.44 \pm 0.01\%$ of the fresh mass (FM) in *U. lactuca*. Trace levels of DMSP (0.14 ± 0.00 DM, $0.02 \pm 0.00\%$ FM) were found in *Cladophora* sp., and DMSP was not detected in *C. prolifera*. DMSP concentrations (%DM) were greater in *U. lactuca* than in both *Cladophora* sp. and *C. prolifera* (Kruskal–Wallis ANOVA $H = 26.852$, $P \leq 0.001$).

Fig. 5 The number of squares consumed by *E. lucunter* in paired artificial feeding assays. Acrylic acid was incorporated into artificial food at natural concentration (1.38% DM) and twice the natural concentration (2.76% DM). Then, it was offered, in conjunction with acrylic-acid-free controls, to sea urchins. Treatments were compared by paired *t*-tests. Error bars represent standard error and letters above bars denote significant differences

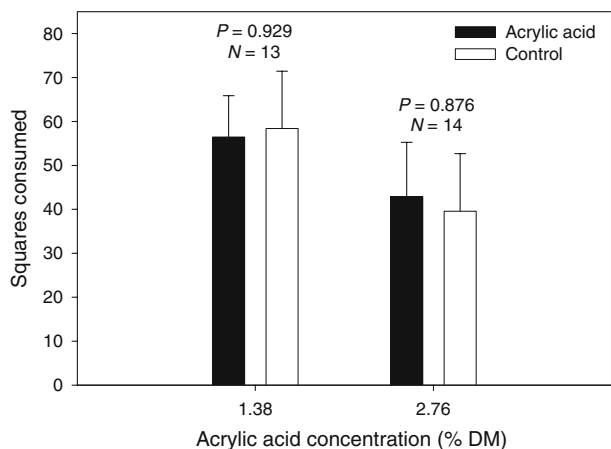
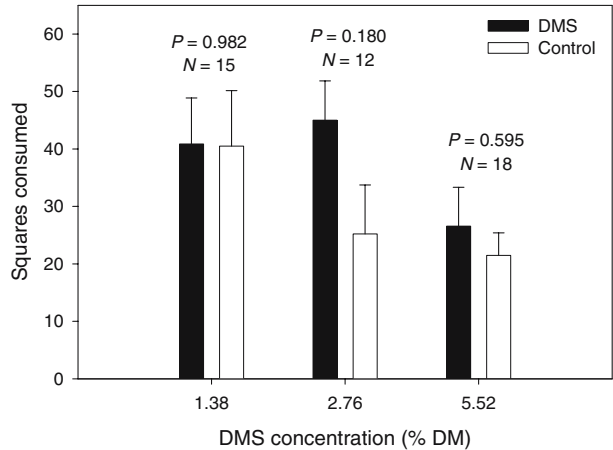


Fig. 6 The number of squares consumed by *E. lucunter* in paired artificial feeding assays. DMS was incorporated into artificial food at natural concentration (1.38% DM), and twice (2.76% DM) and four times (5.52% DM) the natural concentration. Then, it was offered, in conjunction with DMS-free controls, to sea urchins. Treatments were compared by paired *t*-tests. Error bars represent standard error and letters above bars denote significant differences

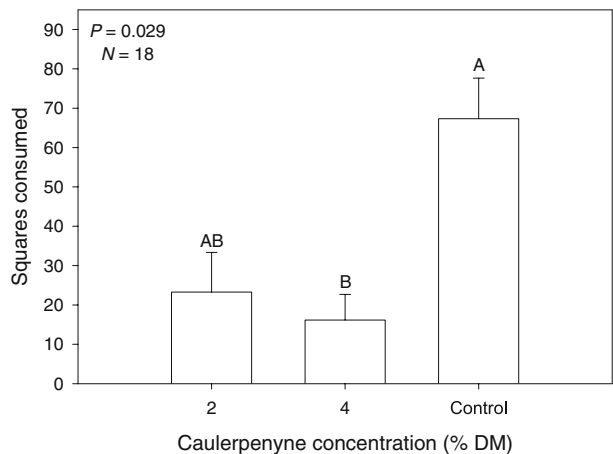


There was no difference in consumption between controls and artificial food containing either DMS or acrylic acid, the cleavage products of DMSP. Acrylic acid at natural (1.38% DM) and twice the natural concentrations failed to reduce consumption by *E. lucunter* (paired *t*-test $t = 0.092$, $P = 0.929$; paired *t*-test $t = -0.159$, $P = 0.876$; respectively; Fig. 5). DMS at natural (1.38% DM), twice, and four times natural concentrations also failed to reduce consumption (paired *t*-test $t = -0.023$, $P = 0.982$; paired *t*-test $t = -1.432$, $P = 0.180$; paired *t*-test $t = -0.542$, $P = 0.595$; respectively; Fig. 6). There was no significant difference in consumption of artificial food containing caulerpenyne at 2% DM with controls, whereas artificial food containing caulerpenyne at 4% DM was fed upon approximately one third as much as controls (Friedman's $\chi^2_r = 7.115$, $P = 0.029$; Fig. 7).

Discussion

This study examined feeding preferences of the sea urchin *E. lucunter* and whether preferences were chemically mediated. The green algae *U. lactuca*, *C. prolifera*, and

Fig. 7 The number of squares consumed by *E. lucunter* in an artificial feeding assay. Extract from the caulerpenyne-containing fraction was incorporated into artificial food at concentrations of 2% and 4% DM, which approximates what is naturally found in *Caulerpa* spp. Then, it was offered, in conjunction with caulerpenyne-free controls, to sea urchins. Treatments were compared by paired *t*-tests. Error bars represent standard error and letters above bars denote significant differences



Cladophora sp., and the brown alga *L. variegata* were unpalatable to *E. lucunter* relative to other algal species. Given that three of four species avoided were green algae, *E. lucunter* may be more sensitive to chlorophyte defenses than those of other types of algae. Feeding responses to these algae, and related species, in past studies have been herbivore specific. For instance, *Caulerpa* spp. were avoided by the surgeonfish *Zebrasoma flavescens* (*C. prolifera*, *C. racemosa*, *Caulerpa serrulata*, *C. sertularioides*; Wylie and Paul, 1988) and the sea urchins *Paracentrotus lividus* (*Caulerpa taxifolia*; Lemee et al., 1996), and *Lytechinus variegatus* (*C. prolifera*, Lowe, 1974; Vadas et al., 1982). In contrast, many reef fish, including parrotfish, surgeonfish, and rabbitfish, consumed *Caulerpa* spp. (*Caulerpa cupressoides*, *C. prolifera*, *C. racemosa*, and *C. sertularioides*; Paul and Hay, 1986; Paul et al., 1990). The percentage of *C. prolifera* individuals eaten by reef fish has ranged between 8% and 70% in preference experiments (Paul and Hay, 1986). *E. lucunter* also preferred *C. racemosa* var. *laetevirins* (Fig. 1b). Alternatively, *Cladophora* spp. were avoided by parrotfish and some surgeonfish (Paul and Hay, 1986), yet were consumed by *Z. flavescens* (Wylie and Paul, 1988) and the amphipod *Amphithoe longimana* (Hay et al., 1987a). *Ulva* spp. were avoided by the sea urchins *Strongylocentrotus droebachiensis* (Van Alstyne and Houser, 2003), *P. lividus* (Lemee et al., 1996), and *L. variegatus* (Lowe, 1974), the spottail pinfish *Diplodus holbrooki* (Hay et al., 1987a), and *A. longimana* (Duffy and Hay, 1994). However, *Ulva* spp. were fed upon by reef fish (Littler et al., 1983), the sea hare *Aplysia californica* and the shore crab *Pachygrapsus crassipes* (Sousa, 1979), the gastropods *Littorina striata*, *Osilinus atratus* (Granado and Caballero, 2001), *Turbo undulatas* (Davis et al., 2005), and the amphipod *Gammarus mucronatus* (Duffy and Hay, 1994).

It is likely that algal chemistry influenced feeding by *E. lucunter*. In this study, nonpolar *U. lactuca* extract reduced, and polar extract stimulated, feeding by *E. lucunter*. The average DMSP concentration in *U. lactuca* was $0.44 \pm 0.01\%$ FM, which was lower than for deterrent *Ulva* spp. from the Pacific northwest ($0.49 \pm 0.22\%$ FM to $1.58 \pm 0.31\%$ FM; Van Alstyne et al., 2001). Previous studies demonstrated that DMS strongly deterred feeding by *S. droebachiensis* at concentrations between 0.04% and 2% FM (Van Alstyne and Houser, 2003), and acrylic acid deterred feeding by *Strongylocentrotus purpuratus* and *S. droebachiensis* at concentrations between 0.1% and 2% FM (Van Alstyne et al., 2001). Although the DMSP concentrations in *U. lactuca* were high enough to generate sufficient DMS and acrylic acid to deter feeding by sea urchins in past studies (Van Alstyne et al., 2001; Van Alstyne and Houser, 2003), no differences in feeding by *E. lucunter* resulted between controls and artificial food containing either acrylic acid or DMS at natural and elevated concentrations. Similarly, no effect of high DMS concentrations was found on feeding by the fish *Thalassoma bifasciatum* (Pawlik et al., 2002), whereas *Ulva* spp. extracts deterred feeding by the amphipod *Gammarus palustris* (Borowsky and Borowsky, 1990), and acrylic acid stimulated feeding by the isopod *Idotea wosnesenskii* at 0.1% to 1% FM (Van Alstyne et al., 2001). DMS may have diffused out of artificial food during this experiment, preventing differences in feeding on experimental food versus controls. However, established DMS diffusion rates (Van Alstyne and Houser, 2003) suggest that DMS should have been present at the end of the assay. *E. lucunter* may be less sensitive to acrylic acid and DMS than temperate sea urchins, as the effects of metabolites can be herbivore specific (Hay et al., 1987b; Paul et al., 2001).

The secondary metabolite caulerpenyne can be converted through activation, degradation, and oxidation to a variety of additional compounds that may have greater toxicity than the precursor, including oxytoxins (1 and 2), epoxycaulerpenynes (6, 7, and 10, 11), 7,7-C-

didehydro-6-hydroxy-6,7-dihydrocaulerpenyne, taxifolials (A–D), and taxifolione (Guerriero et al., 1993; Guerriero and D'Ambrosio, 1999; Jung and Pohnert, 2001). During activation, the 1,4-diacetoxybuta-1,3-diene(bis-enol acetate) moiety is hydrolyzed by esterases in seconds and is replaced with 1,4 dialdehydes, which are suggested to be responsible for significant bioactivity. In this study, the chemistry of *C. prolifera* influenced feeding by *E. lucunter*. Artificial food with polar *C. prolifera* extract stimulated feeding by *E. lucunter*; however, there was no effect of food with nonpolar extract on feeding. Despite the inactivity of the nonpolar extract, caulerpenyne deterred feeding by *E. lucunter* at natural concentrations (>2% and 4% DM). Although sea urchins (McConnell et al., 1982) and a few reef fish (Targett et al., 1986; Paul et al., 1987) have been deterred by caulerpenyne, most reef fish were not deterred by *Caulerpa* spp. extracts or caulerpenyne (Paul et al., 1987; Wylie and Paul, 1988; Paul et al., 1990, 1992).

Variation in herbivory may relate to caulerpenyne concentration, which varies within and among species (Paul and Hay, 1986; Amade and Lemee, 1998; Dumay et al., 2002). For instance, in *C. prolifera*, it varies from 20% to 40% of the nonpolar extract (Paul and Hay, 1986), and *C. taxifolia* and *C. prolifera* have twice the caulerpenyne concentration of *C. racemosa* (Jung et al., 2002). The discrepancy between the deterrent live algae and nondeterrent, nonpolar *C. prolifera* extract may have resulted from activation of caulerpenyne in the live algae. Conversion to oxytoxins or other metabolites requires active algal esterases no longer present in artificial food. While partial activation may have occurred with extraction (Jung et al., 2002), the effect may not have been as great as that of natural activation through feeding. Finally, the discrepancy between the deterrent caulerpenyne and nondeterrent, nonpolar *C. prolifera* extract may have resulted if the extract contained less caulerpenyne than 4% DM, which was tested. Although feeding assays have not been conducted with isolated algal oxytoxins, due to their unstable and reactive nature, some studies have tested the effect of oxytoxins, mucus, and additional compounds derived from caulerpenyne-sequestering sacoglossans. Compounds and mucus inhibit microbial and protistan growth and feeding by freshwater and carnivorous marine fishes (Cimino et al., 1990; Guerriero et al., 1993; Gavagnin et al., 1994).

Chemical defenses against herbivory have not been explored as well in *Cladophora* spp. Some studies have found numerous sterols, free fatty acids and esters, terpenes, aldehydes, betaines, halogenated, and nitrogen-containing compounds in *Cladophora* spp. (Elenkov et al., 1995; Kamenarska et al., 2004). In addition, some studies have demonstrated antibacterial, anti-inflammatory, and cytotoxic activity of individual compounds or extracts (Kamenarska et al., 2004).

The unpalatable green algae used in this study, and related species, have been implicated in macroalgal blooms and invasions globally (Valiela et al., 1997; Jousson et al., 2000; Meinesz et al., 2001). The presence of such species has led to phase-shifts, where dominant structural species of an ecosystem, such as seagrass and corals, have been replaced by algae (Meinesz et al., 1993; Valiela et al., 1997; Thomas and Bell, unpublished data), leading to concomitant alterations in community and ecosystem parameters. Consequences of phase shifts include habitat loss, reduced biodiversity, and altered environmental parameters, species dominance, and trophic dynamics (Valiela et al., 1997; Thomas and Bell, unpublished data).

Algal chemical defenses have been proposed as a mechanism that promotes phase shifts and invasions due to deterrent, toxic, and allelopathic effects (Guerriero et al., 1993; Paul et al., 2001; Jung and Pohnert, 2001). Toxic effects have influenced a wide range of taxonomic organisms. *Ulva* spp. extracts and caulerpenyne are toxic to larval and adult stages of many marine invertebrates and vertebrates (Paul and Fenical, 1986; Lemee et al.,

1993; Pedrotti et al., 1996; Nelson et al., 2003). Ulvoid extracts have reduced the growth and germination of seagrass epiphytes and macroalgal zygotes (Nelson et al., 2003). Caulerpenyne is also toxic to bacterial and fungal pathogens (Paul and Fenical, 1986). Although studies often test for toxicity by bathing organisms in algal extracts, which may not accurately assess potential for deterring settlement or feeding, these studies demonstrate that bloom-forming and invasive species have the potential to impair or kill organisms if they release metabolites into the water column. Given the toxic and deterrent effects of these algae, it appears that their chemistry has the potential to promote bloom persistence and invasion success.

In summary, algal chemistry is partially responsible for feeding patterns observed in this study. Nonpolar extracts of *U. lactuca* and caulerpenyne reduced feeding by *E. lucunter*, whereas polar extracts stimulated feeding. Further analysis is needed to determine (1) which nonpolar *U. lactuca* compounds deter herbivory; (2) whether nonpolar compounds of *C. prolifera*, in addition to caulerpenyne, deter herbivory; and (3) other chemical defenses in *Cladophora* spp. Finally, it is important to understand how algal chemistry influences herbivory, thus indirectly influencing algal communities, given the current rise in algal blooms and invasions.

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Introduction

Plants have evolved a broad spectrum of inducible defense mechanisms to resist damaging insects and pathogens (Karban and Baldwin, 1997). Induced plant defenses may act directly against herbivores or microorganisms, and comprise mechanisms as diverse as the strengthening of plant cell walls, hypersensitive cell death, or the production of toxic and deterrent substances. In addition, plants may employ indirect defenses for instance by emitting volatile compounds in response to feeding or oviposition by arthropods (Dicke and Sabelis, 1988; Hilker and Meiners, 2002). These volatiles can serve as long-range signals for parasitoids and predators, by indicating the presence and location of their often inconspicuous prey.

A central question in the study of indirect plant defenses is how specific the plant-provided volatile cues are (Vet and Dicke, 1992; Dicke, 1999; Turlings et al., 2002). It has been argued that ideally volatile cues should not only be easy to detect but, should also be specific enough to provide information on the identity of the herbivore and its suitability as a host or prey (Vet and Dicke, 1992). Plants can vary considerably in the volatile blends they emit, both in terms of the chemical composition (quality) and in the quantity of volatile compounds. Depending on what factors cause it, this variability may either interfere with or enhance the specific information that the signals contain. Evidence has been found for both, existence and absence of specificity, depending on the studied system (reviewed by Dicke, 1999). At least in some plant–herbivore systems it has been shown that different insect species and instars may elicit different odor blends, resulting in preferential attraction of natural enemies to plants on which their specific (De Moraes et al., 1998; Guerrieri et al., 1999) or preferred host stages (Takabayashi et al., 1995) were feeding. It remains to be resolved, however, how this specificity in plant signals can exist in the face of considerable variability in induced volatile blends caused by other factors. Major differences in the chemical composition can be found among different plant species (Turlings et al., 2002), but within a species genotypic effects have also been found to be important (Loughrin et al., 1995; Peacock et al., 2001; Gouinguéné et al., 2001; Hoballah et al., 2002). Moreover, abiotic factors such as humidity, temperature, light intensity, light cycle, and nutrient availability all can have an effect on the quantity and the quality of herbivore-induced plant odors (Gouinguéné and Turlings, 2002).

An additional complicating aspect is that plants live in environments in which they face the possibility of multiple, synchronous attacks by insects and pathogens. Host finding with the aid of plant-provided volatiles may prove difficult for parasitoids if plant pathogens significantly alter the chemical composition of herbivore-induced odor blends. Pathogen-derived or pathogen-induced odors could mask the host-induced blend or reduce the emission of important compounds. On the other hand, odor blends resulting from simultaneous herbivore and pathogen attack may provide useful information for natural enemies if their hosts are less or better suitable due to poorer or better development on a diseased plant. So far, cross effects between plant-feeding insects and microorganisms have been investigated almost exclusively in the context of induced direct defenses (Hatcher, 1995; Agrawal et al., 1999; Rostás et al., 2003). Whether induced indirect defense against herbivores is compromised by pathogen attacks remains almost unexplored, with the exception of the studies conducted by Cardoza et al. (2002, 2003a,b), who found a significant effect of fungus infection on direct and indirect defenses in peanut plants (see Discussion).

At the biochemical level, pathogen infection commonly induces the salicylic acid (SA) defense pathway, whereas insect attack triggers a defense based on jasmonic acid (JA).

Cross talk between these pathways, however, may occur (Felton and Korth, 2000; Thaler et al., 2002; Devoto and Turner, 2003). It is conceivable that the induction of the SA pathway may also interact with the JA-dependent induction of volatiles in response to herbivory. If so, a change in the emission of many compounds should be the result, and members of the third trophic level may adapt their responses to optimize the exploitation of the signals.

The induced indirect defense mechanism has been extensively investigated in the tritrophic system maize, *Spodoptera* spp., and several larval endoparasitoids (Turlings et al., 1995; Alborn et al., 1997; Hoballah et al., 2002). Feeding by *Spodoptera* larvae leads to the release of green leaf volatiles and induces the accumulation of JA and ethylene. These phytohormones are responsible for the emission of indole, terpenoids, and other compounds (Schmelz et al., 2003a,b; Ruther and Kleier, 2005). Parasitic wasps are highly attracted to these odors (Turlings et al., 1990), and this attraction may benefit the attacked plant (Hoballah and Turlings, 2001). In contrast to some plants, the induction of the SA pathway does not lead to any detectable release of volatiles in maize (Turlings et al., 2002; Van Poecke and Dicke, 2004). Because of the available information on caterpillar-induced emissions of maize and its attractiveness to parasitoids, this tritrophic system lends itself well to studies on the effects of pathogen infection on indirect defense.

In this study, we assessed the effect of the necrotrophic fungus *Setosphaeria turcica* (Leonard et Suggs) on the emission of maize volatiles induced by *Spodoptera littoralis* (Boid.), and the consequences for the third trophic level. The ascomycete *S. turcica* causes the foliar disease known as northern corn leaf blight and is a serious problem for maize growers worldwide (Borchardt et al., 1998). The fungus cooccurs with *S. littoralis* and its parasitoid *Microplitis rufiventris* (Kok.) in Egypt and the countries of the Middle East (Gerling, 1969; Hegazi, 1977; CAB International, 1988). It also cooccurs with *Cotesia marginiventris* in the United States and Latin America (CAB International, 1988; Molina-Ochoa et al., 2003). This parasitoid species uses *S. exigua* and *S. frugiperda* larvae as hosts. These two hosts trigger the release of the same inducible compounds in maize when compared to *S. littoralis* (Turlings et al., 1995; Hoballah, 2001). In a six-arm olfactometer, we tested the responses of the parasitoids *M. rufiventris* and *C. marginiventris* (Cresson) to odors of maize seedlings attacked either by the fungus, the herbivore, or by both. Simultaneously, all odor blends were sampled and subsequently analyzed for comparison. Furthermore, the performance of *S. littoralis* feeding on fungus-infected maize plants and the performance of its natural enemy, *M. rufiventris*, developing in larvae that fed on diseased maize was evaluated.

Methods and Materials

General Methods

Maize (*Zea mays* var. Delprim) plants were grown in polypropylene pots (11 cm high, 4 cm diam) containing commercial soil mix (Coop, Basel) in a climate chamber (23°C, 60% r.h., and 16:8 hr L/D, 550 $\mu\text{mol m}^{-3} \text{sec}^{-1}$). This maize variety was chosen because it is partially resistant to *S. turcica*. Stronger and/or faster induced responses to fungal attack as well as less necrotic tissue that does not produce volatiles can be expected from partially resistant compared to susceptible varieties. In addition, “Delprim” is a variety that emits exceptionally high amounts of herbivore-induced volatiles.

Eggs of *S. littoralis* (Lep., Noctuidae) were supplied by Syngenta (Stein, Switzerland). Newly hatched larvae were reared in transparent plastic boxes on a wheat germ-based artificial diet until used. Colonies of the solitary endoparasitoids *M. rufiventris* and *C. marginiventris* (both Hym., Braconidae) were maintained in the laboratory. For the rearing, 25 *S. littoralis* caterpillars (3–4 d) were offered to a single mated female (4–7 d) for 3 hr in a plastic box (5 cm high, 9.5 cm diam). The parasitized caterpillars were kept in an incubator (25°C, L:D 16:8 hr) until the parasitoids formed cocoons. The cocoons were kept in Petri dishes until adult emergence. Emerging adults were sexed and kept in plastic cages (30 × 30 × 30 cm; Bugdorm I, MegaView Ltd, Taichung, Taiwan) in the same incubator (*C. marginiventris*) or under ambient laboratory conditions (*M. rufiventris*). Cages were supplied with moist cotton wool and droplets of honey.

The necrotrophic fungus *S. turcica* (anamorph: *Exserohilum turcicum*, Dothideaceae) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and cultivated on V8 agar in darkness under laboratory conditions.

Plant Inoculation and Volatile Induction

Spores of *S. turcica* were harvested prior to plant inoculations. A Petri dish culture was flooded with 5 ml 0.05% aq. Tween 20, and then brushed gently with a small paintbrush to detach the spores from the mycelium. The density of the spore suspension was determined with an improved Neubauer chamber and adjusted to 6×10^4 spores ml⁻¹. Maize seedlings (7 d) were inoculated by applying 100 µl spore suspension to the second and third leaves, respectively. Spores were spread homogeneously with a paintbrush. Control plants were mock-inoculated in the same manner with 0.05% aq. Tween 20. All seedlings were placed into two cool boxes with wet tissue papers laid out on the bottom. Plants were kept in darkness for 16 hr (17:00–09:00) at >90% r.h. and ambient temperatures. The following morning, all plants were transferred to a climate chamber (23°C, 60% r.h., and L/D 16:8 hr, 550 µmol m⁻² sec⁻¹). Disease symptoms were allowed to develop for 72 hr after which the plants were used in the experiments. Strength of infection was calculated by scanning the diseased leaves ($N = 9$) and measuring the necrotized areas with Photoshop 7.0 (Adobe) and Surface (© C. Thiemann, Berlin, Germany). On average, 11% of the leaf surface was visibly affected by *S. turcica*. This was the highest achievable disease rate for the partially resistant variety Delprim in the seedling stage.

Volatile Collections

Volatiles from maize seedlings were collected with a six-arm olfactometer, a device allowing for simultaneous odor collection and testing of parasitoid host location behavior (described by Turlings et al., 2004). A single maize plant was placed into one of the six odor source vessels of the olfactometer. Trapping filters were attached to each vessel consisting of glass tubes (7 cm) containing 25 mg of 80–100 mesh Super Q adsorbent (Alltech, Deerfield, IL, USA) that was kept in place by two fine mesh metal screens (described by Heath and Manukian, 1992). Filtered and humidified air was pushed into the odor source vessels at a rate of 1.2 l min⁻¹ vessel⁻¹ originating from a central in-house compressor. Half of the air flow (0.6 l min⁻¹) was pulled through the trapping filter with a vacuum pump (ME2, Vacuubrand, Wertheim, Germany), whereas the other half was allocated to the olfactometer choice chamber. Before each experiment, traps were rinsed with 1 ml methylene chloride. Collections lasted 3 hr after which traps were removed, extracted, and analyzed.

Chemical Analysis

Volatile traps were eluted with 150 μl methylene chloride after each collection, and two internal standards (*n*-octane for green leaf volatiles and nonyl acetate for terpenoids and others, each 200 ng in 10 μl methylene chloride; all chemicals were purchased from Sigma-Aldrich, Germany) were added to these samples. Aliquots (3 μl) of the samples were analyzed by gas chromatography/mass spectrometry (GC: HP 6890 N, MSD: Agilent 5973) equipped with a split/splitless injector and an HP-1 ms column (30 m \times 0.25 mm ID, 0.25 μm film thickness). Samples were injected in pulsed splitless mode. The oven was held at 40°C for 3 min and then programmed at 8°C/min to 230°C, where it was maintained for 9.5 min. Helium (24 cm sec⁻¹) was used as carrier gas. Compound identities were confirmed by comparison with mass spectra of the National Institute of Standards and Technology (NIST) library and mass spectra of commercially available standards. Quantification of compounds was based on comparison with the internal standards. Only those compounds that were reliably found in each sampled plant of the same treatment were quantified. The evaluated compounds comprised >90% of the total amount of the analyzed volatile blends.

Host Location Behavior of Parasitoids

Attraction of *M. rufiventris* and *C. marginiventris* was assessed in the six-arm olfactometer, and simultaneously a part of the volatiles released from the plants was collected for subsequent analyses. Mated 2- to 4-d-old female wasps were used in all experiments. Insects were naïve in a sense that they had no contact to host insects or plants during the adult stage. Six wasps were removed from the cage with an aspirator and released into the central choice chamber of the olfactometer. Previous experiments had shown that female wasps do not interfere with each other in their choices (Turlings et al., 2004). Wasps initially walked up to the top, attracted by the light above the choice chamber. Most would walk into an arm with an attractive odor until the path was blocked by a stainless steel screen. Eventually, they walked up into a glass trapping bulb where they could easily be counted and removed. Each group of insects was given 30 min to make a choice, after which they all were removed, and a new group was released. Five groups of six wasps were tested on a given day. Each olfactometer experiment was replicated on 8 d with a new set of plants ($N = 8$). The position of the plants was changed clockwise after each day of testing. Different sets of plants were used for each parasitoid species. *M. rufiventris* and *C. marginiventris* were tested to simultaneously presented odors of: (1) three undamaged maize plants, (2) one herbivore-damaged maize plant, or (3) one fungus-infected maize plant, or (4) one herbivore-damaged/fungus-infected maize plant. The three undamaged plants were alternated with attacked plants and arranged in a circle. Herbivory treatment was achieved by transferring the plants into the odor source vessels and then placing ten 2nd instars of *S. littoralis* into the whorl of a healthy or infected maize plant (10 d old) the evening (17:00) before an experiment was performed. All other plants were placed in the olfactometer at the same time. The subsequent olfactometer assays were carried out between 09:00 and 13:00 hours.

Development of *S. littoralis* on Fungus-Infected Maize

The performance and mortality of *S. littoralis* caterpillars on *S. turcica*-infected maize plants was tested in two separate experiments. In the first assay, fifteen 4-d-old caterpillars

were selected for equal weight (2.6 ± 0.15 mg) and then placed singly into the whorl of a potted maize seedling. The host plants had previously been inoculated with the fungus or mock-inoculated with Tween 20. Symptoms of the fungus were present on the first three leaves, but not on the youngest fourth leaf. This reflects the heterogenic distribution of fungal infection in nature allowing the herbivore to choose between locally and systemically induced leaves. A cellophane bag (Celloclair, Liestal, Switzerland) over each plant prevented caterpillars from escaping while permitting gas exchange. After 5 and 10 d of feeding, larvae were weighed and placed on a new host plant. Finally, the pupal weight was calculated 2 d after pupation. In another experiment, we measured leaf consumption and survival of *S. littoralis* on infected and healthy leaves. A group of 10 neonate caterpillars was placed into each of 15 Petri dishes (9 cm diam) with moist filter paper. As a food source, each group of caterpillars received either a piece of (1) *S. turcica*-infected leaf bearing symptoms (third leaf), (2) healthy leaf (third leaf), (3) symptom-free leaf (fourth leaf), or (4) healthy leaf (fourth leaf). All Petri dishes were kept in an incubator (25°C, 16:8 hr L/D). The leaf area removed by *S. littoralis* was evaluated as described above for lesion area measurement after 2 d, and the number of surviving caterpillars was recorded.

Development of *M. rufiventris* inside Caterpillars Feeding on Fungus-Infected Maize

We assessed whether *S. turcica* infection had an indirect effect on the parasitoid *M. rufiventris*. Two groups of either five fungus-infected or five healthy maize plants were placed into four insect rearing tents (Bugdorm 2, Megaview, Taiwan). Ten neonate *S. littoralis* caterpillars were transferred onto each plant with a small paintbrush and allowed to feed for 3 d. All caterpillars were collected from the plants and placed into a transparent plastic box. From this pool, 20 caterpillars were randomly chosen and placed in a Petri dish into which a female parasitoid was introduced. The wasp was allowed to oviposit into six caterpillars. Wasp and parasitized caterpillars were then removed and the procedure was repeated five times with new wasps and herbivores. This yielded 36 parasitized caterpillars from fungus-infected and healthy maize plants, respectively. The *S. littoralis* larvae were allowed to continue to feed on the same type of plant they had originated from, either infected or healthy maize plants, for 6 d, i.e., 2 d before the first parasitoid hatched from its host. Caterpillars were then individually placed in Petri dishes (5.5 cm diam) containing a piece of filter paper and artificial diet. After emergence, the hatching rate, survivorship, pupal weight, developmental time, and longevity of *M. rufiventris* were measured. Parasitoid longevity was assessed by measuring the time from egg deposition until the adult died. Adults were supplied with water but were not fed.

Statistics

Data obtained from the six-arm olfactometer were analyzed by modified G-statistics for comparison of log linear models based on a quasi-Poisson distribution and thus fitted to overdispersed data. The software package R (<http://stat.ethz.ch/CRAN/>) was used. For detailed explanations, see Turlings et al. (2004). Two-way ANOVA with treatment and compound as main effects was performed for comparison of volatiles. Herbivore and parasitoid performances were also compared by using Student's *t*-test for independent samples. However, numbers of surviving *S. littoralis* on healthy and diseased plants were analyzed by Mann–Whitney *U* test, and hatching rate and survivorship of parasitoids were assessed via chi square tests.

Results

Effect of *S. turcica* on Volatile Emission

Fungal infection had a quantitative but no detectable qualitative effect on the odor bouquet of maize seedlings, i.e., no new compounds were detected (Fig. 1). Healthy and fungus-infected maize seedlings, both exclusively released linalool at the same rate (Newman–Keuls test after ANOVA, Treatment effect: $P = 0.794$). In contrast, caterpillar feeding triggered the release of large amounts of green leaf volatiles, monoterpenes, indole, and sesquiterpenes. Plants double-treated with *S. turcica* and *S. littoralis* emitted the same blend as plants damaged by *S. littoralis* alone, but most of the induced volatiles were found in significantly lower amounts. Fungus- and herbivore-damaged plants released 48% less of the total amount of volatiles than herbivore-only damaged plants (Newman–Keuls test after ANOVA, Treatment effect: $P < 0.001$). Compounds not reduced in their amounts due to pathogenic infection were (Z)-3-hexenal, (Z)-3-hexen-1-yl-acetate, and β -caryophyllene (Newman–Keuls test after ANOVA, Compound \times Treatment, P values > 0.05). The ratios between a given volatile compound and the total emission did not differ significantly between double-treated and herbivore-only treated plants with one exception: fungal infection lowered the indole/total amount ratio from 14% to 5% ($t = 3.467$; $P = 0.002$).

Host Location Behavior of Parasitoids

Parasitic wasps *M. rufiventris* and *C. marginiventris* preferred the odor of plants that were damaged by *Spodoptera* larvae when compared with healthy (*M. rufiventris*: estimate \pm SE: -2.72 ± 0.31 , $P < 0.001$; *C. marginiventris*: estimate \pm SE: -2.33 ± 0.23 , $P < 0.001$) and fungus-infected maize (*M. rufiventris*: estimate \pm SE: -2.45 ± 0.45 , $P < 0.001$; *C. marginiventris*: estimate \pm SE: -3.10 ± 0.54 , $P < 0.001$) (Fig. 2). However, they did not

Fig. 1 Plant volatiles emitted from seedlings of *Zea mays* (var. Delprim) after single or combined herbivore feeding and fungal infection. Means \pm SE are shown. $N = 10$ –13. Asterisks denote significant differences between treatments: * $P < 0.05$, ** $P < 0.01$, n.s. = not significant. Newman–Keuls test after two-way ANOVA. 1 = (Z)-3-Hexenal; 2 = (Z)-3-hexen-1-yl acetate; 3 = linalool; 4 = (3E)-4,8-dimethyl-1,3,7-nonatriene; 5 = indole; 6 = geranyl acetate; 7 = β -caryophyllene; 8 = (E)- α -bergamotene; 9 = (E)- β -farnesene

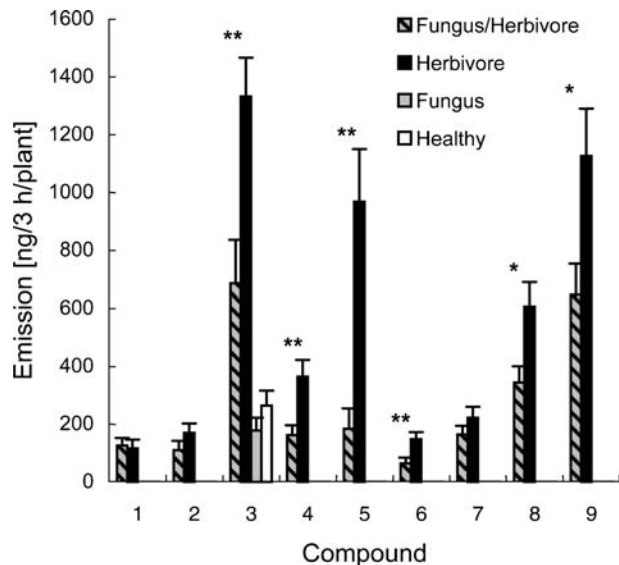
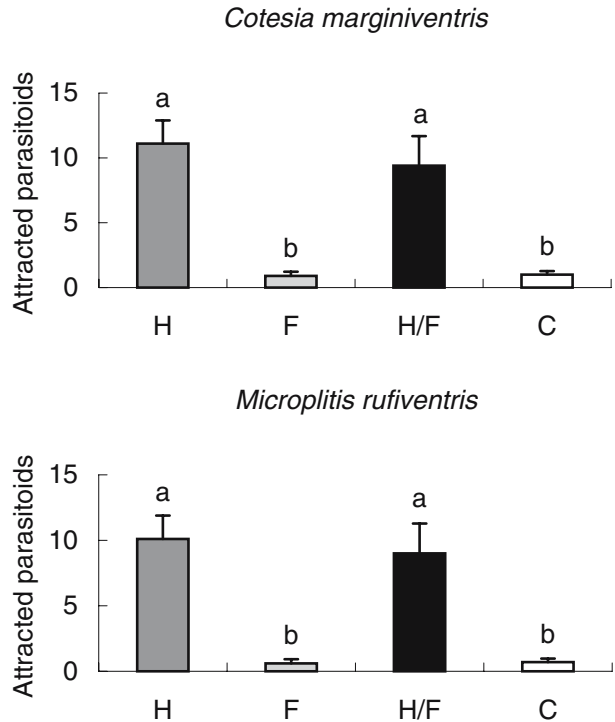


Fig. 2 Responses of naïve female parasitoids to odors emanating from: H = herbivore-infested, F = fungus-infected, H/F = herbivore- and fungus-attacked, or C = healthy maize seedlings in a six-arm olfactometer. The experiments were replicated on 8 d with different sets of plants for each parasitoid species. Per day 30 wasps were released in groups of six. Responding wasps: 80% of *Cotesia marginiventris*, 81% of *Microplitis rufiventris*. Bars represent mean number of parasitoids (\pm SE) per experimental day responding to an odor source. Significant differences between treatments are indicated by different letters above bars ($P < 0.05$)



respond differently to the odors of plants that had been damaged by *S. littoralis* alone and those that had been inoculated with *S. turcica* in addition (*M. rufiventris*: estimate \pm SE: -0.12 ± 0.18 , $P = 0.522$; *C. marginiventris*: estimate \pm SE: -0.14 ± 0.16 , $P = 0.377$). No significant differences in preference were found between fungus-infected and healthy maize seedlings (*M. rufiventris*: estimate \pm SE: 0.27 ± 0.51 , $P = 0.597$; *C. marginiventris*: estimate \pm SE: -0.77 ± 0.56 , $P = 0.172$).

Fig. 3 Performance of *Spodoptera littoralis* on healthy or *Setosphaeria turcica*-infected maize plants. One caterpillar fed on a single plant. All plants were exchanged after 5, 10, and 15 d of feeding. Means \pm standard errors are given. No significant differences were found between treatments. Student's *t*-test for independent samples

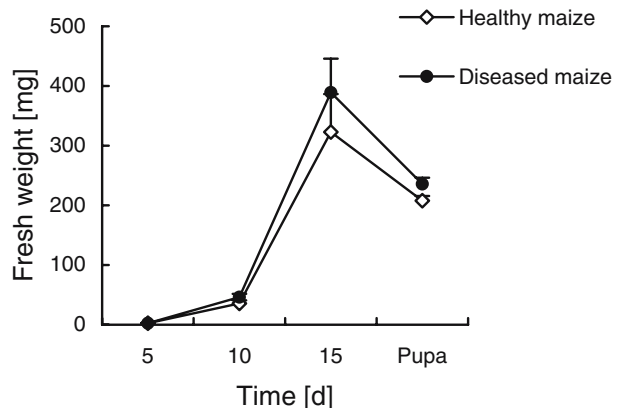


Table 1 Performance of *Spodoptera littoralis* (L₁) on *Setosphaeria turcica*-infected or healthy maize

	Fungus ^a		Healthy ^a		<i>P</i> ^c
	Local	Systemic	Local	Systemic	
Area fed [mm ²] ^b	102 ± 7.0	139 ± 11.0	103 ± 9.3	125 ± 15.4	n.s.
Surviving larvae/leaf	9.6 ± 0.17	9.2 ± 0.27	9.4 ± 0.20	9.6 ± 0.15	n.s.

^aTen caterpillars were kept on detached leaves (local = second leaf, systemic = fourth leaf) for 2 d.

^bMeans and standard errors are given for fed leaf areas ($N = 14-15$).

^cn.s. = Not significant, $P > 0.05$. Student's *t*-test for independent samples and Mann–Whitney *U* test.

Development of *S. littoralis* on Fungus-Infected Maize

Feeding on fungus-infected whole maize plants had no negative impact on the development of *S. littoralis* when compared with caterpillars feeding on healthy plants. No significant differences in larval fresh weights (after 5 d: $t = -1.666$, $P = 0.116$; 10 d: $t = -1.209$; $P = 0.245$, 15 d: $t = -1.21$; $P = 0.245$) or pupal fresh weights ($t = -2.071$, $P = 0.056$) were found at any time point of measurement (Fig. 3). Also, the duration of development was not affected by pathogen infection ($t = 0.355$, $P = 0.728$). Neonate larvae survived equally well on locally or systemically induced leaves of infected maize as on the corresponding leaves from healthy plants (local: $U = 166$, $Z = -0.137$, $P = 0.891$; systemic: $U = 109.5$, $Z = 0.954$, $P = 0.340$) (Table 1). Caterpillars on healthy leaves did not consume significantly different amounts of leaf material than those on diseased leaves (local: $t = 0.219$, $P = 0.828$; systemic: $t = -0.900$, $P = 0.376$).

Development of *M. rufiventris* inside Caterpillars Feeding on Fungus-Infected Maize

No indirect effect of *S. turcica* on the development of *M. rufiventris* was found (Table 2). Larvae of the parasitoid developed equally well in *S. littoralis* caterpillars feeding on infected maize as in caterpillars feeding on control plants. Hatching rate ($\chi^2 = 1.19$, $P = 0.276$), developmental speed (egg–pupa: $t = -0.398$, $P = 0.693$), pupal weight ($t = -1.692$, $P = 0.097$), and survivorship ($\chi^2 = 0.89$, $P = 0.345$) did not differ between treatments.

Table 2 Performance of *Microplitis rufiventris* in caterpillars of *Spodoptera littoralis* that had been reared on either fungus-infected or healthy maize plants

	Fungus	Healthy	<i>P</i> ^d
Hatched pupae ^a [%]	69	80	n.s.
Hatched adults ^b [%]	47	58	n.s.
Developmental time (egg to pupa) [d]	8.7 ± 0.14	8.7 ± 0.12	n.s.
Pupal weight ^c [mg]	3.2 ± 0.04	3.2 ± 0.04	n.s.
Longevity ^c (egg to adult) [d]	25.9 ± 1.10	26.3 ± 0.57	n.s.

^aParasitoid larvae that left the host and pupated outside.

^bAdult parasitoids emerging from cocoon.

^cMeans and standard errors are given.

^dChi square test and Student's *t*-test for independent samples. $N = 36$. n.s. = not significant.

Discussion

The results demonstrate that fungal infection had an impact on the emission of caterpillar-induced plant volatiles. Herbivore-damaged maize seedlings emitted lower amounts of the most abundant volatiles if they were previously infected by *S. turcica*. Pathogen infection alone did not result in the emission of detectable amounts of any compound other than linalool, which is also emitted by healthy plants. Typical fungus volatiles, such as 3-octanone, were not found. We also found no methyl salicylate that could have resulted from the induction of the SA pathway by *S. turcica* (Rostás et al., unpublished), despite using more sensitive methods (MS in single ion mode) to detect this compound. Although it was not possible to achieve a higher infection rate in this maize variety, it is doubtful that a heavier disease rate would have led to the emission of additional volatiles: treatment with a high dose (5 mM) of the SA mimic BTH did not lead to any differences in volatile emission (Rostás et al., unpublished data). Attenuated volatile emission due to double infestation has also been reported by Rodriguez-Saona et al. (2003), but in their case both plant antagonists were insects. It was found that cotton plants damaged by caterpillars of *S. exigua* emitted 60% less volatile compounds if simultaneously infested by the phloem-feeding insect *Bemisia argentifolii*. This is noteworthy because herbivores with a sucking–piercing–feeding mode can induce the SA pathway and, consequently, plant responses that are comparable with defenses against pathogens (Walling, 2000). Concerning the underlying mechanism, we hypothesize that fungal infection could reduce plant volatile emission as a result of the negative cross talk between the pathogen-induced SA pathway and herbivore-induced JA signaling. This antagonistic interaction has been shown for direct defenses in several plants (Fidantsef et al., 1999; Preston et al., 1999; Thaler et al., 2002).

Our observations contrast with reports on the only other plant–fungus–herbivore system investigated so far: in peanut plants, the emission of volatiles induced by *Spodoptera exigua* was not attenuated by the fungus *Sclerotium rolfsii*. However, methyl salicylate (MeSA), an attractive compound for a number of natural enemies (James, 2003a,b; de Boer and Dicke, 2004), was emitted by fungus-infected and double-attacked plants (Cardoza et al., 2002, 2003a,b). This difference in odor emission between maize and peanut is also reflected in the interactions with the second and third trophic level. On peanut plants, *S. exigua* eat more leaf tissue and perform better when the plant is diseased, thus leading to increased volatile emission (Cardoza et al., 2002). More individuals of *C. marginiventris* were found to land on fungus- and herbivore-attacked peanut plants than on plants infested by *S. exigua* alone (Cardoza et al., 2002, 2003a,b). These observations suggest that the wasp's response may be adaptive. However, it needs to be verified whether *C. marginiventris* performs better in *S. exigua* feeding on diseased compared to healthy plants.

In our study, neither *C. marginiventris* nor *M. rufiventris* preferred herbivore-damaged plants to double-treated maize seedlings, although the latter emitted less volatiles. We expected both parasitoid species to be more attracted by plants that were damaged by the herbivore alone, as they emitted about 50% more in total than maize seedlings attacked by both antagonists. Both *C. marginiventris* (Turlings et al., 2004) and *M. rufiventris* (C. Tamó, personal communication) respond in a dose-dependent manner in the six-arm olfactometer. As a general rule, the stronger the volatile emission, the stronger the attraction of the wasps. However, the odor blends in our experiments were not directly comparable to blends offered in dose–response assays where all compounds in the odor blends were equally reduced or increased. In contrast, in double-damaged maize not every compound was significantly reduced: the green leaf volatiles (*Z*)-3-hexenal and (*Z*)-3-hexen-1-yl acetate, as well as the sesquiterpene β -caryophyllene, were not affected by *S. turcica*

infection. The unaltered emission of green leaf volatiles in double-treated plants and their significance for both parasitoid species could be a sufficient explanation as to why the wasps did not change their preference. Hoballah (2001) and D'Alessandro and Turlings (2005) found that green leaf volatiles from maize are important attractants for naïve *M. rufiventris* and, in particular, for *C. marginiventris*. For the latter species, green leaf volatiles and/or related compounds were more attractive than induced terpenoids, as long as wasps had had no oviposition experience in the presence of induced maize odors. Further experiments will have to reveal whether *C. marginiventris* will respond differently to herbivore-infested maize plants with or without fungal infection after associative learning.

Alternatively, the unaltered responsiveness of wasps to caterpillar-damaged plants with fungus infection may be adaptive in the sense that there appears to be no selection pressure on female *M. rufiventris* to avoid *S. turcica*-infected maize. The offspring of *M. rufiventris* developed equally well in caterpillars feeding on healthy or *S. turcica*-infected maize leaves. It is conceivable that for this reason the wasps mainly use those volatiles for host location that are not affected by the fungus. To our knowledge, this is the first study to relate the potential impact of a phytopathogen to parasitoid performance.

Our research shows (1) that fungal infection is another factor that leads to variability in herbivore-induced odor emissions, and (2) that certain variability in the odor bouquet does not necessarily disrupt the mutualistic relationship between parasitoid wasps and plants.

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Introduction

Plants commonly respond to herbivore damage through the synthesis of volatile compounds, a process generally assumed to be detrimental to the herbivore through indirect defense mechanisms (Paré and Tumlinson, 1997, 1999). Induced plant volatiles attract natural enemies of herbivores (Dicke and Sabelis, 1988; Turlings et al., 1990, 1995), indicate the presence of potential competitors, and coincide with the mobilization of direct plant defenses (Karban and Baldwin, 1997). As semiochemicals, induced volatiles may alter the recruitment of herbivores to the damaged host plant, not only by providing chemical cues for host plant location, but also information on the damage status of the host plant (Bernasconi et al., 1998). Behavioral responses of foraging lepidopterans to these cues have been examined primarily in adults because selection of an appropriate host plant for offspring is largely a consequence of female oviposition preferences rather than the consumer (caterpillar) itself (Thompson, 1988). Several species of noctuid moths preferentially oviposit on undamaged rather than damaged plants in response to volatile cues, presumably thus limiting exposure of their offspring to risks of higher predation, parasitism, and competition from older larvae, and performance costs incurred with induced plant defenses (Landolt, 1993; Anderson and Alborn, 1999; De Moraes et al., 2001). Alternatively, some adult coleopterans exploit the relatively greater volatile output of induced plants to locate hosts and conspecifics (Harari et al., 1994; Lougrin et al., 1995; Bolter et al., 1997; Landolt et al., 1999). In both cases, the greater mobility of adults allows for expanded search capabilities for suitable host plants with reduced search time, minimal search costs, and limited exposure to mortality factors (Stamps and Krishnan, 2005; Stamps et al., 2005).

Although adults are the primary life stage for dispersal of most lepidopterans, caterpillars of some species disperse from the original oviposition site and search for new host plants by ballooning (as neonates; Zalucki et al., 2002) or crawling. These larvae forage when suitable food sources are exhausted (Singer and Stireman, 2001), thus avoiding natural enemies (Bernays, 1997; Singer and Stireman, 2003), reducing competitive interactions (Kakimoto et al., 2003), limiting exposure to toxins or balancing nutrients (Singer and Stireman, 2001; Singer et al., 2002), or compensating for suboptimal oviposition choices by females (Beredegué et al., 1992; Roitberg and Mangel, 1993; Doak, 2000). These herbivores rely, to varying degrees, on visual and olfactory cues to orient to new host plants. Despite the fact that volatiles are induced in response to caterpillar attack, their reciprocal effects on the host location behaviors of the same foraging herbivores are poorly understood. The high volatile emissions associated with induced plants may serve as better olfactory cues for host plant location by slow-moving larvae in part through greater chemical apparency (Landolt et al., 2000). Conversely, larvae that select a damaged host plant usually suffer more direct and severe exposure to the deleterious effects of induced defenses and a more restricted ability to move to an alternative host plant than adults (Van Dam et al., 2001). Given the limited choices and greater consequences faced by foraging caterpillars, we investigated larval orientation responses to odors from herbivore-damaged and undamaged host plants in a highly mobile lepidopteran that disperses frequently as a caterpillar.

The fall armyworm (FAW) *Spodoptera frugiperda* (Smith) is a polyphagous noctuid that feeds on over 60 species of plants and is a periodic, but serious, pest of maize, rice,

sorghum, turf grasses, cotton, and peanuts (Luginbill, 1928; Sparks, 1979). Larvae often disperse from the original host plant, in part because of frequent and negative effects of overcrowding. Females lay up to 200 eggs in an egg mass on a single host, often exceeding the resources available to offspring (Sparks, 1979; Pitre et al., 1983; Chapman et al., 1999b). Individuals that remain on the original host plant face competition for resources and the threat of cannibalism from conspecifics (Chapman et al., 1999b). At high densities, FAW can completely defoliate their host plants and migrate in search of new hosts *en masse*, hence the name armyworm (Martin et al., 1980). To compensate, larvae disperse from the oviposition site both within and between host plants. Both neonates and older larvae will move off their host plant if food resources are exhausted or severely degraded by the presence of conspecifics. Despite the polyphagous nature of the species as a whole, selection of an appropriate new host plant can impact larval growth and development. Larval performance of individual FAWs from different host strains varies considerably among host plant species (Pashley, 1988; Meagher et al., 2004) and also can be impacted by previous damage from other herbivores (Bultman and Conard, 1998; Tindall and Stout, 2001). Given that volatiles from herbivore-damaged and undamaged plants differ quantitatively at a minimum, foraging FAW are presented with differences in olfactory cues when making a choice.

We examined FAW responses to herbivore-damaged plants in maize, *Zea mays* L., a crop plant frequently attacked by this pest. FAW caterpillars preferentially feed on leaf whorls and young seedlings (Davis et al., 1999; Meagher and Nagoshi, 2004), and stimulate the release of copious amounts of induced volatiles in response to caterpillar attack (Gouinguéné et al., 2003; Hoballah et al., 2004). The volatile profile of caterpillar-damaged maize changes quantitatively over time as individual volatile components are induced at different rates (Turlings et al., 1998). Roughly speaking, “fresh” damage odors rapidly released by mechanical damage (i.e., green leaf volatiles) can be distinguished from “old” damage odors consisting of terpenoids and other compounds slowly synthesized by the attacked plant (Turlings et al., 1998; Hoballah and Turlings, 2005). We intentionally examined old damage odors because these emissions include induced volatiles that are specifically triggered by insect attack rather than mechanical damage. We also asked whether variation in a single volatile component of old damage odors could alter responses to FAW-damaged plants. The monoterpene linalool is a common constituent of floral and foliar odors that is induced in maize seedlings some hours after initial feeding damage (Turlings et al., 1998). Linalool is attractive to *Spodoptera* spp. (Carlsson et al., 1999; Jonsson and Anderson, 1999; Deng et al., 2004) and responded to by receptor neurons of noctuid adults (Jonsson and Anderson, 1999; Malo et al., 2004; Røstelién et al., 2005). Given the similarities in adult and larval odorant binding proteins essential to olfaction reception (Laue, 2000) and the profile of FAW-induced volatiles from maize, we evaluated sixth instar FAW responses to linalool both as an individual volatile and a component of whole plant odors.

Methods and Materials

Plants

We used *Z. mays* var. Golden Queen, a commercially grown sweet corn variety that, like other sweet corn varieties, is frequently attacked by FAW in Florida (Foster, 1989; Nagoshi and Meagher, 2004). Maize seeds were planted in professional growers mix potting soil (Piedmont Pacific, GA, USA) in 4-in. square pots and maintained under ambient greenhouse light supplemented with high-pressure sodium lamps on a 12 hr light/12 hr

dark photoperiod. Supplemental lighting was used from 7:00 AM to 7:00 PM. Eleven to 14-d-old seedlings with 3–4 leaves were used in all experiments.

Insects

Fall armyworms were obtained from a predominantly corn strain colony at Center for Medical, Agricultural, and Veterinary Entomology, U.S. Department of Agriculture-Agricultural Research Service (Gainesville, FL, USA), maintained on a pinto-bean-based artificial diet (King and Leppla, 1984). One of our main concerns was that the larvae obtain sufficient feeding experience on damaged maize before the behavioral bioassays. Previous studies have indicated that prior feeding experience strongly influences both larval orientation and feeding preferences in polyphagous noctuids (Anderson et al., 1995; Carlsson et al., 1999). In preliminary experiments, we noted that naive (pinto-bean-diet-fed) sixth instars had different olfactory preferences toward damaged plants from experienced (maize-fed) sixth instars; however, one night of feeding on maize was sufficient to switch olfactory preferences. To provide larvae with adequate experience, larvae reared on pinto bean diet were transferred at the end of the fifth instar to Golden Queen maize and allowed to feed overnight. Larvae were intentionally placed on maize in sufficient numbers to ensure near total defoliation of host plant material, thereby gaining exposure to heavily damaged plants as might occur at outbreak densities. All larvae were reared at 25°C under a 16 hr light/8 hr dark photoperiod.

Treatment of Herbivore-Damaged Maize Seedlings

To obtain maize seedlings emitting “old” damage odors during the early peak feeding hours of the FAW, each plant was fed upon by 10 sixth instar FAW at 2:00 PM, 6 hr before their use in bioassays or volatile collections later in the evening (8:00 PM). Damage was inflicted under normal light conditions (previously described) to obtain maximal induction of photosynthate-dependent volatiles. Larvae were removed after 60 min to limit both the extent and the time frame of feeding damage.

Plant Volatile Collections

Because FAW is primarily nocturnal in its feeding activity (Miranda-Anaya et al., 2002), all plant volatile collections were conducted at night several hours after initial damage. Collections of volatiles emitted by maize seedlings were conducted in large glass chambers by using an automated volatile collection system (Analytical Research Systems, Gainesville, FL, USA) modified after Heath and Manukian (1994). Sampling was restricted to above-ground plant structures through the use of a guillotine base. An excess of filtered air (1 l/min) relative to the vacuum pull was added to the chamber to ensure that outside air did not contaminate volatile sampling. Volatiles were sampled for 1 hr by pulling air at 300 ml/min over the plants and through a Super Q absorbent filter (80–100 mesh, Alltech Associates, Inc., Deerfield, IL, USA). Volatiles were eluted from the Super Q absorbent with 200 μ l dichloromethane and 400 ng nonyl acetate as an internal standard.

Identification and Quantification of Plant Volatiles

Volatile analysis by positive ion electron impact gas chromatography–mass spectrometry was performed on an HP 6890 gas chromatograph coupled to an HP 5973 MS detector. One μ l of the sample was injected (240°C) onto an Agilent HP-5MS dimethylpolysiloxane

column [30 m × 250 μm (i.d.) × 0.25 μm, Agilent Technologies, Inc., Palo Alto, CA, USA] and separated by temperatures programmed from 35°C (1.0 min hold) to 230°C at 10°C/min. Helium was used as a carrier gas at 1.2 ml/min. Volatiles were identified by comparison of mass spectra with NIST and Department of Chemical Ecology, Gothenburg University (Sweden) mass spectra libraries and retention times with authentic standards.

Quantities of plant volatiles were estimated by using an Agilent 6890 GC coupled to a flame ionization detector (250°C). One microliter of the sample was injected (splitless, 220°C) onto an Agilent DB-1 dimethylpolysiloxane column [15 m × 250 μm (i.d.) × 0.25 μm, Agilent Technologies Inc., Palo Alto, CA, USA] and separated with temperatures programmed from 40°C (0.50 min hold) to 180°C at 12°C/min followed by a 220°C post run (2 min). Helium was used as a carrier gas at 1.2 ml/min.

Y-tube Olfactometer

The olfactory orientation responses of sixth instar FAW to plant and individual volatile odors were evaluated with a y-tube olfactometer. The experimental choice arena consisted of a horizontal glass y-tube (7.5 cm for each arm and base, 15 cm total length, 10 mm i.d., 12.7 mm o.d.; Chemglass, Inc., Vineland, NJ, USA) with different odor sources introduced separately at the distal end of each arm and pulled through the y-tube past the larva by a vacuum line attached at the base. Volatiles were obtained in the airstream by passing prehumidified, carbon-filtered air over the source of the odor. Volatiles were relayed from the distal odor sources to the y-tube arms by 1/4-in. OD corrugated Teflon tubing. Total airflow was maintained and monitored at 150 ml/min through each arm (0.08 km/hr) and 300 ml/min (0.16 km/hr) out of the base of the y-tube by a flowmeter (Aalborg Instruments, Orangeburg, NY, USA)-regulated Tygon vacuum line. To securely connect the glass y-tube to odor source and vacuum lines of differing diameters, rigid Teflon tubing sections of an intermediate diameter were employed as tight-fitting unions. Larvae were prevented from crawling into the odor source lines by a nylon mesh barrier secured between the Teflon union and the glass y-tube. To minimize ambient vibrations that could disturb the larva, each y-tube was secured to a heavy laminate base consisting of white foam board overlaying a brick.

Whole plant odors were sampled by the methods previously described for volatile collection. (–)Linalool (97% pure, Fluka Chemie AG, Buchs, Switzerland) was introduced into the odor source airstream by capillary release, a method that allows for predictable and stable release of a volatile from a capillary tube based on physiochemical characteristics (Weatherston et al., 1985). Appropriate volatile concentrations were obtained by diverting off and diluting (with filtered air) part of a concentrated odor before it entered the y-tube. The odors were filtered through multiple nylon mesh baffles to thoroughly mix the various volatile components before introduction to the y-tube.

Bioassays

Because FAW is primarily nocturnal in its peak-feeding activity (Miranda-Anaya et al., 2002), all bioassays and volatile collections were conducted at night. Early sixth instars that had molted within the previous 24 hr were used in the bioassays. These caterpillars were starved for 2 hr before the bioassay to obtain hungry, but mobile, larvae with feeding experience on damaged maize. We initially encountered problems with larvae that did not respond due to handling or ambient vibrations. To minimize direct handling, larvae were transferred to the olfactometer by using a detached piece of the apparatus. A single larva

was allowed to crawl into the Teflon tube union between the y-tube base and vacuum line, which was reattached in-line. The larva was then given an hour to make a choice between the odors emanating from each arm. Most larvae began moving into the airflow within a few minutes of introduction to the olfactometer. A choice preference for an odor was scored when a larva touched the mesh barrier separating the distal end of the y-tube arm from the odor source line. Larvae that remained completely motionless for 30 min were assumed to be disturbed by handling and were replaced by a new larva in a fresh Teflon tube union. Larvae that moved but did not make a choice within 1 hr were scored as neutral nonresponders and were excluded from the statistical analysis. Few larvae that moved failed to make a choice (<5%)—This low rate probably occurred because the method used to introduce each larva (crawling into a tube) favored actively mobile caterpillars. Up to 10 larvae were run in parallel in separate y-tube olfactometers.

All parts of the y-tube apparatus between the odor source and vacuum line were cleaned or replaced between bioassay trials to remove contaminants. Both the glassware and Teflon parts were thoroughly rinsed with successive washes of acetone, ethanol, ethanol-water, and Milli-Q-filtered water (Millipore, Inc., Billerica, MA, USA) to remove volatiles. Glassware was dried for 1 hr in a 160°C oven while Teflon parts were dried for at least 1 hr in a strong laminar air flow (90 l/min).

Statistics

The preference ratios obtained in the bioassays were compared for departure from a random distribution by a two-tailed binomial exact test. Neutral responses were excluded from the

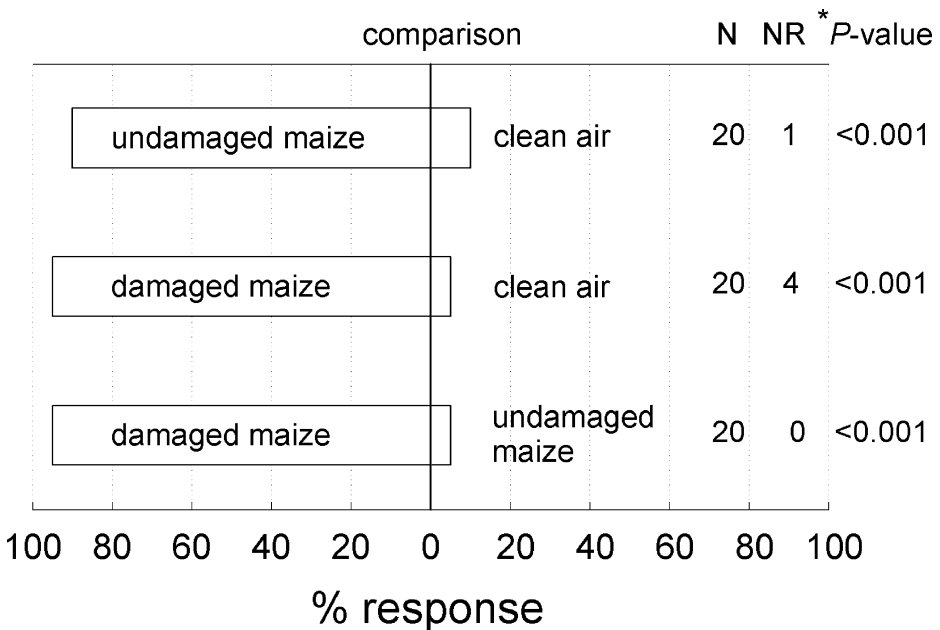


Fig. 1 Orientation responses of sixth instar *S. frugiperda* to FAW-damaged and undamaged maize seedling odors and clean air controls presented in a y-tube olfactometer. NR—number of neutral or nonresponding larvae that moved but did not score a preference for one of the odor within 1 hr. *, probability of ratio departure from a random distribution, two-tailed binomial exact test (neutral responses excluded)

analyses. Volatile component release rates were compared by a Student's *t*-test (JMP 4.0.4, SAS Institute Inc., Cary, NC, USA).

Results

Sixth instars were strongly attracted to plant odors in the olfactometer bioassays, regardless of damage status (Fig. 1). In a direct comparison, FAW preferred odors from damaged plants over those of undamaged plants (Fig. 1).

In a comparison of volatiles collected from attacked and undamaged plants 6 hr after damage, the major induced compounds were linalool and (3*E*)-4,8-dimethyl-1,3,7-nonatriene (Fig. 2). Among the 13 volatiles quantified, (*E*)-2-hexenal, myrcene, (*Z*)-3-hexenyl acetate, (*E*)- β -ocimene, linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, bergamotene, α -humulene, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene were significantly induced (Fig. 2). Other volatiles may be induced at different times after damage. Lipoxygenase products, collectively known as the green leaf volatiles, were not prominently induced at this late time point because their peak induction in maize occurs immediately after damage, then rapidly declines (Turlings et al., 1998; Hoballah and Turlings, 2005).

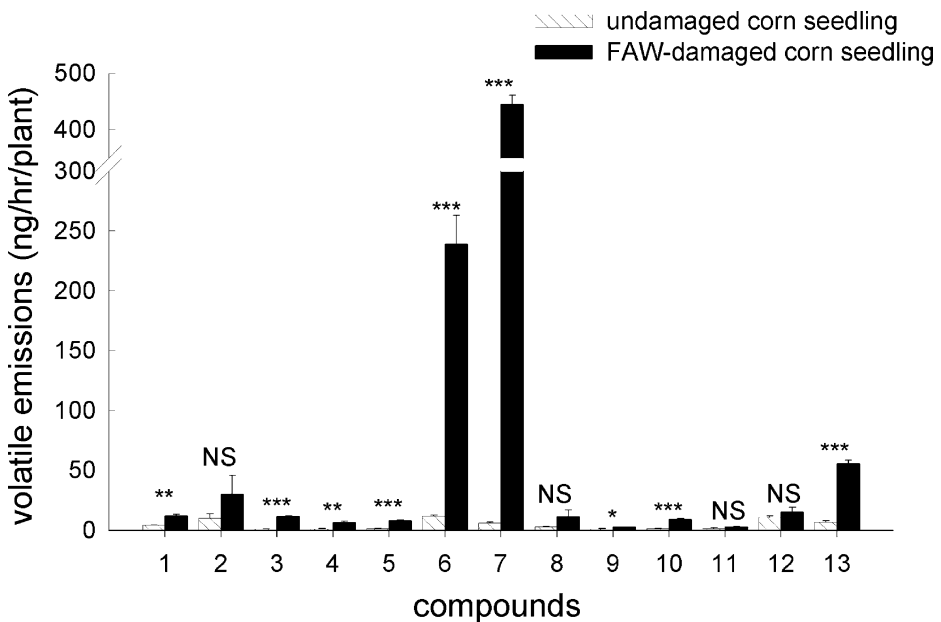


Fig. 2 Average nighttime volatile emission rates (+S.E., $N = 4$) of 13 compounds from undamaged and FAW-damaged maize seedlings (*Z. mays* var. Golden Queen) 1 hr into the scotophase. Damaged plants were fed on for 1 hr by 10 sixth instar FAWs, 5 hr before the onset of scotophase. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS (not significant) $P > 0.05$ by Student's *t*-test. (1) (*E*)-2-hexenal; (2) (*Z*)-3-hexenyl-ol; (3) β -myrcene; (4) (*Z*)-3-hexenyl acetate; (5) (*E*)- β -ocimene; (6) linalool; (7) (3*E*)-4,8-dimethyl-1,3,7-nonatriene; (8) indole; (9) (*E*)- α -bergamotene; (10) α -humulene; (11) (*E*)- β -farnesene; (12) (*E*)-nerolidol; (13) (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

Linalool was an olfactory attractant to sixth instars in the olfactometer bioassays. They were attracted to linalool rather than clean air over a broad range of volatile emissions (Fig. 3). When two release rates were presented in the olfactometer, FAW preferred the higher amount (Fig. 3). These results indicate that FAW is capable of discerning between and responding to different aerial concentrations of linalool, such as would be encountered between induced and uninduced plants.

Direct manipulation of an odor source airstream with capillary-released linalool was sufficient to alter, and in some cases, supplant orientation preferences toward whole plant odors. We first evaluated the effect of linalool supplementation on FAW responses to two split-flow airstreams originating from an undamaged plant. FAW did not demonstrate a significant preference for either airstream without supplementation, both sides representing equivalent proportions of emissions from the common source (Fig. 4). By contrast, FAW preferred a linalool-supplemented plant odor rather than an unsupplemented one (Fig. 4). In a direct comparison of odors from an undamaged maize seedling against linalool introduced at release rates equivalent to an undamaged plant, FAW significantly preferred linalool (Fig. 4).

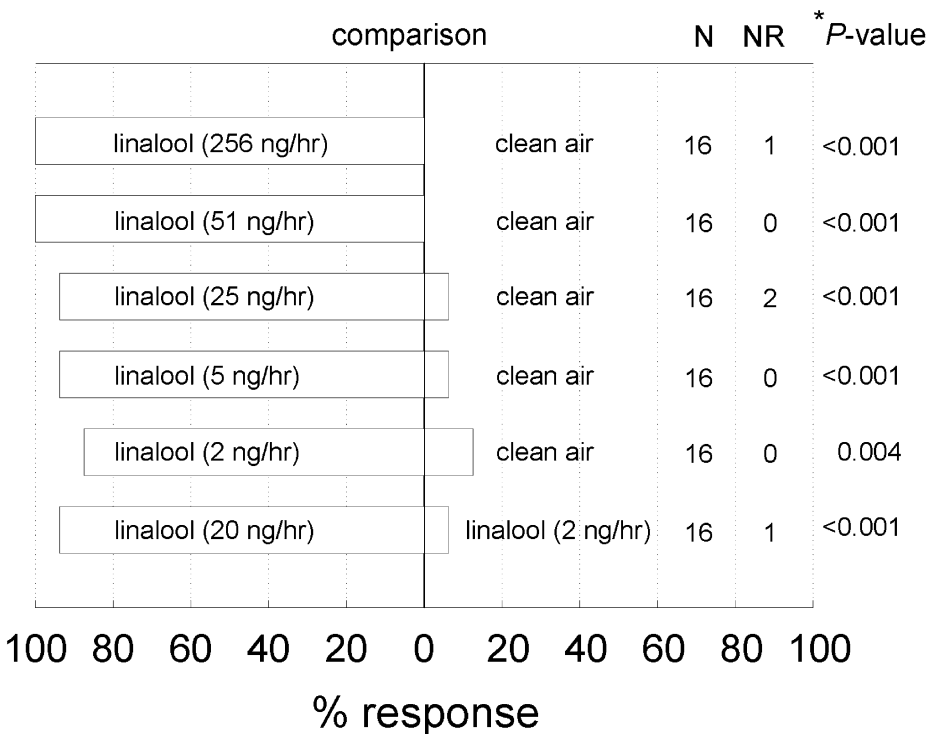


Fig. 3 Orientation responses of sixth instar *S. frugiperda* to linalool and clean air controls presented in a y-tube olfactometer. Linalool release rates (ng/hr) measured for each odor source immediately before the bioassay are given in parentheses. NR—number of neutral or nonresponding larvae that moved but did not score a preference for one of the odor within 1 hr. *, probability of ratio departure from a random distribution, two-tailed binomial exact test (neutral responses excluded)

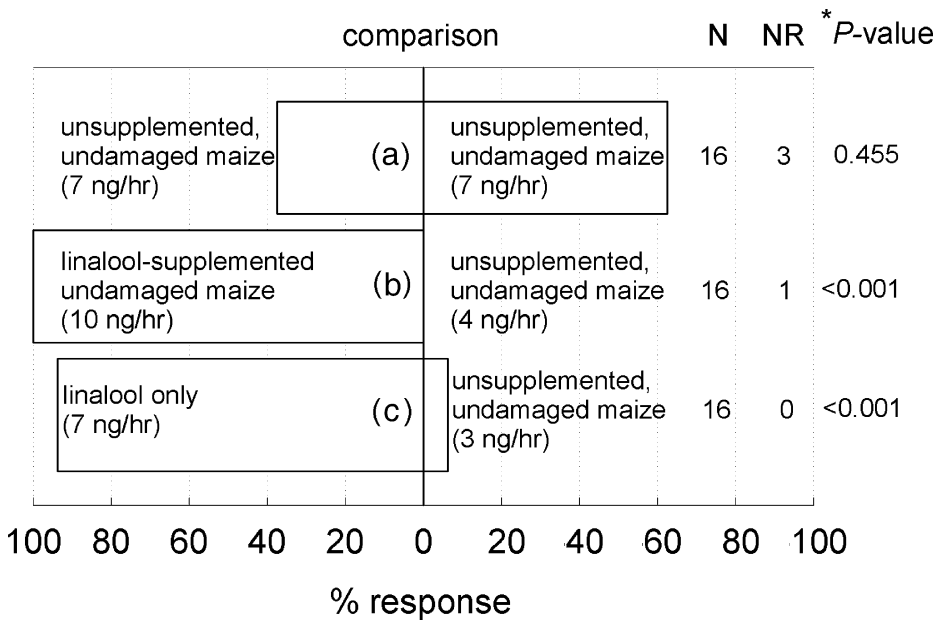


Fig. 4 Orientation responses of sixth instar *S. frugiperda* in y-tube olfactometer trials to (a) equivalent undamaged maize seedling odors originating from a single source divided by a split air flow apparatus; (b) linalool-supplemented, and unsupplemented, undamaged maize seedling odors originating from a single source divided by a split air flow apparatus; or (c) linalool and an unsupplemented, undamaged maize seedling odor. Linalool release rates (ng/hr) measured for each odor source immediately before the bioassay are given in parentheses. NR—number of neutral or nonresponding larvae that moved but did not score a preference for one of the odor within 1 hr. *, probability of ratio departure from a random distribution, two-tailed binomial exact test (neutral responses excluded)

Discussion

The olfactory orientation preferences of sixth instars for linalool over whole undamaged plant odors indicate that a single volatile component can alter preferences toward damaged-plant odors. These preferences could be attributed to attraction to higher amounts of linalool in damaged plants (against undamaged plants) and in the supplemented airstream (against undamaged plants). Our bioassays demonstrated that sixth instars responded favorably to a 10-fold difference in capillary-released linalool concentration (2 ng/hr against 20 ng/hr), of relevance to the average amounts released by undamaged (12 ng/hr) and induced (239 ng/hr) maize seedlings. Our split flow bioassays demonstrate that a preference developed as a result of the addition of an attractant to otherwise equivalent, if already attractive, odor streams. If strong orientation biases for individual volatile components over the plant odor exist, such olfactory preferences could be exploited in trap crop strategies designed to move insects away from valuable crop tissues to expendable plants (Shelton and Badenes-Perez, 2006).

However, linalool is unlikely to be the only volatile from maize seedlings that could evoke behavioral responses in sixth instar FAW, due in part to the role of feeding experience in orientation behaviors of generalist noctuid larvae. Olfactory preferences of polyphagous noctuids, such as FAW, are strongly influenced by exposure to odors during feeding (Anderson et al., 1995; Carlsson et al., 1999; Glendinning, 2002). Experience-based preferences are thought to confer a certain degree of behavioral plasticity on the development of host location

and feeding cues in individual generalist larvae that feed preferentially on specific hosts. Carlsson et al. (1999) demonstrated that either linalool or geraniol could serve as olfactory attractants to third instar *Spodoptera littoralis*, provided that the larvae had previous feeding experience in the presence of the volatile. We contend that while linalool is an olfactory attractant to experienced sixth instars, other induced volatiles may serve a similar role in preferences for damaged maize seedlings and should be evaluated as such in future studies.

Perhaps the most striking aspect of our results is that sixth instars are strongly attracted to herbivore-damaged rather than undamaged host plant odors, despite the disadvantages of association with a damaged host plant (Bolter et al., 1997) and the intensity of density-dependent mortality factors in this species. Like many caterpillars, FAW experiences significantly higher predation rates in association with severely damaged plants (Chapman et al., 2000). Several major parasitoids of FAW have been shown to be specifically attracted to volatiles from caterpillar-damaged maize (Turlings et al., 1995; Gouinguéné et al., 2005; Hoballah and Turlings, 2005). Furthermore, intraspecific competition and conflict are intense in later FAW instars. FAW frequently engages in cannibalism even when food resources are adequate, resulting in an estimated 40% to 60% mortality rate among larvae (Chapman et al., 1999a). Consequently, later instars rarely cohabit in the same leaf whorl and readily attack conspecifics, particularly younger instars. Chapman et al. (1999b) noted that the mere presence of conspecifics reduced larval performance, even when cannibalism was prevented. One consolatory benefit of sixth instar preferences for damaged plants is that asymmetrical encounters between foraging sixth instars and any earlier instar occupying the damage site is likely to favor the older instar (Chapman et al., 1999b; Kakimoto et al., 2003). Otherwise, the advantages of being attracted to a damaged plant when there is an undamaged alternative remain unclear.

One mechanistic constraint that favors attraction to damaged plant odors is the extent of prior feeding experience on attacked plants. Caterpillars feed primarily on attacked plant tissues previously damaged by themselves. Induction of monoterpenes in maize is highly localized to the wounded leaf rather than the whole plant (Turlings and Tumlinson, 1992), so a sixth instar feeding on different leaves experiences repeated inductions of monoterpenes, including linalool, as part of its olfactory experience. Future studies should examine whether plant-reared larvae that are prevented from experiencing induced volatiles (i.e., by frequent movement or use of a mutant host plant deficient in induced volatile responses) prefer herbivore-damaged plants over undamaged plants. Due to their reliance on experience as a mechanism for preference, polyphagous caterpillars such as FAW may fail to realize an optimal choice (orient to a undamaged seedling) because of preferences for more familiar host odors.

Because volatiles of attacked and unattacked Golden Queen maize seedlings vary quantitatively, but not qualitatively, it is not clear whether the observed responses indicate a true orientation preference for damaged plants or simply attraction to a greater source of host volatiles. Orientation to higher amounts of volatile attractants may serve as a mechanism for assessing how close two volatile sources are to the foraging caterpillar, with damaged plants appearing “closer” by virtue of higher volatile output. Other foraging caterpillars use appearance for relative assessment, rather than direct measurement, of distance to select between two competing stimuli. Diurnally foraging caterpillars that orient visually to host plant shapes cannot distinguish between nearby small objects and distant large objects that appear to be of the same size (share the same visual angle; Saxena and Khattar, 1977; Marchand and McNeil, 2004).

Paradoxically, a preference for odors from suboptimal, but detectable, damaged hosts might be a behavioral strategy to cope with the uncertainties of host plant location. Foragers that experience high mortality and costs while searching for ephemeral hosts can be expected to have host-seeking behaviors that emphasize detectability over quality (Ward,

1987; Stamps and Krishnan, 2005; Stamps et al., 2005). In contrast to highly mobile flying adults, the set of potential hosts available to a foraging caterpillar is greatly reduced and widely dispersed due to the “small-scale” search capabilities imposed by the slow speed of crawling (Singer and Stireman, 2001; Stamps et al., 2005). To a foraging caterpillar with limited mobility and search capabilities, the costs encountered with not locating an appropriate host plant may outweigh the risks associated with an induced host plant. Caterpillars searching for a new host plant face mortality from predation, exposure, and starvation as well as sublethal opportunity costs incurred by slow search capabilities and uncertain quality of the new host plant (Sih, 1993; Zalucki et al., 2002; Kaitaniemi et al., 2004). Foraging neonate codling moth *Cydia pomonella* prefer odors from apples damaged by conspecifics over undamaged fruits, in part because damaged fruit release greater amounts of the olfactory attractants, such as α -farnescene, that increase detectability (Landolt et al., 2000). Induced volatiles from an attacked maize seedling may be more apparent, and, therefore, more reliable, olfactory cues for a foraging FAW caterpillar. An emphasis on reliability and detectability at the expense of quality may also explain olfactory preferences for widely produced but relatively nonspecific volatile cues such as linalool by FAW (Stamps and Krishnan, 2005; Stamps et al., 2005). Attraction to induced volatiles may be particularly advantageous to foraging FAWs in the location of host plants that occur in patchy distributions, including agroecosystem and pasture monocultures. Enhanced foraging success may occur directly through perception of the greater aggregate volatile output produced by several neighboring damaged plants, or indirectly through the association of undamaged plants with induced volatile cues from highly detectable herbivore-damaged conspecifics (associational susceptibility; Rodriguez-Saona and Thaler, 2005).

Attraction to a damaged host plant may also be advantageous if the caterpillar can evade some of the consequences associated with induced tissues by feeding away from the damage site. Although our results indicate that sixth instar FAW preferentially orient to the odors of herbivore-damaged plants over undamaged plants, it remains unknown if these preferences hold once the caterpillar locates and comes into contact with the plant. The olfactory preferences reported here may represent a mechanism for host plant location, but not necessarily host acceptance and feeding. Orientation to a distant host plant by definition is restricted to sensory modalities that do not require contact. By contrast, acceptance of a host plant and establishment of a feeding site on a plant involves feeding stimuli received from olfactory, gustatory, and tactile assessment of the tissue (Schoonhoven and van Loon, 2002). Host plant location and acceptance behaviors toward damaged plants are partially decoupled in other lepidopterans. Studies on related noctuid caterpillars in other plants indicate that larvae tend to move away from herbivore-damaged or induced plant tissues, if they move at all on the plant (McAuslane and Alborn, 2000; Anderson et al., 2001). Female *Trichoplusia ni* moths orient toward herbivore-damaged rather than undamaged plants, but preferentially oviposit on undamaged plants when given a choice (Landolt, 1993, 2001). Our preliminary observations indicate that sixth instar FAWs do not display strong preferences for damaged leaves over undamaged leaves when they select an initial feeding site. Such a switch in behavioral responses to damage would be consistent with changes in risk assessment expected for dispersers (Stamps and Krishnan, 2005; Stamps et al., 2005)—Once a host plant is contacted by a foraging caterpillar, the risks associated with host plant location and foraging diminish, leaving only the disadvantages inherent in an herbivore-damaged plant.

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Introduction

Iridoids, cyclopentanoid terpenes, are an important group of secondary defensive plant compounds known to deter feeding or decrease growth rates in many generalist insect herbivores (Bowers and Puttick, 1988; Harvey et al., 2005); they are also bitter tasting and have emetic effects on vertebrates (Seigler, 1998). However, iridoids are often feeding stimulants for the larvae of specialist butterfly species. The sequestration of iridoids by these larvae and their subsequent retention during the adult phase (e.g., Bowers, 1991; Dyer, 1995) have been shown to provide larvae with chemical defense mechanisms for deterring generalist predators, such as birds, ants, wasps, and spiders (e.g., Camara, 1997; Theodoratus and Bowers, 1999).

In addition, iridoids have also often been considered as oviposition cues for specialist butterfly species, which can benefit from the negative effects these compounds have on predators and parasitoids (Pereyra and Bowers, 1988). Recently, it was shown that *Plantago lanceolata* plants chosen for oviposition by the specialist butterfly *Melitaea cinxia* have higher concentrations of iridoids than plants not chosen (Nieminen et al., 2003). Although these results may indicate that ovipositing females select plants with the highest iridoid concentrations, it is also possible that it is the act of oviposition that induces iridoid glycoside production (Nieminen et al., 2003). The induction in plants of other chemical defenses—for example, volatile emissions—has been described elsewhere (Hilker et al., 2002, 2005; Colazza et al., 2004). There is also some indication that the production of iridoid glycosides in *Plantago lanceolata* leaves may be induced by herbivory, although the variation in concentrations with leaf age appears to be much greater (Darrow and Bowers, 1997; Stamp and Bowers, 2000).

Our objective was to test the hypothesis that the specialist butterfly *Euphydryas aurinia* (Lepidoptera: Nymphalidae) lays eggs on leaves of *Lonicera implexa* plants with higher iridoid concentrations. We conducted the study in a Mediterranean site under field conditions with naturally occurring egg clusters. We compared iridoid concentrations in leaves with and without eggs from *L. implexa* plants that had been chosen for oviposition with those in leaves of plants with no *E. aurinia* egg clusters.

Methods and Materials

Studied System: Butterflies, Plants, and Experimental Design

Euphydryas aurinia (Lepidoptera: Nymphalinae, Melitaeini) is a univoltine butterfly occurring throughout Europe, temperate Asia, and North Africa (Tolman and Lewington, 1997). In most of northern and central Europe, it is monophagous on *Succisa pratensis* (Dipsacaceae), whereas in the southern part of its range it uses a diversity of hosts, mainly from the families Dipsacaceae (e.g., *S. pratensis*, *Knautia arvensis*, *Scabiosa columbaria*, and *Cephalaria leucantha*) and Caprifoliaceae (*L. implexa* and *Lonicera etrusca*; Mazel, 1986; Kankare et al., 2005). Despite this diversity of hosts, most populations are monophagous on a local scale (Singer et al., 2002). For instance, in Catalonia, most populations from typical Mediterranean habitats (such as the one studied in this article) are monophagous on *L. implexa*.

During the flight period from mid April to early June, females lay large egg batches of about 200–300 eggs on the underside of leaves of *L. implexa*. Larvae hatch in synchronization within 3 wk and spin a large silken web around the leaves, within which

they feed for about 3 wk. Immediately after their third molt, larvae enter diapause within a winter web (usually at the base of the plant) and do not resume feeding until early next spring. Pupation takes place at the end of the sixth instar, normally by the end of March or early April. This life cycle resembles most other Melitaeini butterflies (see Kuussaari et al., 2004 for a comprehensive review).

During the growing season in June 2004, *L. implexa* plants bearing *E. aurinia* egg clusters were searched for in Olesa de Bonesvalls in the Garraf Natural Park (Barcelona province). It was known from previous research that a good population of *E. aurinia* occurs in this area, which is dominated by evergreen oak (*Quercus ilex*) forest and holly oak (*Quercus coccifera*) shrubland. There is also a high density of *L. implexa*, an evergreen climber usually with several shoots that grows either in dense scrub and forests or as an isolated plant in hedgerows and on slopes.

In 2004, the flight period of *E. aurinia* occurred between the second week of May and the second week of June (as assessed by weekly counts following the standard methodology of the Catalan Butterfly Monitoring Scheme; see Stefanescu, 2000). In early June, right at the end of the flight period, we searched for plants of *L. implexa* bearing egg clusters laid by *E. aurinia*. For this purpose, all plants along a 1-km transect were monitored. Previous observations had indicated that females rarely oviposit on plants growing in dense clumps of scrub, and so searching was restricted to plants growing more or less in isolation (e.g., on the edge of paths). In all, 18 plants were investigated, half of which bore egg clusters. The egg masses were between 1 and 2 wk old.

Once an egg cluster was found, half of the leaf bearing the eggs (the half without eggs to avoid damaging the egg batches) was taken. Then, half of the leaf immediately opposite the leaf with the egg cluster was also sampled. In addition, four to five half leaves from the same plant, all with similar morphological traits (age, size, and color) as the leaves with egg clusters but with no signs of oviposition, were also sampled. Likewise, adjacent plants without any oviposition were also sampled.

In the field, we placed the leaf samples in paper bags and then left them in open plastic boxes between sheets of tissue to dry. In the laboratory, we measured the iridoid content of the four groups: leaves from *L. implexa* plants with no oviposition (9 groups), leaves without oviposition from plants with oviposition present (9 groups), leaves with egg clusters from these same plants (9), and the leaves immediately opposite those leaves with egg clusters (9).

Iridoid Analyses

Air-dried leaf samples were ground with a mill, and stored at -27°C until extraction. We used the hot water extraction method described by Suomi et al. (2000) and Nieminen et al. (2003). Five ml of hot water (90°C) were added to 50 mg of ground sample and then placed for 30 min in an ultrasonic bath with temperature control (TRANSSONIC 570H, Elma group, Pforaheim, Germany). Extract solutions were filtered (Millipore, in $0.22\ \mu\text{m}$ nitrocellulose), and the filtered liquid was evaporated with a rotavapor model LABO ROTA B300 (Resona Technics, Gossau, Switzerland). The residue was diluted to 2 ml with water.

Iridoid compounds were detected by combined high-performance liquid chromatography (HPLC)/mass spectrometry (MS) analysis and fully characterized by high-resolution multidimensional nuclear magnetic resonance (NMR) spectroscopy. We used two different HPLC elution modules to establish the experimental elution method and to validate it. We used a WATERS 2695 separation module with temperature furnace with PDA detector WATERS 2996, and an AGILENT 1100 separation module with PDA AGILENT 1100. The

column utilized was a XTERRA MS C185 (4.6 × 100 mm) of WATERS in the two HPLC equipments. On-line filters (of 0.5 μm) were used as precolumns. Twenty-eight different HPLC elution conditions were tested with a reverse phase column. The method that provided the best separation of the eight highest peaks that were present in all analyzed samples had the following elution conditions: The carriers were A (0.1 KH₂PO₄ in water) and B (acetonitrile), the elution process was 0–9 min gradient of 96% A–4% B until 91% A–9% B at 0.8 ml min⁻¹, 9–27 min isocratic 91% A–9% B at 1.2 ml min⁻¹, 27–29 min gradient 91% A–9% B until 25% A–75% B at 1.2 ml min⁻¹, 29–33 min isocratic 96% A–4% B isocratic at 0.8 ml min⁻¹.

All NMR measurements were carried out at 500.13 MHz on a DRX500 BRUKER spectrometer (Bruker Biospin, Rheinstetten Germany) equipped with a 5-mm triple-resonance inverse TXI probe. All data were acquired and processed using the TOPSPIN v1.3 software package (Bruker Biospin). NMR analysis and assignments of complex mixture samples were also performed at 298 K by concerted use of the following modern 2D NMR measurements: 2D ¹H-¹H COSY, 2D ¹H-¹H TOCSY, 2D ¹H-¹H NOESY, 2D ¹H-¹³C HSQC with multiplicity editing, and 2D ¹H-¹³C HMBC. When undesired overlapping effects occurred, chemical shift assignments and coupling constant determinations were individually confirmed by performing the analogous and better-resolved selective 1D NMR measurements. Solvent presaturation techniques were used in all measurements when required to minimize the residual solvent signal.

The iridoid quantification was conducted by using a calibration curve of loganin standard. The loganin standard was injected at 0, 50, 100, 150, 200, 500, and 1000 ppm. We used the mean of four chromatograms for each concentration.

The acetonitrile (Panreac, Barcelona, Spain) was of HPLC grade, while the water was purified on a Milli-Q system from Millipore (Bedford, MA, USA). The KH₂PO₄ was purchased from Fluka (Buchs, Switzerland). All solvents were previously filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA). Loganin standards were purchased at Extrasynthesis (Genay, France). Secologanin was purchased at Phytoconsult (Gorlaens Lab., Leiden, The Netherlands).

Statistical Analyses

We used independent and paired *t*-tests, one way ANOVAs, and Bonferroni *post hoc* tests (Statistica, StatSoft Inc., Tulsa, OK, USA) to compare the leaf iridoids of the different groups of leaves (9 leaves with egg clusters, 9 leaves directly opposite the previous ones, 9 groups of leaves with no egg clusters from plants with oviposition, and 9 groups of leaves from plants without oviposition).

Results and Discussion

We detected eight different iridoids in the sampled plants (Fig. 1), of which secoxyloganic, loganic, and, above all, secologanic acids were the most abundant (Figs. 1 and 2).

Iridoid concentrations in the leaves of plants with no oviposition (1.00% dry weight ± 0.11 S.E.) and in leaves without oviposition from plants with oviposition (2.2% dry weight ± 1.52 S.E.) were in the range previously described from Finland and the United States in other studies of related plant species [aucubin concentration 1.6–2.7% in Bowers (1991), 0.5–5% in Darrow and Bowers (1997), or 0.6–2.2% in Nieminen et al. (2003); catalpol concentrations 0.4–3.6% in Bowers (1991), 0.2–2.2% in Darrow and Bowers (1997), 0.7–2.0% in Nieminen et al. (2003), or 0.01–1.41% in Willinger and Dobler (2001)].

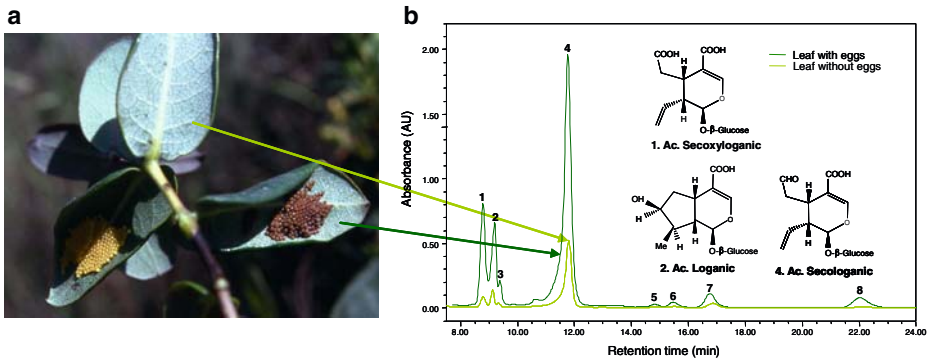


Fig. 1 Increased iridoid concentrations in *L. implexa* leaves used for oviposition by the butterfly *E. aurinia*. The picture (a) shows 1-wk-old (light) and 2-wk-old (dark) egg clusters. The HPLC chromatograms (b) were obtained from foliar hot water extracts

The leaves with oviposition, however, had mean concentrations of all eight detected iridoids that were on average 15 times higher than those found in the leaves without oviposition from the same plant (including the leaves directly opposite those with oviposition and leaves from neighboring plants with no oviposition; $P < 0.05$, Student's *t*-test; Figs. 2 and 3).

The lack of any significant differences in iridoid glycoside concentrations between leaves from plants that did not receive any eggs and the unused leaves from plants receiving eggs (Figs. 2 and 3) would seem to indicate that *E. aurinia* butterflies do not choose plants for oviposition according to their iridoid content.

Nevertheless, *E. aurinia* butterflies might choose the leaves of a plant with the highest iridoid concentrations, although it is highly unlikely that this huge 15-fold difference in iridoid concentrations between leaves with oviposition and leaves directly opposite without oviposition on the same stem—which are clearly out of the common range (Bowers, 1991; Darrow and Bowers, 1997; Willinger and Dobler, 2001; Nieminen et al., 2003)—can be explained by any other process than that of the stimulation of iridoid production by oviposition. Previous studies have also reported plant responses to egg deposition, which include volatile emissions (Colazza et al., 2004; Hilker et al., 2005) and changes in photosynthesis rates (Schroeder et al., 2005).

Further studies are needed to discern how eggs or oviposition signal a leaf to increase its iridoid production; in other words, we need to identify the elicitors responsible for the apparently oviposition-induced change or at least to find out what the kinetics are that are operating behind the changes in iridoid content that occur after oviposition.

Whatever the mechanism, leaves with oviposition were full of defenses (15% leaf dry weight) that might be used by hatching larvae, because iridoids have been shown to be sequestered by caterpillars in other *Euphydryas* species and to act as a chemical defense against natural enemies (e.g., Bowers, 1980, 1981; Bowers and Puttick, 1986; Stermitz et al., 1986, 1994; Franke et al., 1987; Gardner and Stermitz, 1988; Belofsky et al., 1989; L'Empereur and Stermitz, 1990). First instars are the most prone to be attacked by the summer generations of specialist parasitoids such as *Cotesia melitaeorum* and *C. bignellii* (M. R. Shaw, S. van Nouhuys and C. Stefanescu, pers. obs.), which may cause important mortality rates in butterfly populations (Porter, 1983). These parasitoids, however, seem to avoid or perform poorly in host larvae in other Melitaeini species with high iridoid contents

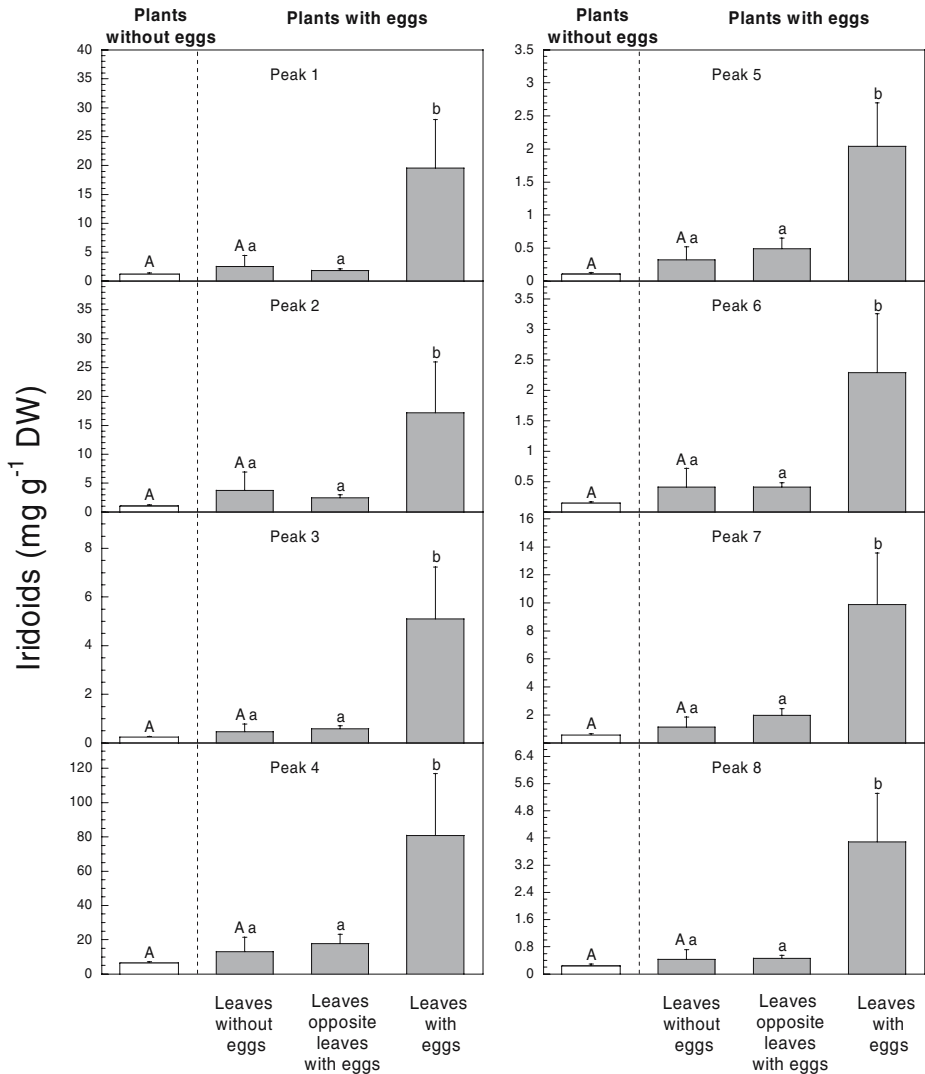
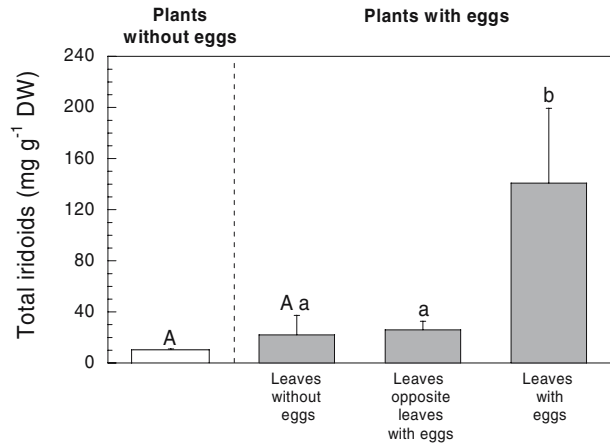


Fig. 2 Concentrations of the different iridoids [the most abundant were fully characterized by high-resolution multidimensional NMR spectroscopy, secoxyloganic (peak 1), loganic (peak 2), and secologanic (peak 4) acids; mean + S.E., $N = 9$] found in *L. implexa* plants with no egg clusters and in leaves with and without egg clusters from plants with oviposition by *E. aurinia*. The letters a and b above the bars indicate statistically significant differences ($P < 0.05$, paired *t*-test) between leaves from the same plants with and without eggs. The capital letter A indicates statistically nonsignificant differences ($P > 0.05$, *t*-test) between plants with and without egg clusters

(Nieminen et al., 2003). Because specialist herbivores seem to be able to sequester secondary compounds from their host plants and incorporate them in their own defense systems, the results presented here may mean that herbivores are able to change their hosts' physiology to make them more suitable for their own defensive purposes. Further studies are needed to assess whether any of the iridoids found in *L. implexa* are actually sequestered by *E. aurinia* larvae, especially in light of Bowers and Williams' (1995)

Fig. 3 Total iridoid concentrations (mean + S.E., $N = 9$) in *L. implexa* plants with no oviposition and in leaves with and without egg clusters from plants with egg clusters. The letters a and b above the bars indicate statistically significant differences ($P < 0.05$, paired *t*-test) between leaves with and without eggs from the same plants. The capital letter A indicates statistically nonsignificant differences ($P > 0.05$, *t*-test) between plants with oviposition and plants without



findings that larvae of *E. gillettii* feeding on another *Lonicera* species were unable to sequester its secoiridoids.

In any case, there are other possible positive effects of the huge amounts of iridoids in leaves with egg clusters. Even if they do not sequester the toxin, larvae could benefit from the host's defense mechanisms by using a leaf that will not be used by generalist herbivores; that is, they would avoid competition for food by using a toxic host.

There are also, however, possible negative effects for the specialist butterfly *E. aurinia*. It is possible that this leaf response—an extremely high and localized production of iridoids—is specifically in response to *E. aurinia*, although it is a specialist on the plant. Iridoid levels must be reached at some point that are too much even for specialist herbivores and will overcome their sequestration capacity and their toxin tolerance capacity. If defenses against generalists are used against a specialist, it will not be cost-effective for the plant to have systemic response mechanisms, and so responses may be only local. This was what we found. Although, the iridoid contents of leaves without oviposition from plants with oviposition tended to be greater than those of leaves from plants with no oviposition, thereby providing a slight indication of systemic reaction (Figs. 2 and 3), as reported, for example, in the induction of plant synomones after oviposition by a phytophagous insect (Meiners and Hilker, 2000), the differences were small and statistically not significant.

Like all plant secondary compounds (e.g., Bowers et al., 1992; Herms and Mattson, 1992; Adler et al., 1995; Peñuelas and Estiarte, 1998), iridoid presence and concentrations often vary considerably in space and time between leaves, plants, and populations in accordance with genetic, developmental, and environmental drivers such as time of day, weather, and soil nutrient conditions (e.g., Teramura, 1983; Bowers et al., 1992; Adler et al., 1995; Darrow and Bowers, 1997; Seigler, 1998; Marak et al., 2000). These results show that oviposition may also be a strong driver of increased foliar iridoid concentrations.

In summary, these results show that *L. implexa* leaves bearing egg clusters had dramatically greater (over 15-fold) concentrations of iridoid glycosides than directly opposite leaves on the same plant and leaves on plants that bore no eggs. These huge and localized foliar iridoid concentrations were likely the result of a plant response to egg deposition. Further studies are needed to definitively prove the induction, to elucidate the elicitor mechanism of the increased production, to check the possible sequestration of iridoids by the larvae, to study the responses of the herbivore predators to this possible

sequestration, and to figure out whether such huge amounts of iridoids are beneficial or not to herbivores.

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take the seeds to sites with improved conditions for germination and survival (Andersen, 1988; Bond et al., 1991; Espadaler and Gomez, 1996, 1997). Within the ant nest, seeds may be protected from fire and predators (Handel and Beattie, 1990; Bond et al., 1991; Christian and Stanton, 2004).

However, not all ants that carry seeds are plant mutualists. Granivorous ants take a wide range of seeds and consume the majority, although they may have a minor dispersal role by dropping some seeds (Hulme, 2002; Retana et al., 2004). Granivores tend to have a specialized seed diet (Hölldobler and Wilson, 1990), while mutualistic ants are generally scavengers with a predominantly invertebrate diet (e.g., *Myrmica*, *Formica*, *Lasius*) and collect only seeds with an elaiosome, which they remove and leave the seed unharmed (Hölldobler and Wilson, 1990; Gomez and Espadaler, 1998).

Thus, elaiosomes are a specific adaptation that facilitates non-predatory seed dispersal. A wide variety of tropical and temperate plants are myrmecochores (i.e., have an elaiosome)—currently 2100–3000 species in 60–80 families (Brew et al., 1989; Hughes and Westoby, 1992; Gomez and Espadaler, 1998)—and four of the seven ant families distribute seeds (Beattie, 1991). Elaiosomes are usually white or yellow appendages on the seed coat (Marshall et al., 1979) and are nutrient-rich, containing lipids, proteins, starch, sugars, vitamins (Kusmenoglu et al., 1989; Beattie, 1991; Lanza et al., 1992; Ohkawara et al., 1997), and essential nutrients that cannot be synthesized by ants, such as linoleic acid and sterols (Gammans et al., 2005).

Many studies have concluded that the elaiosome alone attracts mutualistic ants and induces them to carry the seed. Seeds with the elaiosome removed are often ignored in comparison with intact seeds or separated elaiosomes (Kjellsson, 1985; Skidmore and Heithaus, 1988; Brew et al., 1989). However, the mechanism by which the elaiosome affects ant behavior is not well understood. One hypothesis is that ants are responding simply to a food stimulus (Brew et al., 1989), but the chemical behavior-releaser hypothesis proposes that one or more chemical compounds from the elaiosome induces seed-carrying behavior (Sheridan et al., 1996). Several studies have suggested that this chemical inducer may be the fatty acid oleic acid or the diglyceride 1,2-diolein (Wilson et al., 1958; Marshall et al., 1979; Gordon, 1983; Skidmore and Heithaus, 1988; Brew et al., 1989; Kusmenoglu et al., 1989; Lanza et al., 1992; Table 1). For example, Marshall et al. (1979) investigated the interaction between the ant *Aphaenogaster rudis* and the plant *Viola odorata*. Ants were found to react most strongly to lipid fractions in bioassays, and further fractionation of lipids into fatty acids, sterols, and diglycerides showed that the last induced the greatest reaction from *Aphaenogaster*. An alternative hypothesis to chemical cues is that the brightly colored elaiosome acts as a visual attractant (Davidson and Morton, 1981), but this has not been tested.

In general, studies of elaiosome attraction of ants have not differentiated between granivorous and mutualistic ants. In fact, certain ant species tested, such as *Pogonomyrmex*, *Pheidole*, *Rhytidoponera*, and *Messor* (see Rice and Westoby, 1986; Gomez and Espadaler, 1998), are usually seed eaters. If elaiosomes attract all ants equally, there is clearly a trade-off between the benefit of attracting mutualists and the disadvantage of attracting predators. There should be a selective advantage in being able to attract only mutualists. However, there has been no comparison of the attractiveness of elaiosome chemicals to mutualistic vs. granivorous ants.

Myrmica ruginodis is a mutualistic disperser of a wide range of myrmecochores (Kjellsson, 1985; Mark and Olesen, 1996; Heinken, 2004), including *Ulex* species on European lowland heaths (Stokes et al., 2003; Gammans et al., 2005). *Tetramorium*

Table 1 Studies that have suggested elaiosome chemicals may attract ants

Author	Plant species	Ant species	Chemical identified
Bresinsky (1963)	<i>Melampyrum</i> spp. <i>Melica nutans</i> <i>Luzula pilosa</i> <i>Veronica hederifolia</i> <i>Viola odorata</i> <i>Viola ambigua</i> <i>Viola lutea</i> <i>Viola elatior</i>	<i>Lasius fuliginosus</i>	Ricinoleic acid
Marshall et al. (1979)	<i>Viola odorata</i>	<i>Aphaenogaster rudis</i>	Diglyceride (suggest 1,2 diolein)
Skidmore and Heithaus (1988)	<i>Hepatica americana</i>	<i>Pogonomyrmex rugosus</i>	Diglyceride
Brew et al. (1989)	<i>Acacia myrtifolia</i> <i>Tetraloche stenocarpa</i>	Ten unnamed species	1,2-diolein
Kusmenoglu et al. (1989)	<i>Jeffersonia diphylla</i> <i>Sanguinaria canadensis</i> <i>Trillium sessile</i> <i>Dicentra cucullaria</i>	No ant species tested	Diglyceride (suggest 1,2-diolein)
Lanza et al. (1992)	<i>Trillium erectum</i> <i>T. grandiflorum</i> <i>T. undulatum</i>	No ant species tested	Oleic acid

caespitum is a granivore that eats seeds of many plants including *Ulex* spp. (Brian, 1977; Skinner, 1987). In this article, we used laboratory colonies of *M. ruginodis* and *T. caespitum* to test the following hypotheses. (1) Chemicals on the surface of the elaiosome attract the mutualist *M. ruginodis* to the seed, but the granivore *T. caespitum* does not respond to these chemical cues. (2) *M. ruginodis* responds most strongly to the diglyceride fraction. (3) The yellow elaiosome on *Ulex* seeds is a visual cue for *M. ruginodis*. Our expectation was that patterns of response of either *M. ruginodis* or *T. caespitum* to elaiosome chemical extracts should be similar for both *Ulex* species.

Methods and Materials

Study Species

In southern England, *Ulex minor* produces seeds from the end of May for approximately 2 wk and *U. europaeus* from the middle of June until the end of July. The seeds are ejected explosively from pods that contain one to two (*U. minor*) or three to six (*U. europaeus*) seeds. Ants collect the seeds where they fall. *U. minor* seed length without the elaiosome is 2.0 ± 0.1 mm (mean \pm S.E.). The elaiosome length is 1.2 ± 0.1 mm. *U. europaeus* seeds are slightly longer, 2.2 ± 0.1 mm, and the elaiosome is 1.2 ± 0.1 mm.

Myrmica ruginodis is a red ant 3–6 mm in length that forms polygynous (multiple queen) colonies with between 18 and 6560 workers (Wardlaw and Elmes, 1996). *Myrmica* species are generally scavengers with ~80–90% of their diet made up of homopterans, hemipterans, dipterans, and arachnids, but they are also known to collect plant matter (Brian, 1977). In heathland habitats, nests of *M. ruginodis* are found under small heather (*Calluna*, *Erica*) or gorse (*Ulex*) bushes, and they forage in close proximity to *Ulex* species

(Gammans et al., 2005). In contrast, *T. caespitum* eats seeds of grasses, *Calluna vulgaris*, *Erica cinerea*, and *Ulex* spp. on English heaths, although it will occasionally eat insect prey (Brian, 1977). It is a small black ant, typically 2–3 mm in length, which forms colonies of up to a few thousand workers (Skinner, 1987) in open habitats on southern English heaths (Brian, 1964).

Collection of Study Species

In 2004, ripe seed pods of *U. minor* and *U. europaeus* were collected from three heathland sites in the county of Dorset, southern England. Seed pods were stored in paper bags at 4°C. Seeds were extracted when needed. During March 2004, seven *M. ruginodis* colonies were collected from four Dorset heathlands. These colonies were split to form 40 laboratory nests comprising 50 workers and ~20 larvae. Larvae were added to all nests to encourage the workers to forage for food. Each nest was placed into a nest box of 22 × 22 × 10 cm, which was attached to another smaller “foraging box” of 7 × 7 × 4 cm by a 20-cm-long plastic tube. Six *T. caespitum* colonies were collected from four heathlands in Dorset. Thirty laboratory nests (50 workers with 20 larvae) were set up in the same way, except that the boxes were slightly smaller at 12 × 8 × 2 cm and 7 × 4 × 2 cm, due to the smaller size of this species.

All colonies were fed each week with a standard diet of white sugar and *Drosophila melanogaster* larvae placed into the foraging box, and were watered regularly (Brian and Abbott, 1977; Elmes et al., 2004; Gammans et al., 2005). All colonies survived the experiment and there was a low mortality of ants, similar to other studies (Wardlaw et al., 1998; Gammans et al., 2005).

Standard Procedure for Laboratory Bioassays

All colonies were allowed to habituate to the laboratory nests for 1 wk before the first bioassays. Nests were selected at random, and each nest was used only once in each set of bioassays. When no bioassays were conducted, ants were allowed to roam freely between the nest and foraging boxes. Before each bioassay, any remaining food was removed from the foraging box, which was then cleaned with water to remove any food residue and any possible pheromone trails. Any ants within the foraging box were removed except for a randomly chosen “focus” ant, which was allowed to settle, and the foraging tube was then closed. If the focus ant left the foraging box during the bioassay, the foraging tube was opened and remained so until a new ant entered under its own volition. If no ant was present in the foraging box at the start of the experiment, the tube was left open until an ant entered. This procedure minimized disturbance and ensured that the workers observed were active foragers.

During bioassays, seeds, elaiosomes, or filter paper with chemical extracts were always handled with sterile forceps to prevent contamination. The lid of the foraging box was kept open during the bioassay to prevent build up of any volatile compounds.

Bioassays were conducted during times of day when both ant species normally forage; for *M. ruginodis* 06:00–12:00 and for *T. caespitum* 12:00–18:00 hr. Experiments were conducted with *U. minor* from May 26 to July 9, 2004, and with *U. europaeus* from July 12 to August 2, 2004. Although this approach meant that bioassays were conducted on the two *Ulex* species at different times of the year, it was preferred to ensure that seeds were as fresh as possible for the bioassays. Data analysis reflected this lack of randomization by analyzing results for the two *Ulex* species separately.

Ant Responses to Seeds, Elaiosomes and Diaspores

Bioassays were carried out to assess ant responses to diaspores, separate elaiosomes, and seeds from which the elaiosome had been removed. Three of each “treatment” (diaspore, elaiosome, or seed) were placed into a foraging box, and the number removed to the nest box was counted after 24 hr. Each treatment was replicated in 20 nests for each ant species.

Ant Behavior Toward Diaspore Surface Compounds

Further bioassays were done using extracts of elaiosome chemicals on filter papers. The surface chemicals of 90 *U. minor* or *U. europaeus* diaspores (seed plus elaiosome) were extracted by immersion in diethyl ether for 8 hr. When required, the extract was added to filter paper squares of 2–3 mm, which were then left overnight at room temperature to allow the ether to evaporate. Filter paper was used as the substrate to test reaction to chemical extracts on an object that did not resemble a diaspore. The amount of extract added was equivalent to one diaspore. Control paper squares were immersed in diethyl ether, and then left overnight to allow evaporation. Three control or extract filter paper squares were placed into the foraging boxes of randomly selected nests with only one treatment used per nest. Fifteen colonies were given the extract and 15 the control. Ant behavior was recorded for a period of 20 min. Four distinct ant behaviors were recorded as follows: (1) ignore: the ant paid no attention to the filter paper; (2) antennate: the ant touched the filter paper with its antennae; (3) bite: the ant bit the filter paper with its mandibles; (4) carry: the ant carried the filter paper in its mandibles. We also noted the time between the start of the bioassay and the first antennation of the filter paper. In preliminary experiments, it was noticed that the focus ant would repeat behaviors, especially when showing interest in the filter paper, but might then ignore it for some time, before returning and showing renewed interest. Repetition of behaviors also occurred when a focus ant was replaced by another. Therefore, the total time spent (to the nearest second) and the number of repetitions of all four behaviors was recorded.

Lipid Fractions from Elaiosome Extracts

For each *Ulex* species, 70 elaiosomes were removed from the diaspore and slightly macerated and submerged for 24 hr in 3 ml of chloroform to separate the different lipid fractions of the elaiosomes. The lipid fractions of these extracts were separated with solid phase extraction (1 ml SPE NH₂; Qmx) using the standard methodology described by Kalunzy et al. (1985). Solvents were used in the following sequence: 15% ethyl acetate in hexane to separate diglycerides; hexane for cholesterol esters; 2:1 chloroform/methanol for monoglycerides; 1% diethyl ether in 10% methanol in hexane for triglycerides; 5% ethyl acetate in hexane for cholesterol; a second elution with 5% ethyl acetate in hexane for residual cholesterol; 2% acetic acid in diethyl ether fatty acids; and methanol for phospholipids. The eluent from each wash was stored at –20°C until use in bioassays. The diglyceride fraction (to which *M. ruginodis* reacted) for *U. minor* was sent to the MRS Lipid Analysis Unit in Dundee for quantitative analysis.

For all bioassays, a volume of the lipid fraction equivalent to one elaiosome was added to a filter paper square, and the respective solvent used in the fractionation was added to filter paper as a control. Both sets of papers were left overnight at room temperature to allow the solvent to evaporate. As before, three filter papers of either treatment or control (only one treatment used per nest) were then placed into the foraging box of a randomly selected nest. Thirty nests were used for each ant species; half were given the extract and half the control. All replicates and controls for

each particular fraction were run consecutively. The different fractions were used in random order. The behavior of the focus ant was recorded for 20 min using the protocol described above.

Visual Cues

An experiment was designed to investigate whether visual cues are used by *M. ruginodis* when finding *Ulex* diaspores. *U. minor* diaspores ($N = 270$) were submerged in diethyl ether for 8 hr to remove any surface chemicals. In sets of 30 diaspores, the elaiosomes were painted different colors with nontoxic acrylic paint: brown, yellow, green, red, and blue; one set was left unpainted. A further 30 diaspores were also used without the surface chemicals removed. Three diaspores per color were placed into 10 randomly chosen nests (some nests were repeated), and ant behavior was recorded for 20 min using the protocol described above. Diaspores were left in the foraging arena, and their final location was recorded after 24 hr.

Analysis

All results were analyzed with the Minitab statistical package by using analysis of variance ANOVA where the data were normally distributed. Some data sets had many zero values and remained nonnormally distributed after transformation. These data sets were analyzed by Kruskal–Wallis tests. Treatments and, where relevant, ant species were included as factors in the analyses, with a fully randomized design. Because the *Ulex* species were subjected to bioassays on different dates, they were analyzed separately.

Results

Ant Responses to Diaspores, Seeds and Elaiosomes

The ant *M. ruginodis* moved more elaiosomes (58%; i.e., proportion of the three elaiosomes removed across 20 replicates) than diaspores (18%), and moved seeds without diaspores the

Table 2 Behavioral responses (mean \pm S.E.) of *M. ruginodis* and *T. caespitum* ants to elaiosome surface chemical extracts of two *Ulex* species

Ant species	Plant species	Treatment	Time till antennation (sec)	Duration of antennation (sec)	Number of antennations
<i>M. ruginodis</i>	<i>U. minor</i>	Control	184 \pm 194	1.3 \pm 3.5	1.0 \pm 0.4
		Extract	36 \pm 40 $F_{1,12} = 0.97$ nsd	9.3 \pm 0.6 $F_{1,12} = 30.25^{***}$	7.0 \pm 4.3 $F_{1,12} = 12.81^{**}$
<i>T. caespitum</i>	<i>U. minor</i>	Control	91 \pm 26	24 \pm 20	2.6 \pm 0.6
		Extract	211 \pm 53 $F_{1,24} = 3.35$ nsd	4.0 \pm 1.4 $F_{1,28} = 0.99$ nsd	2.9 \pm 0.6 $F_{1,28} = 0.15$ nsd
<i>M. ruginodis</i>	<i>U. europaeus</i>	Control	188 \pm 62	2.7 \pm 0.8	2.1 \pm 1.2
		Extract	44 \pm 19 $F_{1,15} = 6.67^*$	7.1 \pm 1.7 $F_{1,15} = 4.21$ nsd	4.9 \pm 1.1 $F_{1,15} = 4.90^*$
<i>T. caespitum</i>	<i>U. europaeus</i>	Control	77 \pm 44	0.4 \pm 0.2	0.4 \pm 0.2
		Extract	92 \pm 71 $F_{1,28} = 0.03$ nsd	2.3 \pm 1.1 $F_{1,28} = 3.02$ nsd	1.4 \pm 0.6 $F_{1,28} = 2.87$ nsd

nsd $P > 0.05$, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$. The difference in degrees of freedom reflects the number of times a focal ant was present during the experiment.

least (6%), while the respective percentages for *T. caespitum* were 30%, 8%, and 5%. Therefore, while the strong preference of both species for elaiosomes led to a treatment effect ($F_{2,108} = 29, P < 0.001$), the preference of *M. ruginodis* for diaspores over seeds compared with the relative lack of distinction between the diaspore and seed-only treatments by *T. caespitum* led to an ant species \times treatment interaction ($F_{2,108} = 3.2, P < 0.05$).

Surface Compounds

The major response of ants to filter papers involved antennation. The biting and movement of papers was rare, so the data were not analyzed. ANOVA indicated that *M. ruginodis* antennated the filter papers containing the surface extract for longer and for a greater number of times than the control for both *Ulex* species, although time till first antennation was significantly different between treatments only for *U. europaeus* (Table 2). *T. caespitum* showed no difference in any behavior in response to the extracts of either *Ulex* species (Table 2).

Table 3 *M. ruginodis* and *T. caespitum* behaviors toward the lipid fractions of *U. minor* elaiosomes

Ant species	Solvent vs. lipid fraction	Number of antennations	Duration of antennation	Time until first antennation
<i>M. ruginodis</i>	15% Ethyl acetate in hexane vs. <u>diglyceride</u>	$F_{1,15} = 32.3^{***}$	$F_{1,15} = 13.5^{**}$	$F_{1,13} = 8.4^*$
<i>M. ruginodis</i>	Hexane vs. cholesterol ester	$F_{1,14} = 1.6$ nsd	$F_{1,14} = 2.3$ nsd	$F_{1,9} = 3.1$ nsd
<i>M. ruginodis</i>	2:1 Chloroform/methanol vs. monoglyceride	$F_{1,11} = 0.5$ nsd	$F_{1,11} = 2.4$ nsd	$F_{1,11} = 0.1$ nsd
<i>M. ruginodis</i>	1% Diethyl ether in 10% methanol in hexane vs. triglyceride	$F_{1,13} = 0.9$ nsd	$F_{1,13} = 0.3$ nsd	$F_{1,11} = 0.01$ nsd
<i>M. ruginodis</i>	5% Ethyl acetate in hexane vs. cholesterol residual	$F_{1,12} = 4.3$ nsd	$F_{1,12} = 2.2$ nsd	$F_{1,9} = 1.9$ nsd
<i>M. ruginodis</i>	5% Ethyl acetate in hexane vs. cholesterol	$F_{1,14} = 1.6$ nsd	$F_{1,14} = 1.9$ nsd	$F_{1,11} = 0.4$ nsd
<i>M. ruginodis</i>	2% Acetic acid in diethyl ether vs. fatty acid	$F_{1,6} = 2.84$ nsd	$F_{1,13} = 0.24$ nsd	$F_{1,13} = 0.30$ nsd
<i>M. ruginodis</i>	Methanol vs. phospholipid	$F_{1,13} = 1.7$ nsd	$F_{1,13} < 0.01$ nsd	$F_{1,10} = 0.7$ nsd
<i>T. caespitum</i>	15% Ethyl acetate in hexane vs. <u>diglyceride</u>	$F_{1,8} = 0.05$ nsd	$F_{1,18} = 0.08$ nsd	$F_{1,10} = 1.1$ nsd
<i>T. caespitum</i>	Hexane vs. cholesterol ester	$F_{1,18} = 0.5$ nsd	$F_{1,18} = 0.6$ nsd	$F_{1,12} = 0.07$ nsd
<i>T. caespitum</i>	2:1 Chloroform:methanol vs. monoglyceride	$F_{1,18} = 1.2$ nsd	$F_{1,18} = 2.1$ nsd	$F_{1,12} = 0.06$ nsd
<i>T. caespitum</i>	1% Diethyl ether 10% methanol in hexane vs. <u>triglyceride</u>	$F_{1,17} = 1.2$ nsd	$F_{1,17} = 0.06$ nsd	$F_{1,10} = 13.0^{**}$
<i>T. caespitum</i>	5% Ethyl acetate in hexane vs. <u>cholesterol residuals</u>	$F_{1,18} = 18.0^{***}$	$F_{1,18} = 6.9^*$	$F_{1,11} = 0.9$ nsd
<i>T. caespitum</i>	5% Ethyl acetate in hexane vs. <u>cholesterol</u>	$F_{1,18} = 8.9^{**}$	$F_{1,18} = 2.8$ nsd	$F_{1,18} = 0.5$ nsd
<i>T. caespitum</i>	2% Acetic acid in diethyl ether vs. fatty acid	$F_{1,18} = 1.3$ nsd	$F_{1,10} = 0.02$ nsd	$F_{1,10} = 0.3$ nsd
<i>T. caespitum</i>	Methanol vs. phospholipid	$F_{1,17} = 4.2$ nsd	$F_{1,18} = 4.2$ nsd	$F_{1,9} = 3.7$ nsd

nsd $P > 0.05$, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$. Where behaviors toward the control and lipid fraction were significantly different (in bold), the underlined chemical is that to which the ants responded most strongly. The differences in degrees of freedom reflect the number of times a focal ant was present during the experiment.

Lipid Fractions from Elaiosome Extracts

Sixteen analyses (i.e., eight fractions \times two *Ulex* species) were performed on the data from the lipid fraction experiments for each behavior of each ant species. A 5% significance threshold means $16/20 = 0.8$ tests on average would be significant by chance. However, if the behavioral responses toward a particular fraction are consistent, and biologically meaningful, one would expect an ant species to show responses to a fraction that is similar for the two *Ulex* species. Tests on the two *Ulex* species were independent, and the probability of significant response in terms of a particular behavior to the same fraction of both *Ulex* species is $8/20$ (probability of one test in eight being significant at 5%) \times $1/20$ (probability of the same fraction in the second *Ulex* species having a significant effect at 5%) = 2%.

The comparison of *M. ruginodis* behaviors toward each lipid fraction and its solvent revealed that the only fraction that caused significantly greater reactions than its solvent was

Table 4 *M. ruginodis* and *T. caespitum* behaviors toward the lipid fractions of *U. europaeus* elaiosomes

Ant species	Solvent vs. lipid fraction	Number of antennations	Duration of antennation	Time until first antennation
<i>M. ruginodis</i>	15% Ethyl acetate in hexane vs. <u>diglyceride</u>	$F_{1,9} = 7.6^*$	$F_{1,9} = 13.0^{**}$	$F_{1,9} = 0.9$ nsd
<i>M. ruginodis</i>	<u>Hexane</u> vs. cholesterol ester	$F_{1,14} = 4.2$ nsd	$F_{1,14} = 4.8^*$	$F_{1,14} = 1.4$ nsd
<i>M. ruginodis</i>	2:1 Chloroform:methanol vs. monoglyceride	$F_{1,22} = 2.8$ nsd	$F_{1,22} = 3.5$ nsd	$F_{1,22} = 0.9$ nsd
<i>M. ruginodis</i>	1% Diethyl ether in 10% methanol in hexane vs. triglyceride	$F_{1,8} = 2.2$ nsd	$F_{1,8} = 2.0$ nsd	$F_{1,8} = 0.2$ nsd
<i>M. ruginodis</i>	5% Ethyl acetate in hexane vs. cholesterol residual	$F_{1,12} = 2.5$ nsd	$F_{1,12} = 0.1$ nsd	$F_{1,12} = 0.3$ nsd
<i>M. ruginodis</i>	5% Ethyl acetate in hexane vs. cholesterol	$F_{1,8} = 1.7$ nsd	$F_{1,8} = 2.0$ nsd	$F_{1,8} = 2.2$ nsd
<i>M. ruginodis</i>	2% Acetic acid in diethyl ether vs. fatty acid	$F_{1,14} < 0.01$ nsd	$F_{1,14} = 0.01$ nsd	$F_{1,14} = 0.9$ nsd
<i>M. ruginodis</i>	Methanol vs. phospholipid	$F_{1,13} = 0.05$ nsd	$F_{1,13} = 0.08$ nsd	$F_{1,13} = 0.6$ nsd
<i>T. caespitum</i>	15% Ethyl acetate in hexane vs. diglyceride	$F_{1,18} = 1.0$ nsd	$F_{1,18} = 2.2$ nsd	$F_{1,18} = 1.1$ nsd
<i>T. caespitum</i>	Hexane vs. cholesterol ester	$F_{1,28} = 1.8$ nsd	$F_{1,28} = 3.0$ nsd	$F_{1,28} = 1.7$ nsd
<i>T. caespitum</i>	2:1 Chloroform/methanol vs. monoglyceride	$F_{1,22} = 2.8$ nsd	$F_{1,22} = 3.5$ nsd	$F_{1,22} = 0.9$ nsd
<i>T. caespitum</i>	1% Diethyl ether 10% methanol in hexane vs. triglyceride	$F_{1,28} = 2.3$ nsd	$F_{1,28} = 1.1$ nsd	$F_{1,28} = 2.2$ nsd
<i>T. caespitum</i>	5% Ethyl acetate in hexane vs. cholesterol residuals	$F_{1,24} = 0.6$ nsd	$F_{1,24} = 0.05$ nsd	$F_{1,24} = 1.6$ nsd
<i>T. caespitum</i>	5% Ethyl acetate in hexane vs. cholesterol	$F_{1,23} = 0.2$ nsd	$F_{1,23} = 0.3$ nsd	$F_{1,23} = 1.9$ nsd
<i>T. caespitum</i>	2% Acetic acid in diethyl ether vs. fatty acid	$F_{1,21} = 2.5$ nsd	$F_{1,21} = 2.0$ nsd	$F_{1,21} = 4.2$ nsd
<i>T. caespitum</i>	Methanol vs. phospholipid	$F_{1,22} = 0.3$ nsd	$F_{1,22} = 0.7$ nsd	$F_{1,22} = 0.3$ nsd

nsd $P > 0.05$, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$. Where behaviors toward the control and lipid fraction were significantly different (in bold), the underlined chemical is that which the ants responded to most strongly. The differences in degrees of freedom reflect the number of times a focal ant was present during the experiment.

Table 5 The fatty acid composition of the diglyceride fraction of *U. minor* elaiosomes

Fatty acid	Proportion of total fatty acids (%)
Hexadecadienoic acid (C 16:2)	24.87
Stearic acid (C 18:0)	22.59
Arachidic acid (C 20:0)	21.89
Palmitic acid (C 16:0)	13.49
Oleic acid (n-9; C 18:1)	5.77
Linoleic acid (C 18:2)	3.85
Palmitoleic acid (C 16:2)	2.98
Myristic acid (C 14:0)	1.93
Margaric acid (C 17:0)	1.58
Oleic acid (n-7; C 18:1)	1.05

the diglyceride fraction for both *Ulex* species. The number (*U. minor* extract mean = 4.5, control mean = 0.9; *U. europaeus* extract = 8.0, control = 2.0) and duration (*U. minor* extract = 7 sec, control = 0.9 sec; *U. europaeus* extract = 16.2 sec, control = 3.7 sec) of antennations were greater for both *Ulex* species, and the time till first antennation was faster for *U. minor* (extract = 127 sec, control = 446 sec; Tables 3 and 4). For *U. europaeus*, the hexane solvent was also antennated for significantly longer than the cholesterol ester fraction (Table 4).

Unlike *M. ruginodis*, *T. caespitum* did not show a changed behavior toward the diglyceride fraction (Tables 3 and 4) and there were no significant difference among any fractions and their solvents for *U. europaeus* (Table 4). Comparing the *U. minor* fractions with controls, the time until first antennation was significantly faster for the triglyceride fraction (extract mean = 205 sec, control mean = 734 sec), the cholesterol residual was antennated for a greater duration (extract = 5 sec, control = 0.4 sec) and frequency (extract = 2.7, control = 0.4), and the cholesterol fraction was antennated more times than its solvent (extract = 3, control = 0.4; Table 3).

According to the argument above, the responses of *M. ruginodis* to the diglyceride were greater than expected by chance. However, the *T. caespitum* responses were unconvincing, being inconsistent across the two *Ulex* species and, on average, only slightly more frequent than expected by chance.

The main constituents of the diglyceride fraction were hexadecadienoic acid, stearic acid, and arachidic acid. Oleic acid was present but in small amounts (Table 5).

Visual Cues

ANOVA and Kruskal–Wallis tests showed significant differences in all behaviors of *M. ruginodis* among the five artificial elaiosome colors, the diaspore with surface chemicals removed, and the unaltered diaspore (Table 6). Comparison of means suggested that the unaltered diaspore was more attractive than all the other treatments. When the unaltered diaspore treatment was removed from the analyses, there were no differences among the remaining treatments. The mean values for behaviors to all these other treatments were low, suggesting they were generally unattractive.

Discussion

The data suggest that elaiosomes attract mutualistic ant species through chemical cues. *M. ruginodis* responded both to the general diaspore surface chemicals extracted with diethyl ether, and more particularly to the diglyceride fraction of elaiosome chemicals. Both

Table 6 Behavioral reactions (Mean ± S.E.) of *M. ruginodis* to *U. minor* diaspores with natural and artificial coloration

Behavior	Unaltered diaspore	Diaspore without surface chemicals	Artificial colors				Test with unaltered diaspore		Test without unaltered diaspore	
			Blue	Red	Brown	Green	Yellow	$F_{5,43}$	H_5	$F_{5,43}$
Duration of antennation	81.4 ± 26.2	9.1 ± 3.8	4.8 ± 2.2	7.0 ± 3.5	3.6 ± 1.6	2.4 ± 0.8	3.0 ± 2.0	$F_{5,43} = 6.6^{***}$	$F_{5,43} = 1.0$ nsd	
Number of antennations	194 ± 33	3.2 ± 1.2	3.2 ± 1.6	3.6 ± 0.8	1.4 ± 0.5	2.3 ± 0.8	1.3 ± 0.5	$H_6 = 29^{***}$	$H_5 = 5.9$ nsd	
Number of bites	10.1 ± 2.5	0.7 ± 0.4	0.5 ± 0.5	0.3 ± 0.2	0	0	0.1 ± 0.1	$H_6 = 33.5^{***}$	$H_5 = 6.1$ nsd	
Duration of biting	88.7 ± 23.1	2.1 ± 1.5	2.7 ± 2.7	0.6 ± 0.4	0	0	0.1 ± 0.1	$H_6 = 33.4^{***}$	$H_5 = 6.0$ nsd	
Duration of movement	44.8 ± 31.2	0.2 ± 0.2	3.8 ± 3.8	0	0	0	0	$H_6 = 24.1^{***}$	$H_5 = 5.0$ nsd	
Number of movements	1.6 ± 0.6	0.1 ± 0.1	0.2 ± 0.2	0	0	0	0	$H_6 = 24.6^{***}$	$H_5 = 4.9$ nsd	

Data were analyzed by ANOVA (F) or Kruskal–Wallis (H).

mutualistic and granivorous species carried the diaspore, and both carried elaiosomes more frequently than the diaspore, probably because the elaiosome was easier to carry. The seed with the elaiosome removed produced less of a response, especially from *M. ruginodis*, which suggests the importance of the elaiosome in attracting this ant compared with *T. caespitum*. The lack of reaction of *T. caespitum* to the diethyl ether extracts suggests that the granivore did not respond to chemical cues.

The testing of *T. caespitum* responses to the chemical fractions showed four significant effects. These may be chance events because they were inconsistent across the two *Ulex* species, only slightly more frequent than expected by chance, and they conflict with the lack of response to the complete extract. In contrast, the *M. ruginodis* responses toward the diglyceride fraction were consistent for both *U. minor* and *U. europaeus* and, therefore, probably biologically meaningful.

Our results agree with those of Marshall et al. (1979), Brew et al. (1989), and Kusmenoglu et al. (1989) who found that ants responded to the diglyceride fraction of elaiosomes. However, this response seems to be more specific than has been assumed previously, and our results support the hypothesis that selection has acted on the evolution of cues that attract mutualists but not predators. The granivore detects seeds, possibly by simple mechanical cues, whereas the mutualist has more sensitive and accurate chemically-based recognition. *M. ruginodis* dependence on this chemical cue for finding *Ulex* diaspores was further demonstrated by the experiment on visual cues. It seems that visual cues are not used, as no color produced a greater reaction than another. In contrast, the removal of surface chemicals in this experiment decreased the responses of ants to the diaspore.

It is unclear whether the recognition of chemicals is gustatory, by antennation on or near the elaiosome, or by olfaction of volatiles. Slingsby and Bond (1981) suggested that ants can detect elaiosome chemicals over some distance, but Sheridan et al. (1996) produced evidence that elaiosome chemicals are detected by gustation. We often observed *M. ruginodis* antennating the air within 1–2 cm of elaiosome extracts or diaspores, suggesting olfaction. Diglycerides are not volatile, however, (Sheridan et al., 1996), and an olfactorial response is likely to involve a more volatile and as yet undetected compound(s). However, if it exists, there should be a trade-off between the volatility and the longevity of such a cue. Such trade-offs have been encountered with the parasitoid *Ichneumon eumerus* that attacks caterpillars of the butterfly *Maculinea rebeli* inside host ant colonies. A mimic of the ants alarm pheromone is used. However, mimic compounds have a greater molecular weight and are less volatile than the template, providing the intruders with more time to attack the caterpillar (Thomas et al., 2002).

M. ruginodis rarely bit or picked up filter papers, in contrast to behavior toward the diaspore. This result contrasts with Brew et al. (1989), who found that piths treated only with oleic acid, 1,2-diolein, and triolein were removed by ants at the same rate as elaiosomes. This suggests that in the *Ulex*–*M. ruginodis* system diglycerides act only in the initial stages of attraction, and that the stimulus to remove the diaspore comes subsequently. This may be a simple food cue released when the elaiosome is damaged by biting.

In this myrmecochorous system of *Ulex* seeds and *M. ruginodis* ants, there appears to be a highly evolved relationship between the species. *Ulex* elaiosomes release chemicals that attract *M. ruginodis* and contain essential nutrients, linoleic acid, and sterols, which increase nest productivity (Gammons et al., 2005). It may be that the specific chemical attractant arose because *M. ruginodis* evolved a refined ability to detect such chemicals, or *Ulex* elaiosome chemicals have evolved and become specifically attractive to a mutualistic ant, or both processes have occurred through coevolution.

It is well established that many plant species use chemicals to manipulate insect behavior for their benefit, e.g., through repelling herbivores (De Moraes et al., 2001) or attracting

ants that defend the plant (Raine et al., 2004). The study of chemical attractants is important for understanding biotic interactions that affect populations and communities, and is an area in which further research is needed (Weissburg et al., 2002; Muller and Riederer, 2005).

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Introduction

Avocado (*Persea americana*: Lauraceae) originated in the neotropics where it is naturally pollinated by a wide range of insects, mainly stingless bees and social wasps (Ish-Am et al., 1999; Can-Alonzo et al., 2005). It is an important crop in many tropical and subtropical regions around the world (Knight, 2002). Its open and rather small (1 cm diam) flowers can also be visited by honey bees, which are its main pollinators in agricultural landscapes (Gazit and Degani, 2002). However, even when colonies of honey bees are placed inside the orchards, avocado flowers often suffer from insufficient pollination activity, resulting in low fruit yields (Vithanage, 1990; Ish-Am, 1998; Ish-Am and Eisikowitch, 1998; Gazit and Degani, 2002).

The attractiveness of nectar to pollinators is probably most affected by its taste (Adler, 2000), but may also be affected by odor (Raguso, 2004) and color (Thorp et al., 1975). The taste of nectar is dominated by a high sugar concentration. Several studies have attempted to define whether honey bees prefer nectars that are rich in sucrose or hexose. Wykes (1952) showed that honey bees prefer a sugar ratio of 1:1:1 (sucrose/fructose/glucose) over a pure sucrose solution. Avocado nectar contains almost exclusively sucrose and a low concentration of the unique seven-carbon sugar alcohol, perseitol (Ish-Am, 1994; Liu et al., 1995; Dvash et al., 2002; Dag et al., 2003). Indeed, the high ratio of sucrose in avocado nectar has been suggested as the cause for the low attractiveness of avocado flowers to honey bees (Ish-Am, 1994). A preference for hexoses over sucrose is also suggested by physiological considerations, because sucrose has to be broken down before it can be utilized (Harborne, 1993). Other studies, however, have found that honey bees are indifferent to the ratio of sugars (Southwick et al., 1981), or even prefer a high sucrose concentration (Bachman and Waller, 1977; Hagler et al., 1990). The sensitivity of honey bees to perseitol has never been evaluated.

Other components of nectar, including minerals, phenolic compounds, and amino acids, may make a cardinal contribution to its attractiveness to honey bees. Minerals such as potassium (Waller et al., 1972) and sodium chloride (von Frisch, 1950) deter honey bees. Phenolic compounds affect the taste of nectar even at very low concentrations (Baker, 1977), and in some cases they have been suspected of repelling honey bees (Hagler and Buchmann, 1993; Adler, 2000). Several nectar amino acids have also been shown to affect preference (Kim and Smith, 2000; Gardener and Gillman, 2002; Carter et al., 2006). Ish-Am (1994) found that glycine and histidine are the dominant amino acids in some avocado cultivars; at their naturally occurring concentrations, however, these amino acids do not repel honey bees (Inouye and Waller, 1984) and may even attract them (Kim and Smith, 2000). Avocado nectar is poor in proline, which is attractive to honey bees (Carter et al., 2006), but so is citrus (cv. "Valencia") nectar, and yet it is highly attractive to bees (Ish-Am, 1994). Hence, it does not appear that amino acid composition can explain the low attractiveness of avocado nectar.

Odors guide bees toward flowers and may affect their attractiveness (von Frisch, 1967). The volatile components of nectar, including phenolic compounds, form particular odor bouquets (Anklam, 1998). Some of these compounds are more attractive to honey bees than others (Jay, 1986; Henning et al., 1992; Winston and Slessor, 1993).

Colors also affect the attractiveness of flowers (von Frisch, 1967; Giurfa et al., 1995), and nectar may contribute to their visual display (Thorp et al., 1975). Color differences are especially salient when comparing honeys, which are derived from nectar, and can be used in choice experiments for testing the influence of nectar components on bees' preferences

(Afik et al., 2006). Nectar and honey colors are produced by dissolved light-absorbing compounds. Whereas sugar solution is transparent, dark honeys such as avocado honey have relatively high concentrations of minerals (Petrov, 1970; Terrab and Heredia, 2004; Dag et al., 2006). Other compounds, including flavonoids (Anklam, 1998) and organic acids (Mato et al., 2003), may also affect honey color, in either the visible or ultraviolet spectra, which are visible to bees (Hagler and Buchmann, 1993).

Previous work has revealed that avocado honey is relatively unattractive to honey bees when compared with honey from competing flowers (Afik et al., 2006). These results suggest that some attributes of avocado nectar are responsible for the relatively low attractiveness of avocado flowers. Here, we studied the influence of odor, color, sugar composition, phenolic compounds, and minerals on the selection of sugar source by honey bees.

Methods and Materials

Mineral Composition

We measured mineral composition in avocado honey, nonavocado honey, avocado (cv. “Ettinger”) nectar, and citrus (cv. “Shamouti”) nectar. The honey was extracted from colonies placed in avocado orchards during the blooming season. Its avocado origin was confirmed by sugar analysis, perseitol constituting 2.5% of the total sugars (Dvash et al., 2002). The nonavocado honey was extracted from colonies placed in citrus orchards during the citrus blooming season; it contained no perseitol. Nectar samples were collected from flowers by hand using microcapillary tubes. Flowers were covered with paper bags on the evening before collection to prevent nectar consumption by insects.

Nitric acid (5 ml of 65%, w/w) was added to the two honey and two nectar samples. Samples were prepared for analysis by microwave-assisted digestion, using an MLS 1200 mega microwave digestion unit [Milestone Sorisole (BG) Italy] at 500 W for 10 min. Liquid residues were taken up in deionized water to a final volume of 25 ml. Concentrations of the different elements were determined simultaneously by inductively coupled plasma–atomic emission spectrometry (ICP-AES), according to EPA 6010B (1996), using two ICP-AES systems, models “Spectroflame” and “Spectroflame Modula E” from Spectro (Kleve, Germany).

Behavioral Bioassay—Proboscis Extension Response

Odor

We employed the proboscis extension response (PER) bioassay to test the responses of harnessed bees to the odors of avocado and citrus flowers and honey. In this bioassay, subjects extend their proboscis in response to an odor associated with an appetitive reinforcement (Bitterman et al., 1983). The experiment was conducted in April 2003, in Rehovot, Israel. We tested bees from two colonies. One hive was located in the field, between citrus and avocado orchards, during their simultaneous blooming period. Thus, the foragers from this colony may have visited citrus and avocado flowers before being tested. A second hive was introduced into a 12 × 6 × 3 m enclosure (15 mesh) before the blooming period to avoid preconditioning. This colony was fed sugar solution and pollen patties during the experimental period.

Each morning, 30 bees from one colony were caught in glass vials as they flew out of the hive. The vials were placed on ice for 1 or 2 min until the bees were motionless, and then the bees were strapped into a sectioned hollow plastic tube (6 mm diam), with a 3-mm-wide strip of duct tape that wrapped around the tube and (dorsal) thorax of the bee (Shafir et al., 1999). When they awoke, bees were fed 5 μ l of a 30% (w/w) sucrose solution. Typically, only a few bees did not feed, and they were removed from the experiment. Each day, 24 bees were chosen and allowed to adapt to the harness for 1 hr. We tested a total of 142 bees from the colony in the orchard and 215 bees from the colony in the enclosure.

During the experiment, odor was delivered to each bee for 3 sec. An air pump delivered air through valves controlled by a computer, and a Tygon tubing connected to a 50-ml plastic syringe filled with fresh flowers or a 130-ml glass vial filled with 30 ml honey solution, diluted to a total sugar concentration of 60% (w/w). The vial was placed in a warm water bath (40°C; Abramson and Boyd, 2001). Tubing from the odor source delivered odors to within 2 cm of the bee.

Each bee experienced five different odors with an intertrial interval of 10 min. The first two presented were of avocado flowers (“Ettinger” or “Fuerte” cultivars) and citrus flowers (“Shamouti”). The order of odor presentation was alternated every day. Because these were unrewarded trials, after the first two trials the bees were fed 2 μ l of 30% sucrose solution to avoid starvation. The subsequent three trials included odors of avocado honey, nonavocado honey, and an air control. The order of the two honey sources was alternated every day, but the air control was always last. An extension of the proboscis in response to a particular odor was considered a positive response and the proportion of bees responding to each odor was calculated.

Sugars

The sensitivity of honey bees to increasing concentrations of various sugar solutions was tested in February and July of 2002, respectively, in two sets of PER experiments (Page et al., 1998). The first experiment tested the sensitivity of bees to different sugars ($N = 150$ bees, 37–38 bees for each sugar). The second tested the bees’ sensitivity to different sugar mixtures ($N = 275$ bees, 68–69 bees for each mixture). The bees were caught and harnessed as described for the odor experiment. Each trial with a sugar concentration was preceded by a trial with distilled water, which served as a baseline to which the effect of the sugar component of the solution was compared. The intertrial interval was 4 min.

The experiment was begun by touching the right antenna of each bee with a cotton ball soaked in distilled water. The PER to the touch was recorded, and in the next trial the same antenna was touched again, this time with a cotton ball soaked in a sugar solution. We rotated between antennae so that the next two trials (water and increasing sugar solution) were to the left antenna, and so forth. The total sugar concentrations of the examined solutions were as follows: 0.1%, 0.3%, 1%, 3%, 10%, and 30% w/w (g solute/g solution).

On each day of the experiment, the tested bees were separated randomly into four different groups, which were tested for their response to four different sugar solutions. In the first experiment, each solution contained only a single sugar: sucrose, glucose, fructose, or perseitol, with an added concentration of 4.5% (w/w), the highest concentration that could be reached using perseitol. In the second experiment, four sugar mixtures were used: (1) “avocado nectar”: 95% sucrose and 5% perseitol, to simulate the sugar composition of avocado nectar (Ish-Am, 1994); (2) “perseitol-enriched”: 90% sucrose and 10% perseitol; (3) “citrus nectar”: 50% fructose, 30% glucose, and 20% sucrose, to simulate the sugar composition of citrus nectar (Ish-Am, 1994); (4) sucrose solution, used as a reference.

Behavioral Bioassay—Free-Flying Bees

General Procedure

The effects of honey color, and phenolics and mineral composition, were studied with a cafeteria-style choice paradigm, in which free-flying honey bees could choose from three available feeders. Five-frame nucleus hives were kept in screened enclosures. For the honey color and phenolics experiments, colonies were kept in a $12 \times 6 \times 3$ m (15 mesh) enclosure. Only one colony was tested at a time, and the entrances to the other colonies were closed the evening before each test. The mineral composition experiment was conducted in $5 \times 2.5 \times 2$ m (20 mesh) enclosures, each housing one colony. The bees had *ad libitum* access to a water source and were provided with a pollen patty once a week.

Three different honey solutions were prepared each day by diluting honey or sucrose with distilled water to reach 60% (w/w) total dissolved solids. The concentration was measured by a hand refractometer (REF 114, brix units, 28–62 ATC). Although the tested honeys contain mainly glucose and fructose (Dag et al., 2006), their refractive index is similar to that of sucrose (Kearns and Inouye, 1993). The three different solutions used in the experiments were avocado honey, nonavocado honey, and sucrose.

The solutions were presented to the bees in 200-ml bird feeders. The three feeders were placed in a circle, 15 cm apart, on a carousel that rotated at a velocity of 2 rpm, to prevent a potential location bias. The volume consumed from each feeder was measured every 20 min. The experiment ended when 130 ml were consumed from one of the feeders, or after 4 hr had elapsed. The volume of solution consumed from each feeder was measured at the end of the experiment. The solution from which bees consumed the highest volume was set to represent 100% consumption, and the consumed volumes from the rest of the feeders were compared with this highest volume. The results are presented as relative percentage consumption from the different solutions.

Color

Avocado honey is characterized by its dark brown color (Terrab and Heredia, 2004; Dag et al., 2006). To test whether the bees' preference for nonavocado over avocado honey is influenced by color (Afik et al., 2006), we tested 10 colonies in a preference experiment in which the feeders were covered with an opaque green plastic cover. A narrow opening (marked in yellow) was left at the bottom of the cover, allowing bees to reach the feeder inside. The choice was between avocado honey, nonavocado honey, and sucrose solution.

Phenolic Compounds in Honey

To test the effect of phenolic compounds in avocado honey on preference, an experiment was conducted in which bees had a choice between dilute avocado honey, sucrose solution with a phenolic concentrate produced from avocado honey, and sucrose solution with ethanol (the solvent). The phenolic concentrate was made by dissolving 600 g of avocado honey in 1.2 l distilled water; 200 ml of ethyl acetate was mixed with the honey solution and then separated from the solution using a funnel separator. This process was repeated five times. All ethyl acetate fractions were pooled, and the solvent was evaporated under reduced pressure on ice (Singleton et al., 1999). The residue was dissolved in 2 ml ethanol, because such a low concentration of ethanol is known to have only a minor effect on honey bees (Abramson et al., 2000).

Each feeder of avocado honey solution contained 200 g of honey (200 ml of 60% diluted honey solution; Weast, 1988). Therefore, the 2 ml phenolic concentrate that was extracted from 600 g of honey was divided into three portions of 0.67 ml, which sufficed for three replicates of the preference test, with three different colonies. Similarly, 0.67 ml of ethanol was added to each feeder of sucrose solution.

Potassium

To test the effect of potassium concentration in honey on preference, an experiment was conducted in which bees had a choice between avocado honey, nonavocado honey, and nonavocado honey enriched with potassium. Potassium concentrations were 3768 and 325 mg/kg in the avocado and nonavocado honeys, respectively (Table 1). We, therefore, dissolved 3443 mg potassium in 1 kg nonavocado honey solution to reach a concentration equivalent to that of the avocado honey solution. Four different potassium salts were used: potassium chloride (KCl), potassium hydrogen phosphate (K_2HPO_4), potassium D-gluconate ($C_6H_{11}KO_7$), and tri-potassium citrate ($C_6H_5K_3O_7$). The amounts of potassium salts added were calculated from their molecular weights to reach the desired concentration of potassium. Eight colonies were tested for each of the four salts. Each day, all the colonies were tested for the same potassium salt, with 1 or 2 d between tests. Because the addition of potassium salts did not noticeably change the color or odor of the honey, the feeders were placed on colored cardboards (yellow, blue, or purple) to allow the bees to visually discriminate between the solutions. The colors were rotated between colonies and between days of the experiment to avoid potential color bias.

Statistical Analyses

Differences in the responses of harnessed bees to odors were tested by χ^2 test. The sugar sensitivity experiment was analyzed by two-way ANOVA, including the honey source as a nominal factor, the log of the sugar concentration as a continuous factor, and the proportion of discriminating bees at each sugar concentration as the dependent variable. A discriminating bee was defined as a bee that extended its proboscis in the trial with sugar solution but not in the preceding trial with water. This provided a conservative measure of the sensitivity to the sugar itself. Differences in the preferences of free-flying bees between

Table 1 Concentrations (mg/kg) of detected minerals in avocado and nonavocado honey and nectar

Mineral	Avocado honey	Nonavocado honey	Avocado nectar	Citrus nectar
K	3768.3	324.8	3946.2	184.7
P	651.5	47.0	511.2	18.5
Mg	204.6	18.5	188.3	<5
S	188.3	27.7	170.4	<5
Ca	82.7	75.8	<150	<150
Na	58.9	79.1	53.8	18.5
Si	18.0	7.0	43.9	29.5
Zn	10.9	1.5	<30	<30
B	9.9	7.2	10.8	4.2
Fe	9.3	2.7	13.5	<5
Cu	3.2	0.1	3.1	<0.5
Pb	1.2	2.9	<1	<1

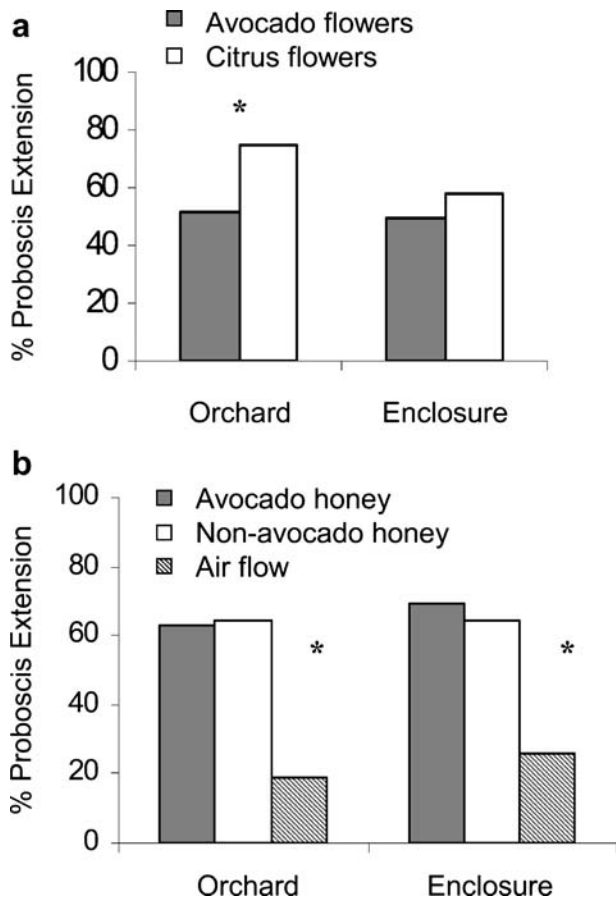
honey sources were tested by one-way ANOVA. Differences between pairs of treatments within the same experiment were tested by Tukey–Kramer test. The arcsin square root transformation was employed on the percentage honey consumption data before analysis (Sokal and Rohlf, 1995). Statistical analyses were performed using JMP 5.0.1 software (SAS Institute, Inc.).

Results

Odor

Approximately half of the bees from the enclosure responded spontaneously to floral odors, and there was no difference between the response rates to avocado and citrus flowers ($\chi^2_{1,428} = 3.03, P > 0.05$; Fig. 1a). The response of the orchard bees to the odor of avocado flowers was similar to that of the bees from the enclosure ($\chi^2_{1,355} = 0.27, P > 0.05$), but a significantly higher proportion responded to citrus flowers than to avocado flowers ($\chi^2_{1,282} = 16.8, P < 0.001$).

Fig. 1 The percentage of bees that responded spontaneously, by proboscis extension, to different odors: (a) floral odors and (b) honey odors. Bees from two colonies were tested. One hive was placed in an orchard ($N = 142$ bees) and the second in an enclosure ($N = 215$ bees). * indicates significant differences between treatments with $P < 0.001$ (χ^2 test)



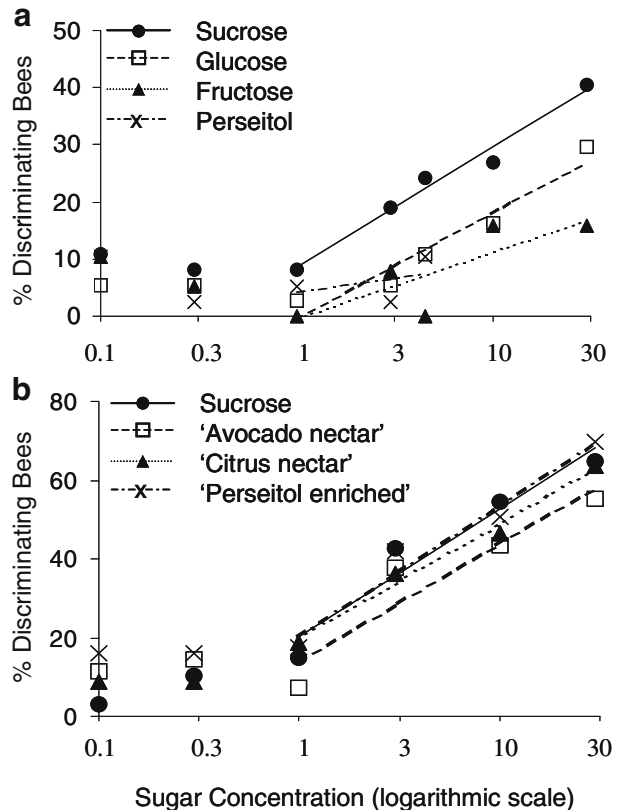
The spontaneous response rate of bees to honey odors was 65%, and it was not affected by honey source ($\chi^2_{1,712} = 0.40$, $P > 0.05$; Fig. 1b) or location ($\chi^2_{1,712} = 0.74$, $P > 0.05$). The spontaneous response to the air flow was lower than the responses to either honey odor ($\chi^2_{2,1067} = 176.7$, $P < 0.001$).

Sugars

Visual inspection shows that bees started detecting sugars only at concentrations $>1\%$ (Fig. 2). Therefore, we analyzed the effect of sugar type and concentration for concentrations of $\geq 1\%$. In the first experiment, which measured the sensitivity of bees to different sugars, bees were most sensitive to the sucrose solution (Fig. 2a). Both sugar type ($F_{3,10} = 14.7$, $P = 0.001$) and concentration ($F_{1,10} = 31.2$, $P < 0.001$) affected discrimination, but the interaction between them was not significant ($F_{3,10} = 1.99$, $P > 0.05$). Nevertheless, separate linear regressions for each sugar showed that the percentage of discriminating bees increased with increased sucrose ($F_{1,3} = 139.5$, $P = 0.001$) and glucose ($F_{1,3} = 41.5$, $P = 0.008$) concentration, while no concentration effect was evident for fructose ($F_{1,3} = 6.21$, $P > 0.05$) or perseitol ($F_{1,1} = 0.23$, $P > 0.05$).

In the second experiment, which measured the bees' sensitivity to four sugar mixtures, bees responded similarly to all sugar mixtures ($F_{3,8} = 1.77$, $P > 0.05$; Fig. 2b). Sensitivity

Fig. 2 The percentage of bees that discriminated between having their antennae touched with cotton balls soaked with distilled water and sugar solution according to their proboscis extension response. (a) The discrimination percent in response to different sugars at increasing concentrations ($N = 150$ bees). (b) The discrimination percent in response to different sugar mixtures at increasing concentrations ($N = 275$ bees). Lines are linear best fits



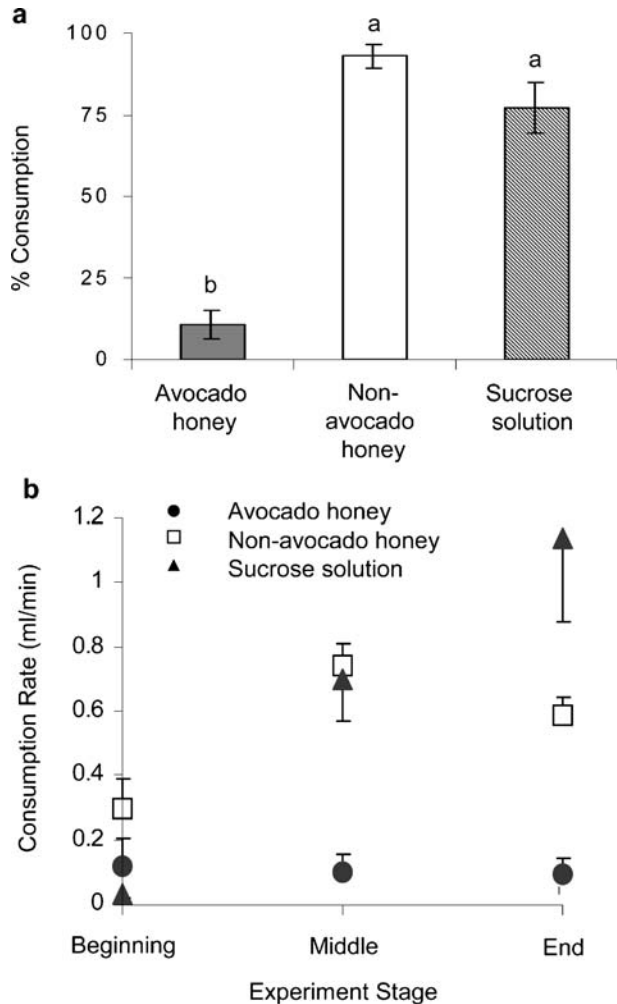
increased with concentration ($F_{1,8} = 127.0, P < 0.001$) in a similar fashion for all sugar mixtures (sugar mixture \times concentration: $F_{3,8} = 0.12, P > 0.05$).

Color

Repellence by avocado honey was observed even when opaque feeders were used, and bees could not discriminate between the solutions by their color ($F_{2,27} = 44.9, P < 0.001$; Fig. 3a). Consumption of avocado honey was lower than that of nonavocado honey and sucrose solution (Tukey’s test, $P < 0.05$). No significant differences were found in final consumption between nonavocado honey and sucrose solution (Tukey’s test, $P > 0.05$).

The consumption rate of the solutions was measured every 20 min. Figure 3b shows the average rate of the first, middle, and last 20-min increments of the experiment for every colony. Consumption rate was affected by the solution source ($F_{2,81} = 18.8, P < 0.001$), by the time point in the experiment ($F_{2,81} = 13.8, P < 0.001$), and by the interaction between

Fig. 3 (a) Bees’ mean (\pm S.E.) relative consumption of different sugar solutions presented in covered feeders ($N = 10$ colonies). Relative consumption represents the ratio between the amount consumed from each solution and the amount consumed from the solution with the highest consumption, for each colony tested. Different letters indicate significant differences between treatments with $P < 0.05$ (Tukey’s test). (b) Consumption rate (ml/min \pm S.E.) as the experiment progressed. Beginning—The rate during the first 20 min of the experiment. Middle—The rate during 20 min in the middle of the experiment. End—The rate during the last 20 min of the experiment



them ($F_{4,81} = 7.46$, $P < 0.001$). Consumption rate of the nonavocado honey was highest after the first 20 min and increased until the middle of the experiment. Consumption rate of the sucrose solution showed a different pattern. Sucrose was consumed the most slowly at the beginning, but the rate increased consistently, and it was consumed at the highest rate toward the end of the experiment.

Phenolic Compounds

Consumption of the sucrose solution enriched with phenolics from avocado honey was higher than that of the sucrose solution or of the avocado honey ($F_{2,6} = 30.6$, $P = 0.001$; Fig. 4), with no significant difference between the latter two (Tukey's test, $P > 0.05$), probably due to the strong attraction to the enriched sucrose solution.

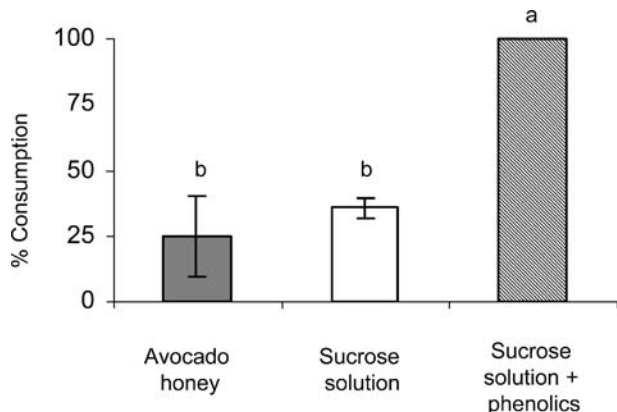
Mineral Composition

Twelve different minerals were found in avocado honey at concentrations higher than 1 mg/kg (Table 1). The concentrations of 10 minerals out of the 12 were higher in the avocado honey than in the nonavocado honey, in some cases by an order of magnitude. Among the minerals that were detected in the honey samples, nine were also detected in avocado nectar, and only five in citrus nectar, all of them with higher concentrations in avocado than in the citrus nectar. Potassium was the most dominant mineral in all samples, with a concentration in avocado honey and nectar that was >10-fold that in nonavocado honey and citrus nectar.

Potassium

The consumption of nonavocado honey enriched with potassium salts was intermediate between those of nonavocado honey and avocado honey (Fig. 5). Three salts significantly reduced consumption relative to nonavocado honey, but were still preferred to avocado honey (KCl: $F_{2,21} = 42.4$, $P < 0.001$; K-gluconate: $F_{2,21} = 101.3$, $P < 0.001$; K_3 -citrate: $F_{2,21} = 234.5$, $P < 0.001$). Only K_2 -phosphate reduced consumption to the level of avocado honey ($F_{2,21} = 85.5$, $P < 0.001$).

Fig. 4 Bees' mean (\pm S.E.) relative consumption of solutions containing avocado honey, sucrose solution, and sucrose solution enriched with phenolics extracted from avocado honey. Different letters indicate significant differences between treatments with $P < 0.05$ ($N = 3$ colonies; Tukey's test)



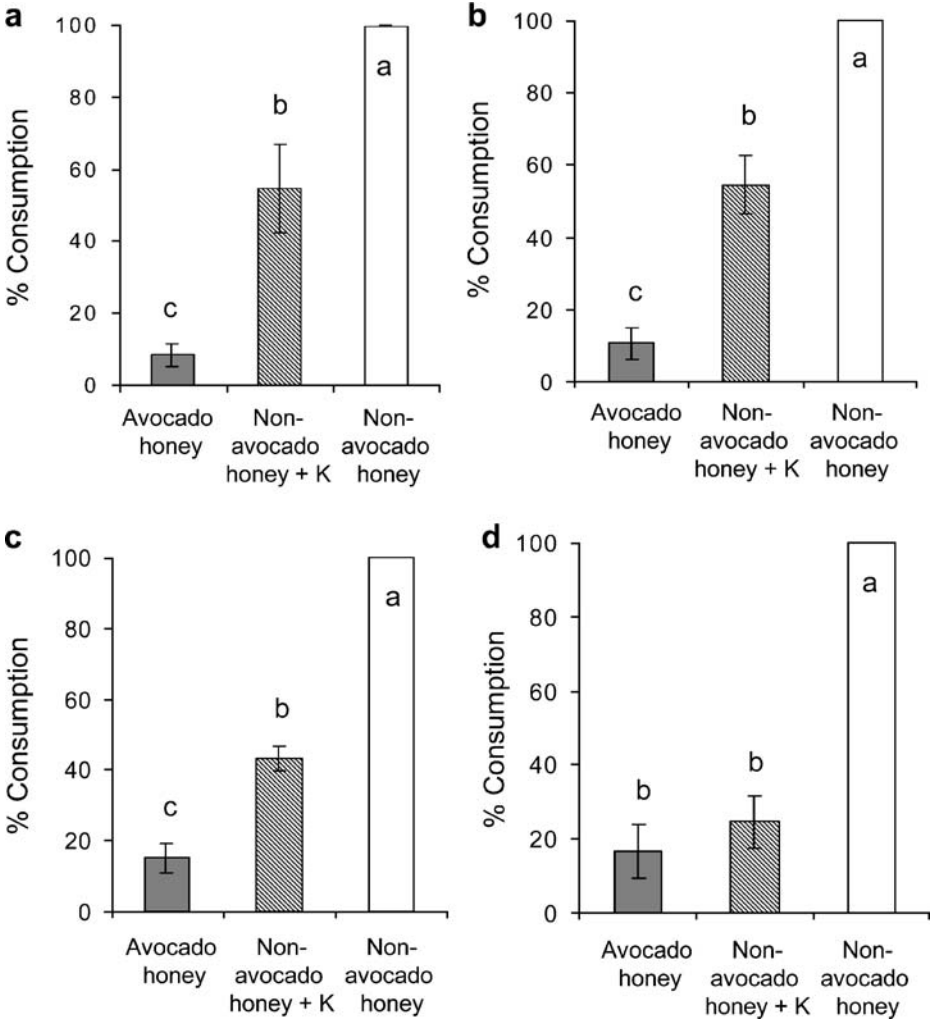


Fig. 5 Bees' mean (\pm S.E.) relative consumption of solutions containing avocado honey, nonavocado honey, and nonavocado honey enriched with different potassium salts: (a) KCl, (b) K-gluconate, (c) K₃-citrate, and (d) K₂-phosphate. Different letters indicate significant differences between treatments with $P < 0.05$ ($N = 8$ colonies for each potassium salt; Tukey's test)

Discussion

The dissolved solids in nectar consist mainly of carbohydrates (Luttge, 1977), but a wide variety of minor components may define its nature (Adler, 2000). This complicates the identification of individual taste and odor compounds that may affect its attractiveness to pollinators. Here, we separated various constituents of nectar and tested their effects on the preferences of honey bees. The response of honey bees to floral and honey odors evaluated the importance of volatile compounds. The odor of citrus flowers was more attractive to experienced bees than that of avocado flowers. These foragers were collected from a colony located among blooming citrus and avocado trees. Because honey bees tend to prefer citrus

over avocado (Vithanage, 1990; Ish-Am and Eisikowitch, 1998; Gazit and Degani, 2002), it is likely that we collected more bees that were foraging on citrus than on avocado. The higher response to the odor of citrus flowers probably reflected the bees' foraging experience in the field. The indifference of naïve bees to the floral odors supports the notion that odors act mainly as signals that bees learn to associate with their respective floral rewards, and do not themselves affect choice behavior greatly. This view was also supported by the indifference of bees to honey odors, while they clearly preferred nonavocado honey in taste assays. Taste, rather than odor, probably affected the choice of honey, because honey bees find it difficult to discriminate between honey odors (Bonod et al., 2003).

The response of bees to different sugar solutions indicated that the sugar composition of avocado nectar cannot explain its low attractiveness. Their sensitivity to sucrose was found to be higher than their sensitivity to glucose or fructose, separately, thus supporting the findings of Wykes (1952), Waller (1972), and Bachman and Waller (1977). Sugar mixtures, however, are perceived differently relative to pure sucrose (Wykes, 1952; Waller, 1972; Bachman and Waller, 1977). The response to a hexose-rich mixture, "citrus nectar" in the present experiments, was similar to that of sucrose. Therefore, it appears that high sucrose content in avocado nectar does not diminish its attractiveness, in contrast to previous assumptions (Ish-Am, 1994). Bees did not respond to perseitol solution, and their response to sucrose solution containing perseitol was similar to their response to pure sucrose solution. Perseitol, which was suspected to deter honey bees from avocado (Ish-Am, 1994; Can-Alonzo et al., 2005), seems to have no effect on preference. Thus, differences in sugar composition between avocado and citrus nectar cannot account for the bees' stronger preference for citrus flowers. Moreover, during the process of honey ripening, sucrose is inverted into glucose and fructose. As a result, the sugar compositions of avocado and nonavocado honeys are similar (Dag et al., 2006). Nevertheless, higher consumption of nonavocado honey in the color and potassium experiments indicated that preference is determined by a component other than the dominant sugars.

Honey color also could not explain the bees' preferences. Bees were repelled by the avocado honey even when feeders were covered. Comparison of the consumption rate as the experiment progressed revealed a potential role of odor in establishing preference. The honey solutions would be easy to locate due to their aromas, whereas sucrose solution is odorless. Bees visiting the nonavocado honey feeder would further mark it with Nasonov pheromone (Winston, 1987), and bees visiting the avocado honey feeder would abandon it. Thus, a rapid preference for the nonavocado honey feeder would develop (Fig. 3b). Eventually, bees finding the sucrose solution would start marking it with pheromone, and by the end of the experiment, it did indeed attract the greatest number of bees, revealing that it is even more attractive than nonavocado honey.

Phenolic compounds have been suspected of being repellent to bees (Baker, 1977; Rhoades and Bergdahl, 1981; Adler, 2000), but actual repellence has seldom been found. Hagler and Buchmann (1993) tested two phenolic compounds and found that they were repellent at high concentrations, but at low concentrations they did not deter bees and even increased attractiveness. They also found repellence to phenolic-rich nectars and honeys from three different botanical sources. One of them was almond honey, which was later found to be repellent due to amygdalin (London-Shafir et al., 2003). Another source was salt cedar, which contains high potassium concentrations (Waller et al., 1972). Our results indicated that phenolics increase the attractiveness of sucrose solution to honey bees, probably by adding odor to the solution, making it easier to locate. A similar increase in

visitation rate to phenolic-rich nectar was found for *Apis cerana* (Liu et al., 2004). Hence, no support was found for a repellent effect of phenolics on honey bees.

Little is known about the effect of nectar minerals on honey bee foraging behavior (Nicolson and W.-Worswick, 1990). A repellent effect of sodium chloride in sucrose solution was demonstrated by von Frisch (1950), who showed that a 0.015 M salt solution deterred honey bees, although a 0.0075 M solution no longer deterred them. The sodium component in the latter solution was 173 ppm. We found lower sodium concentrations than that in all honeys and nectars tested, and they were within a similar range in all samples. Hence, sodium does not seem to be responsible for deterring bees from avocado. Waller et al. (1972) found a repellent effect of potassium in onion nectar, at potassium concentrations similar to those found in the current study for avocado nectar. Our results indicate that, for three out of four potassium salts tested, dissolving 3500 ppm potassium in nonavocado honey to equalize its concentration with that of avocado honey decreases consumption by half. This indicates that potassium is a major cause for the low attractiveness of avocado honey, but not the only one. Cations such as potassium are usually accompanied by anions, although selective secretion to nectar is also possible (Luttge, 1977). In avocado nectar, potassium may be coupled with perseitol (Ishizu et al., 2001). This is supported by the strong correlation between perseitol and potassium in avocado honey (Dag et al., 2006). The most dominant anion found in avocado honey was phosphate, but its concentration was too low to equalize the potassium concentration. Adding K_2 -phosphate to nonavocado honey reduced consumption to a level similar to that of avocado honey. Thus, high mineral concentrations, mainly of potassium and phosphate, seem to be the main cause for the low attractiveness of avocado flowers. The effect of other minerals, such as magnesium, sulfur, and copper, whose concentrations differ between avocado and nonavocado honeys, still remains to be tested. Potassium concentration in avocado nectar may often be higher than that measured in this study and, therefore, high enough to repel honey bees from avocado flowers. Potassium concentration in avocado nectar is high, but not unique (Hiebert and Calder, 1983; Waller et al., 1972). The effect of nectar minerals, potassium in particular, on the foraging behavior of pollinators may be widespread.

The reason for the presence of repellent components in nectar is not clear, but several possible roles have been suggested (Rhoades and Bergdahl, 1981; Adler, 2000). To answer this question for avocado, we have to compare the honey bees' response with avocado nectar, these insects not being its natural pollinators, with the response of avocado's natural pollinators. These natural pollinators may not be as strongly repelled by the nectar. For example, various hummingbird species have been found to differ in their response to mineral-rich nectar (Bouchard et al., 2000). Another approach would be to test whether potassium concentration in the nectar correlates with its abundance in the soil. It is possible that in avocado's natural habitat, the infertile soils of the neotropical rainforest (Wolstenholme, 2002), potassium concentration in the nectar is lower than in cultivated plots, and does not repel pollinators. It would also be interesting to study the influence of the agricultural practice of intensive fertilization of avocado (Lahav and Whaley, 2002) on the pollination effectiveness of bees.

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(Wink and Witte, 1991). Early in the twentieth century, German plant breeders started producing “sweet” lupins with seed alkaloid contents lower than 0.01%, making them suitable as a high-protein animal feed (Wink, 1994). Narrow-leafed lupin, *Lupinus angustifolius*, and white lupin, *L. albus*, comprise, to date, most of the grain lupin produced worldwide (Gladstones, 1998; Berlandier, 2001; Littlewood and Garlinge, 2001). A consequence of reducing alkaloid content in cultivated lupins has been an increased susceptibility to generalist herbivore species such as the aphid *Myzus persicae* (Wink, 1994).

M. persicae is a highly polyphagous species, feeding on plants from more than 50 families (Weber, 1985). However, despite the well-documented polyphagia of *M. persicae* as a species, there is considerable variation among populations in performance on different hosts (Weber, 1985; Hales et al., 1997). Thus, rather than being characterized by a general-purpose genotype (Vorburger et al., 2003a,b), *M. persicae* populations are more often locally adapted to a regional complement of host plants often varying in time and space (Weber, 1985; Nikolakakis et al., 2003). For example, the tobacco aphid described previously as a separate species *M. nicotianae* (Blackman, 1987), is now generally accepted to be a host-adapted subspecies of *M. persicae* (Clements et al., 2000; Zitoudi et al., 2001; Margaritopoulos et al., 2003). In the “wheatbelt” of Western Australia (WA), *M. persicae* populations show little variation in host use. In a previous study, all of 30 WA-collected clones of *M. persicae* tested were capable of successfully feeding on cultivated narrow-leafed lupins (Edwards, 2001). Unlike other generalist legume aphids in WA (i.e., *Acyrtosiphum kondoi*, *Aphis craccivora*), *M. persicae* feeds successfully on the narrow-leafed lupin varieties “Kalya” and “Tanjil” (Edwards et al., 2003), which have been bred for aphid resistance (Littlewood and Garlinge, 2001).

We examined the hypothesis that successful lupin feeding by *M. persicae* in WA is not a species characteristic, but instead indicates local adaptation of WA clones to a novel host plant. This hypothesis predicts that *M. persicae* clones from eastern Australia, where cultivated narrow-leafed lupins are not commonly grown, could not utilize this host as well as clones from Western Australia. To test this, we measured the growth rate and colonization ability on narrow-leafed lupins of nine aphid clones collected off a wide range of host plants from various locations around eastern Australia to a lupin-feeding clone from Western Australia. The plant varieties used have well-characterized levels of resistance to aphids with “Tallerack” being the most susceptible, “Tanjil” showing moderate levels of resistance, and “Kalya” being the most resistant of the three varieties (Cowling, 1999; Littlewood and Garlinge, 2001; Zehnder et al., 2001; Edwards et al., 2003). We tested our hypothesis by comparing the performance of selected clones on artificial diet containing the most abundant phloem alkaloids in these varieties.

Methods and Materials

Experimental Species

Radish plants, *Raphanus sativus* “Long Scarlet”, used to maintain the aphid cultures, were planted in commercially available UC Riverside potting soil mix and were used for aphid rearing at 3–4 wk after emergence. Narrow-leafed lupin seeds for varieties Tallerack, Kalya, and Tanjil were donated by Kadambot Siddique (Center for Legumes in Mediterranean Agriculture, University of Western Australia, WA). Seeds of all varieties were sown in pairs in commercially available topsoil (Soils ain’t Soils, Balcatta, WA) in seedling trays and transplanted, 1 wk after emergence, to 1-l plastic pots containing the same soil. Each pot

received 50 ml tap water three times per week. Plants were used for experiments 15–20 d after transplanting. At this time, they had 5–7 fully expanded leaves. All plants were kept in a temperature-controlled glasshouse maintained at $20 \pm 5^\circ\text{C}$ under natural light (approx. 14:10 hr L:D) until needed.

The clones of *M. persicae* used were provided by Christoph Vorburger and Paul Sunnucks (Department of Genetics, La Trobe University, VIC, Australia) or Dinah Hales (Department of Biological Sciences, Macquarie University, NSW, Australia) except for the lupin-feeding clone (“Lupin” clone), which was collected in September 2002 from *L. angustifolius* in the wheatbelt region of Western Australia (Table 1). Because a previous extensive survey of WA clones showed little variation in feeding ability on narrow-leaved lupins in the wheatbelt area (Edwards, 2001), the lupin-feeding clone was considered representative of the common lupin-feeding phenotype in WA. Because all the colonies could not be successfully maintained on narrow-leaved lupin, experimental consistency was maintained by rearing them for at least three generations on radish before they were used in the experiments. The rearing methods and procedures were as described by Edwards (2001). Additionally, a bulk-rearing of the clones was maintained on whole 3- to 4-wk-old radish plants kept in an incubator at 20°C and 14:10 hr L:D photoperiod. New radish plants were infested with 10 apterae every 7–10 d to maintain a constant supply of suitable insects for the experiments.

Immature Performance

Each aphid clone was tested for performance on each of the 3 lupin varieties. Performance was assayed on eight individual replicate plants of each variety. Individual plants were infested with ten 2nd-instar nymphs (<48 hr old) randomly selected from the pool available, weighed collectively on a microbalance, and placed on the cotyledons or on the lowermost axillary leaf on the plant. Each plant was covered with a mesh sleeve made from Voile material to fit snugly around the pots. Sleeves were held upright over the plant canopy by loops made from electrical wire (25 mm diam) with their ends buried in the soil within the pot. The experiment was terminated after 5 d to ensure that the aphids did not begin to produce nymphs (Edwards, 2001), then the surviving aphids were removed from the plants and again weighed as a group. The mean relative growth rate (MRGR) was calculated as the difference between the logarithms of the weight of aphids put on the plant and the weight of aphids removed from the plants, divided by the number of days the aphids had been on the plant (Edwards, 2001). The experiment was set up in single replicates including

Table 1 Collection and life cycle information of *M. persicae* clones used in the experiments

Clone ID	Locality	Collection date	Original host	Life cycle
BP2	Monbulk, Victoria	March 14, 2002	<i>Capsella bursa</i>	Holocyclic
C61	Shepparton, Victoria	April 1, 2002	<i>Capsicum annuum</i>	Holocyclic
C70	Myrtleford, Victoria	April 2, 2002	<i>Capsicum annuum</i>	Holocyclic
X13	Monbulk, Victoria	March 14, 2002	<i>Solanum nigrum</i>	Holocyclic
23	Inverquarity, Tasmania	October 20, 1998	<i>Prunus persicae</i>	Holocyclic
M53	Bacchus Marsh, Victoria	March 25, 2002	<i>Malva spp.</i>	Holocyclic
Rad72	Myrtleford, Victoria	April 2, 2002	<i>R. raphanistrum</i>	Holocyclic
Y3	Silvan, Victoria	March 14, 2002	<i>S. physalifolium</i>	Holocyclic
Y63	Bright, Victoria	April 2, 2002	<i>S. physalifolium</i>	Holocyclic
Lupin	Jennacubbine, WA	September 10, 2002	<i>Lupinus angustifolius</i>	Anholocyclic

all aphid clones and repeated over time to obtain eight replicates for each plant variety/aphid clone combination. Analysis of variance (ANOVA) was used to test for differences in MRGR among clones within lupin varieties (PROC GLM, SAS Institute, 1996). All data were $\log(x)$ transformed as required to conform to the assumptions of normality before analyses. Significant results obtained by ANOVA were subjected to Tukey's mean separation test (PROC GLM; SAS Institute, 1996).

Colonization Ability of Apterous Females

Based on the MRGR data from the three lupin varieties, we selected five clones of aphids for further testing: C61, BP2, C70, X13, and the lupin-feeding clone. This experiment was designed to determine the ability of newly molted (<24 hr) females to establish a colony on each of the lupin varieties. This assay was selected for more detailed clonal comparisons because, compared to the MRGR assay in the previous section, it provided a greater capacity to distinguish among the clones by measuring reproductive capacity in addition to growth. A single apterous female was placed on the growing tip (shoot) of a plant and confined by using "sock-cages" made out of Voile material as described. Apterous were chosen for this experiment because of their higher fecundity, and because previous studies have shown no difference in host acceptance between apterous and alate *M. persicae* on lupins (Edwards, 2001; Cardoza et al., 2005). Insects were allowed to feed and reproduce for 12 d. The experiment was set up in single replicates consisting of 5 clones \times 3 varieties, and was repeated over time as needed to obtain a total of five replicates for each clone/variety combination. At the end of the experiments, all insects were removed from the plants, and their cumulative weights were determined and recorded. Differences in colonization ability among the clones on the three lupin varieties (PROC GLM, SAS Institute, 1996) were tested by ANOVA. Significant results obtained by ANOVA were further analyzed by Tukey's mean separation test (PROC GLM, SAS Institute, 1996).

Gas Chromatography-Mass Spectrometry Analysis of Lupin Phloem Alkaloids

Plants were grown as described above, and phloem samples were obtained by submerging the stems of two excised lupin plants (approx. 2 g FW) in 3 ml 20 mM EDTA-2 Na for 5 hr. The concentration of ethylenediaminetetraacetic acid (EDTA) was chosen as optimal based on preliminary experiments testing a range of concentrations. A total of 6 samples per plant variety were obtained in this manner. Samples were immediately stored at -80°C and freeze-dried within 24 hr of collection. The dry phloem sample was dissolved in 2 ml double-distilled water. An aliquot of 1 ml of this solution was used for alkaloid analysis. Phloem samples (1 ml) were made basic with 200 μl of $\text{NH}_3\text{H}_2\text{O}$ to pH 10.0, and extracted with dichloromethane twice. The pH was further increased with 200 μl of 10 M NaOH to pH 14.0 and extracted with dichloromethane twice. Dichloromethane extracts were combined, concentrated under nitrogen gas to yield total alkaloid extracts, and dissolved with ethyl acetate before GC-MS analysis.

The presence of lupin alkaloids in phloem was determined by gas chromatography-mass spectrometry (GC-MS) with an HP5986 GC-MS instrument equipped with an HP-5MS column (0.24 μm film thickness, 10 m \times 0.35 mm i.d.) and helium as the carrier gas. Injector temperatures were maintained at 250°C , and oven temperature was programmed from 40°C (isothermal for 2 min) to 290°C at a rate of $10^{\circ}\text{C}/\text{min}$. Injections of 1 μl were performed on-column. Auxiliary temperature was 250°C . The electron impact mass spectra were recorded at 70 eV ionization energy, and at a scan mode covering a mass range (m/z) of 50–500 amu.

Identifications were made by comparison of the mass spectral data and retention times to those of authentic samples (lupanine and 13-OH-lupanine) provided by David Harris (Harris and Spadek, 1986; Brooke et al., 1996). Phloem samples were coinjected with authentic samples. The amounts of individual alkaloids were determined by using standard curves for the appropriate compounds at five concentrations, using eicosane as internal standard. Differences in the amounts of the detected alkaloids among the three lupin varieties were evaluated using ANOVA (PROC GLM; SAS Institute, 1996). Significant results obtained by ANOVA were then subjected to Tukey's mean separation tests.

Artificial Diet Feeding Assays

Alkaloids were isolated from the high-alkaloid (1.8%) *L. angustifolius* cv. Fest by David Harris at the Western Australia Chemistry Centre. Mixed alkaloids were extracted with trichloroacetic acid and then made basic with 10 M caustic. Individual alkaloids were extracted with dichloromethane, then converted to perchlorate salts, and allowed to precipitate from methanol. Salts were purified by recrystallization, and the final products were >99% pure (Harris and Spadek, 1986; Brooke et al., 1996).

Artificial diet sachets were used to evaluate the effect of the individual alkaloids detected in the GC-MS profiles of the lupin varieties. The diet used was that described in Dadd and Mittler (1966). Diet sachets were prepared by dispensing 135 μ l of the diet in between two layers of Parafilm[®] stretched over a Plexiglass ring (20 mm diam; 5 mm high), and insects were confined to the feeding arena within the ring with a round glass coverslip secured with a metal bulldog clip (Ridsdill-Smith et al., 2004). The lupin-feeding clone, along with clones BP2 and X13, was used to test the effect of different alkaloid concentrations on aphid performance, based on MRGR. Two alkaloids (13-OH-lupanine and lupanine) were tested, based on their detection in our phloem sample analyses. Treatments consisted of artificial diet containing 0% (control), 0.001%, or 0.01% for the lupanine alkaloid. In preliminary experiments, amending the diet with similar amounts of 13-OH-lupanine did not cause any significant effect on aphid survival and weight gain; therefore, this compound was tested at 0% (control), 0.01%, and 0.1% concentrations instead. Amended diet solutions were prepared fresh on the day of the experiment. Insects used for this assay were 1st instar nymphs (\leq 48 hr) of BP2, X13, and the lupin-feeding clone. Nymphs were procured by transferring approximately 20 apterous females from the bulk rearing cages to Petri plates containing a single radish leaf. The petiole of the leaf was embedded in an agar slab to prevent desiccation.

Females were allowed to feed and nympho-posit for a period of 48 hr, after which nymphs were randomly collected and weighed in groups of 5 with a Sartorius 3MP microbalance (Göttingen, Germany). Nymphs were then transferred to the different treatment sachets and allowed to feed for 5 d. After this feeding period, surviving nymphs were counted, collected, and collectively weighed. These data were used to determine the MRGR of the different clones on the diet treatments. The experiment was set up in triplicates with each replicate containing three concentrations of the compounds for each of the three aphid clones. The experiment was repeated over time to obtain a total of nine replicates for each compound concentrations/aphid clone combination. All insects and experimental sachets were kept in a 20°C incubator maintained at a 14:10 hr L:D photoperiod for the duration of the experiment. The effect of each alkaloid on *M. persicae* clonal MRGR was statistically analyzed by ANOVA with "clone" as a treatment and alkaloid concentration as a covariate (PROC GLM; SAS Institute, 1996). To test for discriminating dosages, clonal MRGR comparisons were conducted by using single-factor analyses of variance, with Bonferroni correction for multiple comparisons (PROC GLM, SAS Institute, 1996).

Results

Immature Performance

Immature performance (MRGR) of the clones was significantly affected by the plant variety ($F = 19.23$; $df = 2,231$; $P < 0.001$). The greatest variation in performance was obtained in the most susceptible narrow-leaved lupin variety Tallerack, on which 70% of the clones tested achieved a positive MRGR (Fig. 1). The MRGR of the lupin-feeding clone was higher than that of all other clones ($F = 4.28$; $df = 9,71$; $P < 0.001$), except clone C61 (Fig. 1). The lupin-feeding clone had the highest MRGR on the resistant varieties Tanjil ($F = 2.31$; $df = 9,71$; $P < 0.001$) and Kalya ($F = 4.85$; $df = 9,71$; $P < 0.001$) (Fig. 1).

Colonization Ability of Apterous Females

We found a significant effect of variety ($F = 91.3$; $df = 2,70$; $P < 0.001$) and clone ($F = 10.3$; $df = 4,70$; $P < 0.001$), but not their interaction on the insect weight accumulated by *M. persicae*. The highest insect mass was attained by the lupin-feeding clone on all three lupin varieties (Fig. 2). The greatest variability in colonization performance was observed on the susceptible variety Tallerack with the lupin-feeding clone showing a greater increase in overall weight gain ($F = 3.38$; $df = 4,21$; $P = 0.029$), followed by clones C61, X13, C70, and finally BP2 (Fig. 2). In contrast, the total weight gain obtained by clone BP2 on this lupin variety was lower compared to those of all other clones ($F = 3.38$; $df = 4,21$; $P = 0.029$) (Fig. 2). The lupin-feeding clone also outperformed all other clones on the resistant varieties Tanjil ($F = 3.41$; $df = 4,21$; $P = 0.027$) and Kalya ($F = 4.23$; $df = 4,21$; $P = 0.012$) (Fig. 2). Interestingly however, the accumulated weight of the lupin-feeding clone was greater on Kalya than on Tanjil ($F = 5.82$; $df = 2,12$; $P = 0.017$) (Fig. 2).

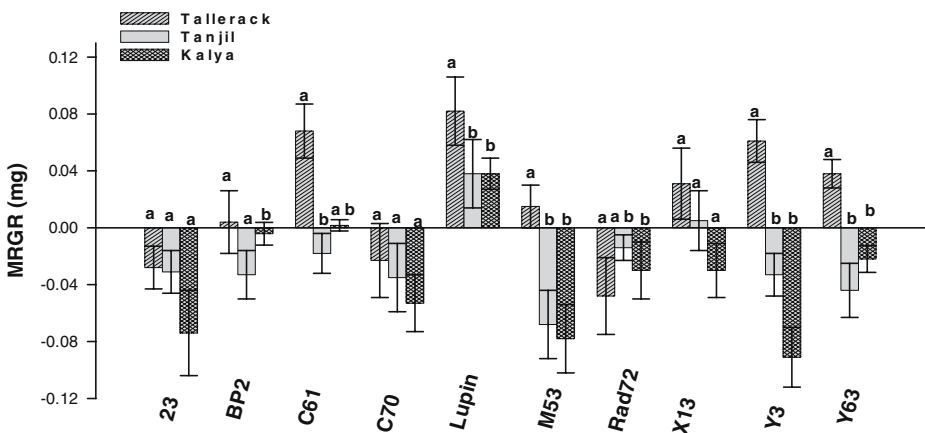


Fig. 1 Mean relative growth rate (MRGR) of 10 Australian *M. persicae* clones on three *L. angustifolius* varieties, “Tallerack” (susceptible), “Tanjil”, and “Kalya” (resistant). “Lupin” refers to the lupin-feeding *M. persicae* clone. Different letters represent significant differences in MRGR for individual clones across the three lupin varieties tested (Tukey’s mean separation test, $P \leq 0.05$). Error bars denote 1 SE

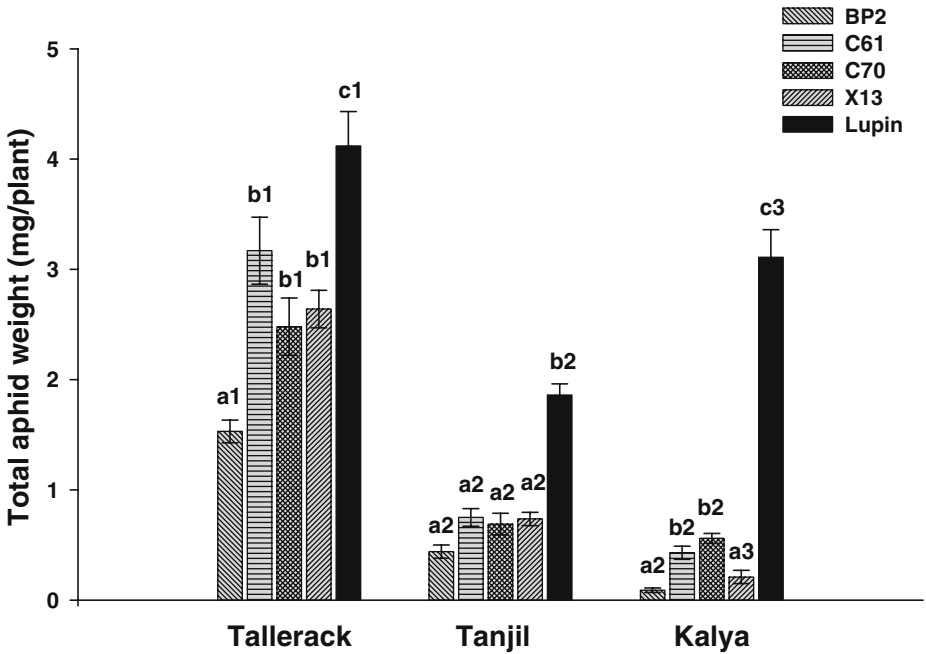
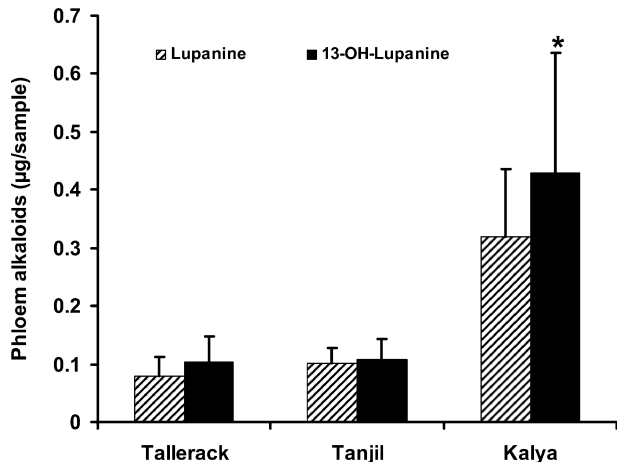


Fig. 2 Accumulated colony weight gain of 5 Australian *M. persicae* clones on *L. angustifolius* varieties, Tallerack (susceptible), Tanjil, and Kalya (resistant). Lupin refers to the lupin-feeding *M. persicae* clone. Different letters represent significant differences between insect clones within plant varieties whereas numbers represent significant differences within clones across the three plant varieties (Tukey’s mean separation test, $P \leq 0.05$). Error bars denote 1 SE

Gas Chromatography-Mass Spectrometry Analysis of Lupin Phloem Alkaloids

Analyses of phloem collected via EDTA chelation showed no qualitative differences between the susceptible variety Tallerack and its resistant counterparts. Furthermore, amounts of the 13-OH-lupanine alkaloid did not differ among lupin cultivars. However, the amounts of the

Fig. 3 Level of alkaloids 13-OH-lupanine and lupanine in phloem collected from *L. angustifolius* varieties, Tallerack (susceptible), Tanjil, and Kalya (resistant), based on GC-MS analyses. Significant differences were found in the levels of alkaloids between the most resistant Kalya and the other two varieties tested (Tukey’s mean separation test, $P \leq 0.05$). Error bars denote 1 SE



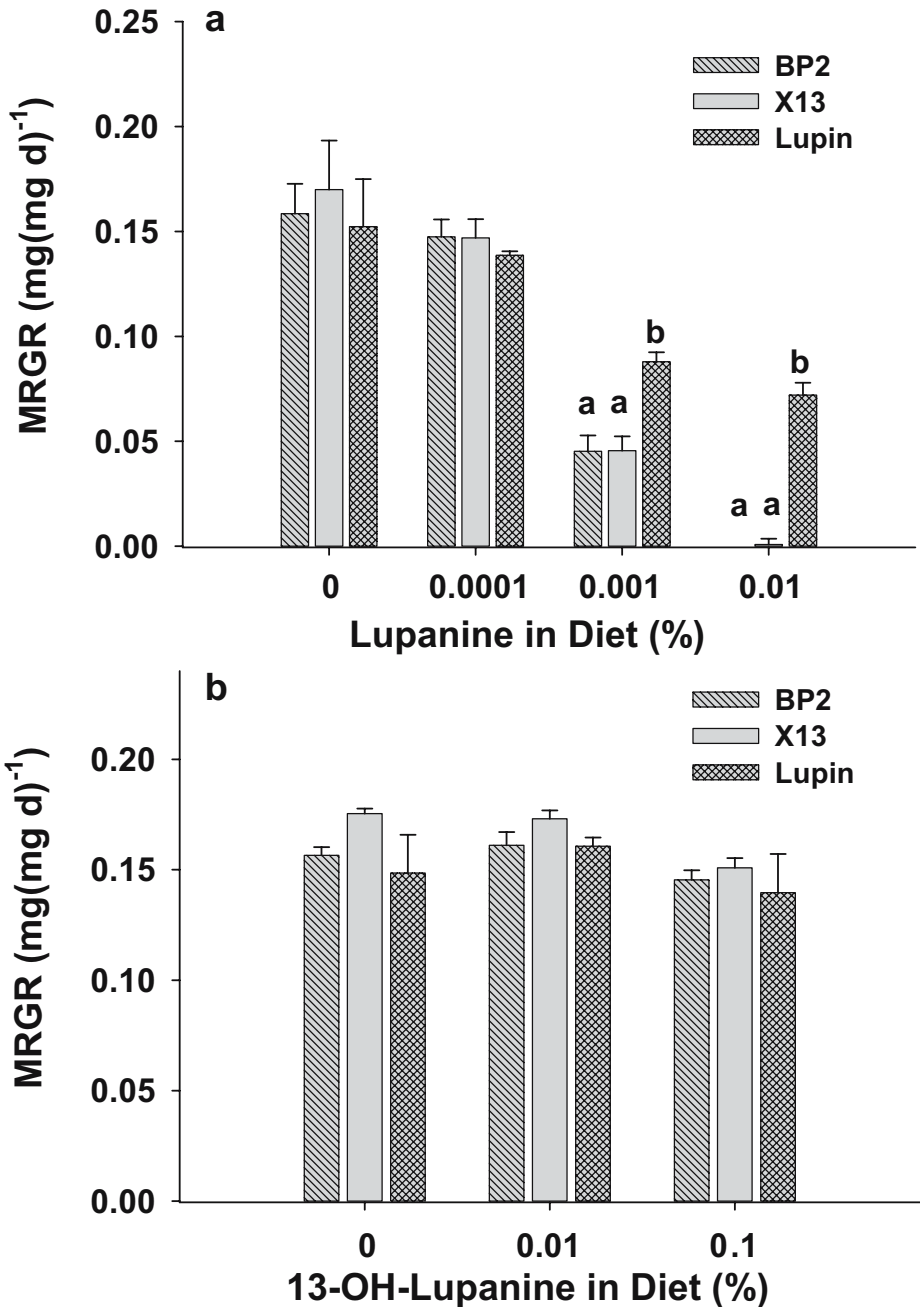


Fig. 4 Performance, based on MRGR, of selected *M. persicae* clones on artificial diet amended with different levels of *L. angustifolius* alkaloids. Lupin refers to the lupin-feeding *M. persicae* clone. (a) Diet amended with 0 (control), 0.0001%, 0.001%, and 0.01% lupanine, and (b) Diet amended with 0 (control), 0.01%, and 0.1% 13-OH-lupanine. Different letters indicate significant differences between insect clones within alkaloid concentrations (Tukey's mean separation test, $P \leq 0.05$). Error bars denote 1 SE

lupanine alkaloid differed among the three lupin cultivars ($F = 4.18$; $df = 2,14$; $P = 0.038$) (Fig. 3). Not surprisingly, Tallerack (the most susceptible variety) yielded the lowest amount of this alkaloid and Kalya (the most resistant variety) had the highest amount. However, differences in phloem lupanine content were only significantly higher for Kalya, compared to the levels of the other two cultivars (Fig. 3).

Artificial Diet Feeding Assays

Aphid MRGR was affected by lupanine concentration ($F = 152.8$; $df = 1,105$; $P < 0.001$), but did not differ among the clones across all concentrations ($F = 0.344$; $df = 2,105$; $P = 0.71$) (Fig. 4a). Aphid MRGR decreased with increasing lupanine concentration in all the tested clones (Fig. 4a). The lupin-feeding clone showed a higher MRGR than the other two clones at a lupanine concentration of 0.01% ($F = 21.046$; $df = 2,24$; $P < 0.001$), but not at 0.001% ($F = 3.840$; $df = 2,26$; $P = 0.036$) (Fig. 4a). Aphid MRGR also differed among the tested 13-OH lupanine concentrations ($F = 7.44$; $df = 1,89$; $P = 0.008$), but did not suppress aphid growth to the same degree as lupanine—even at a higher concentration of 0.1% (Fig. 4b). The tested clones did not respond differentially to 13-OH lupanine.

Discussion

In this study, a lupin-feeding *M. persicae* clone from the Western Australia wheatbelt was shown to significantly outperform nine clones from eastern Australia on both susceptible and resistant narrow-leaved lupin varieties. This suggests that the ability to feed on narrow-leaved lupins in *M. persicae* is not characteristic of the species. Previous work has shown that the ability to feed on lupins by Western Australian *M. persicae* clones is not related to the host plant upon which they were collected, nor does it vary with collection location in the wheatbelt (Edwards, 2001). Aphids, in general, and *M. persicae*, in particular, show phenotypic plasticity that allows them to exploit new host species, overcome novel resistance mechanisms in newly developed plant varieties, and become resistant to the most common insecticidal compounds used for their control. Host plant adaptation frequently occurs in many aphid species. The pea aphid (*Acyrtosiphon pisum*) has been demonstrated to form host races specialized to particular host plant genera (Via, 1991; Sandstroem, 1994). In some generalist aphid species including *M. persicae*, genetic structuring among host plants has been reported (Zitoudi et al., 2001; Ruiz-Montoya et al., 2003). Local selection pressures acting on asexually reproducing aphid populations often result in the predominance of specialized clones (Haack et al., 2000; Vorburger et al., 2003a,b). The abundance of narrow-leaved lupins during the WA growing season may provide sufficient selection pressure for a lupin-adapted clone to predominate.

Lupin alkaloids have been previously implicated in providing defense against insect herbivores, including aphids (Waller and Nowacki, 1977; Berlandier, 1996; Sweetingham et al., 1998). These alkaloids are known to be present in the phloem (Wink and Roemer, 1986), and we have shown, with EDTA chelation, that levels of the bioactive alkaloid lupanine appear to correlate with the levels of aphid resistance in the lupin varieties Tallerack and Kalya. Despite being moderately resistant, the lupin variety Tanjil did not exhibit significantly higher phloem lupanine levels than Tallerack. There is some evidence that resistance in Tanjil involves an induced component (Edwards et al., 2003; Cardoza et al., 2005), so it is possible that phloem lupanine levels could be elevated by aphid feeding. Differences in *in planta* performance among aphid clones were reproduced by using artificial diet supplemented with lupanine,

further implicating this alkaloid in aphid resistance. Thus, it appears that the improved lupin-feeding performance of the lupin-feeding clone from Western Australia is related to its ability to overcome the toxic effects of the phloem alkaloid lupanine.

Insect herbivores can overcome host plant chemical defenses in a number of ways including olfactory discrimination, reduced rates of gut resorption, improved metabolic detoxification, and/or toxin sequestration (Bernays and Chapman, 1994). Polyphagous generalists often select plants, or tissues within plants, in which toxin levels are lower (Schoonhoven, 1972), but aphids appear not to discriminate among narrow-leaved lupin varieties with varying levels of resistance (Edwards et al., 2003). Successful aphid feeding may also involve avoiding the induction of plant defenses (Zhu-Salzman et al., 2004), but there appears to be at most a minor induced component to aphid resistance in resistant narrow-leaved lupin varieties (Cardoza et al., 2005). As such, behavioral avoidance of alkaloid ingestion does not appear to be the mechanism by which *M. persicae* successfully feeds on lupin. Rather, as suggested by the diet bioassays in this study, lupin-feeding *M. persicae* must have a higher tolerance of lupanine in their diet.

The mechanism by which the lupin-feeding clone accomplishes this has yet to be determined. Little is known of the mechanisms underlying host use in aphids (but see Hawthorne and Via, 2001; Via and Hawthorne, 2002; Del Campo et al., 2003). There is no evidence that *M. persicae* has the capacity to sequester alkaloids as is accomplished by the specialist lupin aphid, *M. albifrons* (Wink and Roemer, 1986). It is possible that, like *M. albifrons*, the lupin-feeding clone of *M. persicae* has reduced gut resorption of lupanine, and the majority is excreted in its honeydew (Wink and Roemer, 1986). Pyrrolizidine alkaloids are excreted in the honeydew of *M. persicae* feeding on *Senecio* spp., but the honeydew also contains hydrolyzed by-products including retronecine (Molyneux et al., 1990). The reduced toxicity of 13-OH lupanine compared to lupanine suggests one mechanism by which *M. persicae* could successfully detoxify lupanine. Salivary oxidative enzymes are widely present in aphids (Urbanska et al., 1998; Miles, 1999; Cherqui and Tjallingii, 2000), as are a myriad of enzyme systems (e.g., esterases, glutathione-*S*-transferases) known to function in the breakdown of toxic phytochemicals (Loayza-Muro et al., 2000; Francis et al., 2001). Evidently, further research is necessary to determine the fate of lupanine in the lupin-feeding clone. A comparative study of the lupin feeding physiology between the lupin-feeding clone and clone BP2, which achieved the poorest performance on all narrow-leaved lupin varieties, may shed light on this.

We present evidence of intraspecific variation in Australian *M. persicae* in its ability to feed on lupins with varying degrees of aphid resistance. Our data show high variability in aphid response to resistance factors in narrow-leaved lupins. We believe that the information provided on aphid clonal performance provides the basis for future work on the biochemical and/or genetic factors underlying host-adaptive plasticity in *M. persicae* and other generalist aphid species.

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Introduction

Variation in the concentration of plant phenolics and increased activities of oxidative enzymes in response to insect attack are common phenomena in plants (Felton et al., 1994; Bi et al., 1997; Constabel and Ryan, 1998; Stout et al., 1998; Constabel et al., 2000). The relationship between reduced growth of Lepidopteran larvae and phenolic compounds in attacked plants has been described in several reports (Hartley and Firm, 1989; Stamp 1990; Felton et al., 1992b; Horwath and Stamp, 1993; Stamp and Yang, 1996). However, studies of plant metabolic pathways that are activated in response to insect attack are relatively recent, and the results are far from conclusive.

Phenolic oxidation catalyzed by polyphenol oxidase (PPO) and peroxidase (POD) is potentially a plant defense mechanism against herbivorous insects. Covalent bonds formed between oxidized phenols (quinones) and leaf proteins can alter the availability of amino acids (Felton et al., 1989). Alkylation of amino acids, especially essential amino acids, can result in reduced nutritional value of plant proteins for insects (Felton et al., 1992a; Stout et al., 1998). Also, quinones can be directly toxic to herbivores (Duffey and Stout, 1996), and the cyclic reduction of oxidized phenols can form reactive oxygen species ($\cdot\text{OH}$, H_2O_2 , and $\text{O}_2^{\cdot-}$), that damage essential nutrients and/or complex molecules, such as lipids, proteins, and nucleic acids (Bi and Felton, 1995).

The leaf miner, *Leucoptera coffeella* (Guérin-Méneville, 1842) (Lepidoptera: Lyonetiidae), the main insect pest of coffee plants in Brazil, is also an important primary pest in other coffee-producing countries. It is a microlepidopteran, in which butterflies only lays eggs in the adaxial leaf surface during the night. After a period of approx. 5 d, larvae arise directly from eggs to the leaf mesophyll, without contact with the external environment (Parra 1985). Larvae feed exclusively on palisade parenchyma cells, which in coffee leaves are formed by a single layer of cells (Deddeca 1957). On average, after 10 d the larva leave the leaf mesophyll to pupate, which lasts approx. 5 d. Adults survive on average 10 d under laboratory conditions (Parra 1985). Leaves attacked by this leaf miner usually fall and, depending on the defoliation intensity, yield loss, may account for 50% losses in coffee production (Paulini et al., 1976; Thomaziello et al., 1979).

The genus *Coffea* includes two commercially important species, both susceptible to *L. coffeella*: *C. arabica* L. and *C. canephora* Pierre. Seventy percent of commercial coffee plantations use Arabic-type plants, because of the superior quality of their beans (Pereira 2000).

A strategy for the development of cultivars resistant to leaf miners has been the transfer of resistance genes from the wild species *C. racemosa*, via successive back crosses to *C. arabica*. Although resistance to this pest is conferred by two complementary and dominant genes, little is known about the biochemical nature of the resistance (Guerreiro-Filho et al., 1999). There have been few studies on the biochemical nature of resistance of coffee plants to the leaf miner. Recently, Guerreiro-Filho and Mazzafera (2000) studied plants from 13 *Coffea* Species and *C. arabica*/*C. racemosa* hybrids, and concluded that resistance is not related to leaf concentrations of caffeine. The differences observed in the leaf structures of *C. arabica*, *C. racemosa*, and susceptible and resistant hybrids are not related to their resistance to leaf miners (Ramiro et al., 2004). However, Ramiro et al. (2004) observed reduced insect growth on leaves of resistant plants. This suggests that chemical substances in the palisade parenchyma might interfere with feeding and the consequent development of leaf miners.

Chlorogenic acid is a general term used to identify a series of esters formed by quinic acid with cinnamic acids (Clifford 1985). Among several isomers found in coffee,

5-caffeoylquinic acid is the most abundant in *C. arabica* seeds (Clifford 1985) and leaves (Mazzafera and Robinson, 2000). This phenol is susceptible to the action of PPO and POD and can be rapidly converted by PPO into chlorogenoquinone, a highly reactive molecule capable of bonding covalently to other molecules, principally proteins, making them less available for assimilation by the digestive tract of insects (Felton et al., 1989). The quantity of constitutive PPO in coffee leaves is relatively high compared to that found in other species. However, induction of this enzyme by mechanical wounding and by treatment with methyl jasmonate is limited (Mazzafera and Robinson, 2000).

Our objective was to analyze the physiological importance of phenolic oxidation and its role in resistance of coffee plants to attack by *L. coffeella*, in *C. arabica*, *C. racemosa*, and in some of their hybrids presenting different levels of resistance.

Methods and Materials

Plant Material

Analyses were carried out on healthy leaves of mature coffee plants maintained under field conditions at the Experimental Center of Instituto Agronômico (IAC), Campinas (SP), Brazil. Plants received the same agricultural practices, such as fertilization and weed control. Plants were not subjected to drought or cold stress, and did not receive any form of chemical treatment to control pests in order to prevent any detrimental effect on the experiments. Plants were divided into four groups: (1) *C. arabica* (susceptible); (2) *C. racemosa* (resistant); (3) susceptible hybrids; and (4) resistant hybrids. The hybrids belong to two progenies of advanced generations of crosses between *C. arabica* and *C. racemosa*, obtained through genetic breeding to produce coffee plants resistant to the leaf miner *L. coffeella*.

Five individuals from each group were chosen by using plant vigor and resistance or susceptibility as selection criteria. They were as follows: *C. arabica* cultivar Obatã IAC-1669-20, lot 100 (C 400, C 215, C 393, C 576, C 569); *C. racemosa* (H6608-1, H6611-1, H6593-1, H6593-3, IAC 5057); *C. arabica* × *C. racemosa*—resistant (H14954-7, H14954-29, H14954-37, H14954-45, H14954-46); *C. arabica* × *C. racemosa*—susceptible (H13685-1-10, H13685-1-2, H14949-14, H13376-8, H13685-1-26). The alphanumeric designators in parenthesis refer to the identification of each individual plant.

Resistance Evaluation

Insects were produced in rearing cages, using the method described by Katiyar and Ferrer (1968) and adapted by Parra (1985). Cages made with voile tissue and containing infested plants were maintained in a room with high humidity, and a 10% aqueous sucrose solution was offered to insects by wetting a filter paper placed inside of the cage. Coffee plant resistance was evaluated according to Guerreiro-Filho (1994), who validated the method by comparing laboratory evaluations with nursery and field evaluations. Here, healthy mature leaves were collected from the field, with their peduncles immersed in distilled water in assay tubes. Leaves were exposed overnight to insect oviposition in the cages. The next day, using a stereo microscope, a place on the adaxial leaf surface containing three eggs was chosen, and a leaf disk (1 cm diam) was removed with a cork borer. Nine disks were obtained for each plant, and they were transferred into a plastic box containing foam in the bottom. The box was closed with transparent glass and placed under dim laboratory light at room temperature ($25 \pm 2^\circ\text{C}$). Humidity inside the boxes was maintained by adding

distilled water to the foam. Evaluation of the degree of resistance was made after 10 d of infestation using a scale from 1 to 4 (Guerreiro-Filho et al., 1999): 1 = point lesions (resistant); 2 = small filiform lesions (moderately resistant); 3 = large irregular lesions (moderately susceptible); 4 = large rounded lesions (susceptible). The experimental model adopted for data analysis was a fully randomized allocation, with nine replications (disks), and with the factors “group” and “plants” being analyzed by nested analysis of variance (ANOVA).

Biochemical Analyses

Biochemical analyses consisted of comparative analyses between infested and uninfested leaves in different stages of insect development. For extraction of total soluble phenols, 20 healthy mature leaves were collected from each plant in the field. Ten leaves were infested in the insect rearing cages as previously described, whereas 10 others remained uninfested. Except for exposure to insects, all leaves were maintained under the same conditions. In the case of leaves exposed to leaf miners, eggs deposited on one side of the central leaf vein were eliminated with a scalpel, leaving only those deposited on the other side. Eggs were eliminated under a stereo microscope (Guerreiro-Filho et al., 1999). Infested leaves were maintained in humid boxes until 2 d after eclosion of the caterpillars. The uninfested leaves were maintained in separate boxes under the same conditions and for the same period of time. After eclosion, a 25 mm² leaf disk was removed from each side of the central vein of the leaves (sides with and without lesions). On the infested sides, leaf disks were taken from undamaged areas, near the lesions. Soon after they were removed, the disks were individually identified, weighed, and maintained on ice for further extraction. For phenol extractions, each leaf disk was placed into 3 ml ethanol, in screw-top vials, and maintained in a water bath at 50°C until the leaf tissue was completely discolored. Total soluble phenols (Swain and Hillis, 1959) and chlorogenic acid (5-caffeoylquinic acid) (Mazzafera 1999) were determined in this extract. Chlorogenic acid was used as a standard to build a calibration curve ($y = 0.01028x + 0.043$, $R = 0.9936$) to measure total soluble phenols.

A chromatographic profile of the phenolic compounds was obtained with a Shimadzu high-performance liquid chromatography (HPLC), equipped with a diode array detector. Detection of compounds was monitored between 190 and 400 nm. The detector signal was acquired with a workstation, using the Shimadzu Class VP system. An LC18 reverse phase column (Supelco) was used for separation. The separation gradient was 0–70% methanol in 0.5% sodium acetate for 25 min, progressing from 70% to 100% in 25–26 min, and maintained at 100% methanol until 35 min. The solvent flow was 1 ml/min. Quantification of chlorogenic acid for each treatment was made by calculating the areas of the graphs at 326 nm and compared to those obtained with pure chlorogenic acid (Sigma, St. Louis, MO, USA). The elution time of chlorogenic acid was 10 min. Data were analyzed by using a factorial model (infestation group) in a completely randomized experimental design, with five replications (plants). Chlorogenic acid concentration indices were transformed using the equation $y = \log(x + 1)$.

To determine POD and PPO activities, leaves were removed from each plant, placed into leaf-miner-infested cages, and transferred into humid chambers. They were analyzed 1 d (1 DAE) or 4 d (4 DAE) after eclosion of the larva. Controls were uninfested leaves maintained in the humid chambers during the same periods as the infested leaves. Freshly sampled leaves were also collected for analysis. The leaves of five plants were used for each group.

As eclosion of coffee leaf miner eggs occurs approximately 5 d after egg laying (Parra 1985), leaf segments 1 and 4 DAE were evaluated 7 and 10 d, respectively, after the leaves were collected from the field.

Enzymes were extracted with 100 mM sodium phosphate buffer, pH 7, containing 5 mM dithiothreitol (DTT) and polyvinylpyrrolidone (1:10 w/v) in a Polytron homogenizer (Kinematica AG, USA). Leaves were cut into small pieces and submitted to a 10-sec pulse at speed 3, followed by an additional 20 sec at speed 5. Part of the extracts was transferred into Eppendorf tubes, and centrifuged in an Eppendorf bench-top centrifuge at 14,000 rpm for 15 min. The supernatant was collected for protein concentration determination (Bradford 1976). Extracts were stored at -80°C for later analysis. POD and PPO measurements followed the methods used by Mazzafera et al. (1989). POD activity was measured with 5 μM guaiacol and 0.05% H_2O_2 in 100 mM K-phosphate buffer, pH 7, following the absorbance variation at 420 nm (25°C , 30 min, in the absence of light). PPO activity was measured with 7 μM chlorogenic acid and 45 mM glycine at 100 mM K-phosphate buffer, pH 7, following the absorbance variation at 420 nm (30°C , 30 min, in the absence of light). Data were analyzed by using the factorial model (days after eclosion populations) in a fully random experimental design, with five replications (plants). Rates were transformed using the equation $y = \log(x + 1)$.

ANOVA was determined with the statistical program SANEST (Zonta and Machado, 1992), and the means compared with the Tukey test, at 5% probability.

Results

Damage to Leaf Disks

The expression of resistance by plants is shown in Table 1. ANOVA confirmed the existence of significant differences in the expression of resistance to leaf miners among the coffee groups. However, there were no significant differences among plants belonging to the same group. Comparison of means by the Tukey test did not show significant differences between *C. arabica* and the susceptible hybrids, or between *C. racemosa* and the resistant hybrids.

Table 1 Level of resistance (scale of 1 to 4, with 1 being very resistant) to coffee leaf miner in plants sampled from four groups evaluated for the type of lesion in leaves

Groups	Individuals evaluated ^a					Mean
	1	2	3	4	5	
<i>Coffea arabica</i>	4.0	4.0	4.0	4.0	4.0	4.0a
<i>Coffea racemosa</i>	1.7	2.4	1.2	2.1	2.4	2.0b
Susceptible hybrids	2.7	4.0	2.9	3.2	2.7	3.0a
Resistant hybrids	1.6	1.0	1.1	1.0	1.0	1.1b

$F_{\text{groups}} = 49.41^{**}$
 $F_{\text{Plant (groups)}} = 0.37^{\text{ns}}$

^a Mean of nine leaf disks evaluated in the laboratory.

**Significant at the 1% probability level; n.s., not significant at the 5% probability level.

Total Soluble Phenols

Feeding by the coffee leaf miner did not significantly alter the phenol levels in leaves, either on the damaged side of the leaves, or on the side from which the eggs were removed, in each group of plants (Table 2). However, infested leaves showed a trend toward a decrease in phenolic compounds in all populations, although the decrease was not significant. Infested leaves of *C. arabica*, *C. racemosa*, and susceptible hybrids had 19.8%, 15.2%, and 17.0% decreases in phenol concentrations, respectively, compared to uninfested leaves. Resistant hybrids showed little change (3.0%) when infested (Table 2). This decrease in phenolic content was significant only in the means of the treatments (Table 2), demonstrating that these plants, in general, responded to feeding damage by the leaf miners with a reduction in the total soluble phenols in the leaves.

On average, phenolic compounds represented 5.3% of the fresh weight of the leaves of *C. arabica*, 4.8% in the susceptible hybrids, 5.1% in the resistant hybrids, and 2.3% in *C. racemosa*. The high phenolic content of *C. arabica* leaves seems to be a characteristic of the species, as *C. racemosa*, the resistant parent, showed significantly lower content compared with plants from the other groups (Table 2).

Qualitative Comparison of Phenolic Compounds

HPLC analysis did not reveal qualitative differences in the phenolic compounds in infested leaves compared to uninfested leaves (data not shown). On the other hand, *C. racemosa* had a considerably different elution pattern compared to the other genetic groups (Fig. 1). In this species, a large peak was detected at 20 min, but its identity could not be established. It showed two peaks of maximum absorbance at 270 and 326 nm (data not shown).

The leaf contents of chlorogenic acid were significantly different in all treatments. Uninfested leaves of *C. racemosa* had approximately six times less chlorogenic acid than *C. arabica* and the hybrid populations, which did not differ significantly from each other (Table 3).

Chlorogenic acid concentration showed a similar tendency to that observed for total phenols in the hybrids and in *C. arabica*, i.e., a reduced content in infested tissues. There was a 54.9% reduction in the quantity of chlorogenic acid on the infested side of *C. arabica* leaves compared to uninfested leaves. In the susceptible hybrids, reduction in phenol concentration was less pronounced (27.7%), but it was significant. In the resistant hybrid, there was a 19.4% reduction in chlorogenic acid in infested leaves; however, this trend was not significant. On the other hand, opposite to what was observed for total phenols, *C. racemosa* responded to leaf miner attack with a 66.6% increase in chlorogenic acid concentration.

Table 2 Mean concentrations of phenolic compounds [mass equivalents (mg) of chlorogenic acid per G fresh leaf] in leaves of *C. arabica* and *C. racemosa* and of two hybrids derived from a cross between these species

Groups	UL	USL	IL	Mean*
<i>C. arabica</i>	60.9 ± 10.9	49.2 ± 7.1	48.8 ± 4.6	52.9a
<i>C. arabica</i> × <i>C. racemosa</i> (S)	53.2 ± 13.9	45.7 ± 5.4	45.1 ± 5.5	48.0a
<i>C. arabica</i> × <i>C. racemosa</i> (R)	52.5 ± 2.1	49.9 ± 3.8	51.0 ± 2.9	51.2a
<i>C. racemosa</i>	24.9 ± 9.6	24.2 ± 10.3	20.6 ± 7.8	23.0b
Mean**	47.9A	42.2AB	41.5B	

Mean values for groups (*) and treatments (**) when followed by the same letter, they are not significantly different (Tukey, 5%). S: susceptible; R: resistant; UL: uninfested leaves; USL: uninfested side of the leaf; IL: infested side of the leaf.

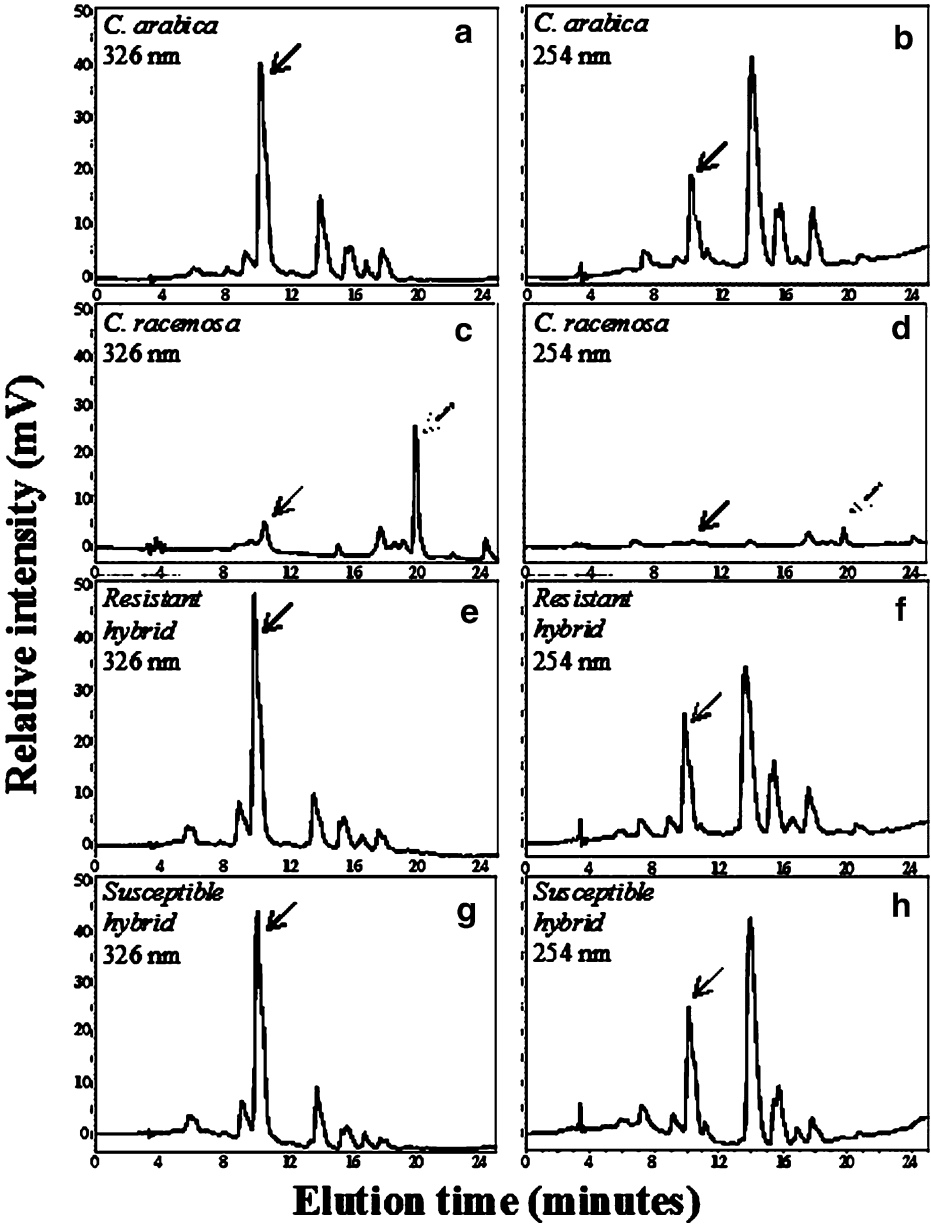


Fig. 1 HPLC chromatogram profiles of alcoholic extracts of leaves of *Coffea arabica* (a, b), *C. racemosa* (c, d), and resistant (e, f) or susceptible (g, h) hybrids to *Leucoptera coffeella*. Profiles obtained at 326 nm (a, c, e, g) and 254 nm (b, d, f, h) are shown. Solid arrows indicate chlorogenic acid and dashed arrow a possible chlorogenic acid isomer in *C. racemosa*

Table 3 Mean concentrations of chlorogenic acid ($\mu\text{g/g}$ fresh leaf) in *C. arabica* and *C. racemosa*, and in two hybrids derived from crosses between them, expressing different levels of resistance to the coffee leaf miner (*Leucoptera coffeella*)

Groups	UL	USL	IL
<i>C. arabica</i>	2092 \pm 411a A	1522 \pm 229b A	944 \pm 202c B
<i>C. arabica</i> \times <i>C. racemosa</i> (S)	1949 \pm 337a A	1674 \pm 197b A	1409 \pm 182b A
<i>C. arabica</i> \times <i>C. racemosa</i> (R)	1935 \pm 201a A	1833 \pm 178a A	1559 \pm 222a A
<i>C. racemosa</i>	355 \pm 18b B	431 \pm 164b B	591 \pm 126a C

Values for each group followed by the same capital letter and treatment followed by the same small letter were not significantly different (Tukey 5%). S: susceptible; R: resistant; UL: uninfested leaves; USL: uninfested side of the leaf; IL: infested side of the leaf.

Evaluation of Oxidative Enzyme Activity

POD activities, measured soon after leaves were collected, were higher in *C. arabica* and the resistant progeny than in the susceptible hybrid. Lowest activities were observed in *C. racemosa*, which had up to 15 times less activity.

Under insect infestation, the groups responded differently to treatments 1 and 4 DAE. There was no induction of POD activity due to insect attack in any of the populations at 1 DAE (Table 4). At 4 DAE, leaf miners provoked a 72.4% increase in POD activity in *C. arabica* and 89.6% in the susceptible hybrids. POD activity induced by *L. coffeella* was positively correlated with the type of lesion provoked by feeding activity (Table 1, $R = 0.46$, $T = 2.17$, $P < 0.05$). In these plants, the damage caused by insect feeding was characterized by a massive and localized destruction of a large number of palisade cells (Ramiro et al., 2004).

PPO activity in recently collected leaves varied significantly among populations (Table 5). *C. racemosa* leaves had twice the activity of *C. arabica* leaves, and was fivefold higher than leaves from hybrid plants. The constitutive PPO concentrations in resistant and susceptible hybrids were lower than in the parental species. At 1 DAE, leaf miner attack did not induce PPO activity in any of the populations. At 4 DAE, PPO was significantly induced by insect feeding only in *C. racemosa*. Activity was lower in leaves of resistant and susceptible hybrids, in all the treatments, when compared to the parents.

Table 4 Mean peroxidase activity ($\Delta A_{470}/\text{hr}/\mu\text{g}$ protein) in recently collected, infested, and uninfested coffee leaves, 1 or 4 days after eclosion of the leaf miner larvae

Groups	RCL	1 DAE		4 DAE	
		UL	I	UL	I
<i>C. arabica</i>	0.569 \pm 0.116b A	0.363 \pm 0.088c B	0.397 \pm 0.078c B	0.566 \pm 0.111b A	0.976 \pm 0.240a A
Susceptible hybrids	0.212 \pm 0.049c B	0.457 \pm 0.090b AB	0.432 \pm 0.129b B	0.355 \pm 0.091b B	0.673 \pm 0.194a B
Resistant hybrids	0.676 \pm 0.097a A	0.591 \pm 0.118a A	0.627 \pm 0.192a A	0.550 \pm 0.183a A	0.720 \pm 0.144a B
<i>C. racemosa</i>	0.047 \pm 0.013c C	0.065 \pm 0.020bc C	0.097 \pm 0.022b C	0.155 \pm 0.024a C	0.168 \pm 0.052a C

Values for each group followed by the same capital letter and treatment followed by the same small letter were not significantly different (Tukey 5%). DAE: days after eclosion of the leaf miner larvae; RCL: recently collected leaves; UL: uninfested leaves; I: infested leaves.

Table 5 Mean polyphenol oxidase activity ($\Delta A_{470}/\text{hr}/\mu\text{g}$ protein) in recently collected, infested and uninfested coffee leaves, 1 or 4 days after eclosion of the leaf miner larvae

Groups	RCL	1 DAE		4 DAE	
		UL	I	UL	I
<i>C. arabica</i>	0.155 ± 0.028a B	0.128 ± 0.015a B	0.132 ± 0.029a B	0.155 ± 0.043a B	0.153 ± 0.045a B
Susceptible hybrids	0.075 ± 0.023a C	0.055 ± 0.019a C	0.070 ± 0.023a C	0.080 ± 0.025a C	0.106 ± 0.029a C
Resistant hybrids	0.068 ± 0.017a C	0.057 ± 0.020a C	0.074 ± 0.027a C	0.048 ± 0.015a C	0.095 ± 0.028a C
<i>C. racemosa</i>	0.303 ± 0.077b A	0.350 ± 0.104b A	0.366 ± 0.054b A	0.336 ± 0.087b A	0.521 ± 0.176a A

Values for each group followed by the same capital letter and treatment followed by the same small letter were not significantly different (Tukey 5%). DAE: days after eclosion of the leaf miner larvae; RCL: recently collected leaves; UL: uninfested leaves; I: infested leaves.

Discussion

Resistance of *C. racemosa* to the leaf miner *L. coffeella* was attributable to expression of two dominant and complementary genes that were transferred to *C. arabica* by successive backcrosses of the interspecific hybrids to the later parent (Guerreiro-Filho et al., 1999). Among the hybrids obtained from these backcrosses, we selected one resistant and one susceptible to the leaf miner to study the role played by phenols and oxidative enzymes in resistance. Although the defensive role of phenolic compounds and oxidative enzymes appear to be more evident in other plant–insect interactions (Felton et al., 1989; Duffey and Stout, 1996), our results suggest that phenol content apparently does not play a central role in the resistance of coffee to the coffee leaf miner. We found differences between parental species in total soluble phenol concentration, chlorogenic acid content, and oxidative PPO activity; however, the hybrids, did not differ for any of these characteristics.

Data obtained here show that feeding by *L. coffeella* is correlated with quantitative alterations in phenol metabolism and in the activity of oxidative enzymes in coffee leaves. In general, plants responded to feeding damage by reducing the total soluble phenols in the leaves (Table 2). High phenol concentration seems to be characteristic of *C. arabica*, whereas *C. racemosa*, the donor of resistance genes, showed significantly lower concentration. However, the values of hybrids were similar to *C. arabica*. Additionally, the chromatographic profiles of the hybrids were similar to *C. arabica*.

C. racemosa showed a large peak on HPLC, not detected in other plants used in this study. Although speculative, this peak might be an isomer of chlorogenic acid because it showed a maximum absorption peak at 326 nm, a characteristic wavelength for this group of compounds (Clifford 1985). Identification of chlorogenic acid isomers has been carried out mostly in *C. canephora* and *C. arabica* (Clifford 1985; Clifford et al., 2003). The only report on *C. racemosa* indicated the presence of 5-caffeoylquinic acid as the main isomer in seeds (Clifford et al., 1989).

The peak was absent in the hybrids and *C. arabica*, suggesting that it might not be related to leaf miner resistance. However, insect attack in *C. racemosa* was correlated with a significant induction of chlorogenic acid and PPO. Consequently, this compound is being isolated for identification and to determine its participation in the resistance of *C. racemosa* to *L. coffeella*.

The significant increase in chlorogenic acid and PPO activity observed only in insect-infested *C. racemosa* suggests a strategy to increase the availability of substrate for the production of quinones via enzyme activity. However, this does not seem to be the key factor in the defense of resistant hybrids against the leaf miner, because neither chlorogenic acid induction nor increased PPO activity was observed in these plants.

In a recent report, Melo et al. (2006) showed that constitutive PPO activity was higher in leaves of *C. racemosa* than *C. arabica*, and that it was induced by methyljasmonate in the former species, but not by mechanical damage. Also, two other hybrids from these species showed PPO activity close to the *C. arabica* values. Additionally, the phenol content was higher in *C. arabica*. In support of our results, infestation of the two hybrids and *C. arabica* plants with *L. coffeella* did not cause an increase in PPO activity, although a discrete drop of the constitutive activity was also observed in *C. arabica* leaves after insect exposure. Melo et al. (2006) also studied several other coffee species, but did not find a relationship between PPO activity and phenolic contents with resistance to coffee leaf miner or coffee leaf rust disease. These authors concluded that coffee resistance may be related to the oxidative potential of the tissue regarding the phenolic composition rather than simply to a higher PPO activity.

Unexpectedly, recently collected leaves of *C. arabica* showed higher constitutive levels of POD than either infested or uninfested leaves. We do not have a reasonable explanation for this decrease in activity after insect exposure except to speculate that it might be related to leaf detachment. This was not observed for other coffee populations. However, it is interesting to note that the resistant hybrid also showed high constitutive POD activity, but did not decrease after detachment. Leaf detachment does not affect resistance level as shown by Guerreiro-Filho (1994), who compared data obtained from infested coffee plants in the field and nursery with data from the leaf test used here.

Induction of POD due to attack by the leaf miner occurred in *C. arabica* and in the susceptible hybrids. Stout et al. (1994) suggested that the plant response to damage could be related to the type of tissue attacked and to the duration (persistence) and/or magnitude of the lesion. The ratio between damage intensity and response magnitude was almost always positive, indicating that the number of damaged cells influenced the response.

Our results showed a significant correlation between the amount of damage caused by caterpillars (level of resistance) and POD activity. Nevertheless, this differential induction of peroxidase may not be a reaction to insect attack, but rather an effort to replace damaged tissue, because this enzyme is responsible for lignification and suberization in vegetal tissues (Goldberg et al., 1985). Additionally, POD activity might also be related to oxidative stress developed during the larvae feeding (Bi and Felton, 1995).

According to Stout et al. (1994), the induction of PPO and POD in response to insect feeding is not definitive evidence that oxidative enzymes are directly involved in plant defenses to restrict insect attack, because of the complex relationship between these enzymes and several chemical compounds in the cell, and their multiple biological activities.

The action of phenols in biological systems seems to be influenced by several different factors, such as cell concentration, environmental conditions, structural variations, and the degree of specificity between the organisms involved (Appel 1993). *L. coffeella* has high specificity for coffee. It feeds exclusively on cells of the palisade parenchyma in susceptible *C. arabica* (Ramiro et al., 2004). Studies to evaluate phenols, carbohydrates, proteins, and other compounds in the palisade tissue of *C. arabica* and *C. racemosa* might bring important information for understanding of the resistance mechanism to this pest.

Assuming that resistant hybrids inherited the two resistance genes from *C. racemosa* (Guerreiro-Filho 1994), these results for phenolic content and POD/PPO activity are not strong evidence to suggest their participation in a direct defense mechanism of coffee

against *L. coffeella*. However, coinduction of chlorogenic acid and oxidative enzymes was observed in *C. racemosa*, indicating that the two resistance genes transferred to *C. arabica* (Guerreiro-Filho 1994) may not be related to such a mechanism.

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Introduction

Intraguild interactions between carnivores that share the same trophic resources include intraguild predation, which has been well documented (Rosenheim et al., 1993; Colfer and Rosenheim, 1995; Rosenheim 1998; Ferguson and Stiling, 1996; Raymond et al., 2000; Snyder and Ives, 2001), but also may involve interactions that serve to avoid intraguild predation (Doubtina et al., 1998; Taylor et al., 1998; Nakashima and Senoo, 2003). Immature stages of parasitoids often become intraguild prey in interactions between predators and parasitoids operating in the same guild, and so adult parasitoids would benefit by developing avoidance responses to cues from intraguild predator species living in the same habitats to reduce predation risks to their offspring. Aphids are associated with a large assemblage of insect natural enemies (Mackauer and Finlayson, 1967; Takada, 1968; Wheeler, 1977; Wratten and Powell, 1991; Ekbom, 1994), and so adult aphid parasitoids would encounter different types and species of intraguild predator during host searching. The more generalist aphid parasitoids, which can forage in a range of habitats, might be expected to develop a generalized response to predator cues.

Semiochemicals mediating intraguild interactions are poorly defined. Hydrocarbons have been reported previously as components of chemical trails left on substrates by seven-spot ladybirds, *Coccinella septempunctata* (Kosaki and Yamaoka, 1996). The same study suggested that large amounts of these chemicals were secreted from the tarsi. Recently, two aliphatic hydrocarbons, *n*-tricosane (C₂₃H₄₈) and *n*-pentacosane (C₂₅H₅₂), were shown to induce avoidance responses by the aphid parasitoid, *Aphidius ervi*, toward *C. septempunctata* (Nakashima et al., 2004). These hydrocarbons were present in the chemical trails left by the ladybirds on plant surfaces. Adult *A. ervi* females spent a significantly shorter time foraging on plants treated with these chemicals, resulting in lower parasitism rates of the pea aphid, *Acyrtosiphon pisum*, than on untreated plants (Nakashima et al., 2004).

In this study, we compare the responses to ladybird trails of three parasitoid species that attack *A. pisum* but which differ greatly in their host and habitat ranges. *A. eadyi* is a specialist on *A. pisum* (Pennacchio, 1989), and forages predominantly in habitats containing legume plants, whereas eight aphid species belonging to seven genera, including *A. pisum*, have been recorded as host species of *A. ervi* (Pennacchio, 1989), allowing it to forage in a wider range of habitats. *Praon volucre* is an even more polyphagous parasitoid, attacking at least 30 species belonging to 13 genera (Stary, 1976), which occur in a greater variety of habitats including forests. Therefore, each parasitoid species may be at risk from different species and numbers of intraguild predators due to these differences in the range of habitats in which they forage. For example, *C. septempunctata* occurs predominantly on herbaceous plants, while the two-spot ladybird, *A. bipunctata*, is found on both herbaceous plants and trees (Honék, 1985), suggesting that *P. volucre* would regularly encounter both *C. septempunctata* and *A. bipunctata*, but the two *Aphidius* species would probably encounter *A. bipunctata* much less frequently than *C. septempunctata*. It is hypothesized that *P. volucre* would detect and avoid a broader range of chemicals in ladybird trails than would the two *Aphidius* species, and that the *Aphidius* species should be more sensitive to the particular chemicals in trails of ladybirds that are dominant species in their preferred habitat. The aims of our study were to compare the avoidance responses of these three parasitoid species to the chemical trails of the two ladybird species, and to measure their sensitivity to the different hydrocarbons present in the trails.

Materials and Methods

Insects

The three aphid parasitoids, *Aphidius eadyi*, *Aphidius ervi*, and *Praon volucre*, were obtained from laboratory colonies that had been initiated with mummies of pea aphids, *Acyrtosiphon pisum*. The first two species were initially collected from pea fields in Harpenden, Hertfordshire, and Sharnbrook, Bedfordshire, UK, during spring 2001, while *P. volucre* was collected in Harpenden during spring 2002. Overwintered, adult, seven-spot ladybirds, *Coccinella septempunctata*, were collected from evergreen shrubs at Rothamsted Research during March and April, 2002. Overwintering, adult, two-spot ladybirds, *Adalia bipunctata*, were collected from window frames at Horticultural Research International in East Malling, Kent, UK, in November 2001. Parasitoids and ladybirds were both kept at 20°C and a light–dark 16:8 hr photoperiod, and provided with *Acyrtosiphon pisum*, reared on broad bean plants, *Vicia faba* L. (Fabaceae; var. Sutton), as food. Parasitoids were removed from colony cages at the mummy stage, and were kept in petri dishes (9 cm diam, 1.5 cm height) containing honey solution on cotton wool as adult food until emergence. Two days after the first adult emergence was observed, females were individually confined in small petri dishes (5.0 × 1.5 cm) with ~50 *A. pisum* and honey solution on cotton wool. All females used in bioassays were 4–5 d old.

Ladybird Chemicals

Extracts of ladybird chemical trails were prepared by confining a single *C. septempunctata* or *A. bipunctata* adult in a glass petri dish (9 × 1.5 cm) for 18 hr at 20°C, then removing the ladybirds and washing the dishes with distilled hexane (10 ml per dish). Glass surfaces provide an ideal substrate for the isolation and collection of insect chemical trails. The extracts were evaporated to 100 µl under a gentle stream of high-purity nitrogen and stored in microvials at –20°C until further use. These procedures were repeated six times for adults of each ladybird species.

Gas Chromatography

The ladybird footprint hexane extracts were analyzed on a Hewlett-Packard 6890A gas chromatograph (GC) equipped with a cool on-column injector, a flame-ionization detector, and a 50 m × 0.32 mm (0.52 µm film thickness) i.d. HP-1 bonded-phase fused-silica capillary column (J & W Scientific). The oven temperature was maintained at 30°C for 0.5 min, then programmed at 5°C/min to 150°C, held at this temperature for 0.1 min, then programmed at 10°C/min to 280°C. The carrier gas was hydrogen. Quantities of *n*-tricosane, *n*-pentacosane, and *n*-heptacosane in the extracts were determined by comparison of GC peak areas with those obtained from authentic samples (100 ng).

Coupled GC–Mass Spectrometry

A capillary GC column (50 m × 0.32 mm i.d. HP-1) fitted with a cool on-column injector was directly coupled to a mass spectrometer (Thermo-Finnigan MAT95XP, Bremen, Germany). Ionization was by electron impact at 70 eV, 200°C. The oven temperature was maintained at 30°C for 5 min, then programmed at 5°C/min to 280°C. The carrier gas was helium. Tentative identifications were made by comparison of mass spectrometry (MS) data

with published spectra (NIST, 2002). Confirmation of tentative identifications was accomplished by peak enhancement on GC with authentic samples obtained from commercial sources (Pickett, 1990).

Chemicals

n-Tricosane (C₂₃H₄₈), *n*-pentacosane (C₂₅H₅₂), and *n*-heptacosane (C₂₇H₅₆; all 99% purity) were purchased from the Aldrich Chemical Company (Gillingham, UK). For behavioral studies, individual solutions of these chemicals were prepared in distilled ethanol at the following concentrations: 10, 1, 0.1, and 0.01 µg/ml. All solvents were distilled before use.

Application of Chemicals to Plants

Pure authentic compounds in ethanol were applied to broad bean plants, *V. faba*, for use in behavioral bioassays, as previously described (Nakashima et al., 2004). These solutions were applied to the plants (six- to eight-leaf stage, one plant per pot) by using a rotary atomizer mounted on a multispeed track. The atomizer operated at 4500 rpm and produced a drop size of approximately 110 µm VMD. Applications were made at a velocity of 0.4 msec⁻¹ at a height of 25 cm above the plants, providing an application rate of 1.04 ml m². Control leaves were prepared in a similar manner with distilled ethanol. By using the ethanolic solutions of compounds prepared at the concentrations described above, and based on the application rate and the dimensions of the leaf squares (see below), the leaf squares generated (1.5 × 1.5 cm) were coated with 4, 0.4, 0.04, and 0.004 ng test compound, respectively.

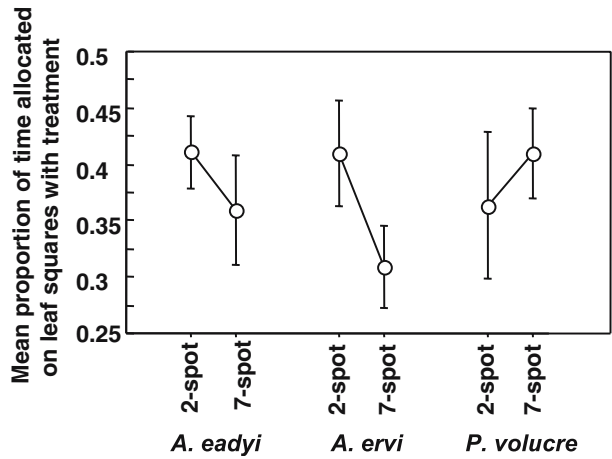
Behavioral Bioassays

The effects of *C. septempunctata* and *A. bipunctata* trails and trail extracts on aphid parasitoid responses were investigated by using a dual-choice bioassay. Treatment leaves with ladybird trails were prepared by inserting a leaf from a bean plant (six- to eight-leaf stage) into a plastic container via a slit, releasing a ladybird adult into the container, allowing it to walk upon the leaf for 24 hr, and then removing it from the plant. Control leaves were left untouched for a similar length of time. Leaves were used for experiments immediately after exposure ended. Treated and control leaf squares (1.5 × 1.5 cm) were taken from the plants and placed 0.5 cm apart in a petri dish (5 cm diam), into which a single parasitoid female was then released. After allowing the parasitoid to settle (1 min), the time spent foraging on each leaf square was measured for a period of 10 min. Each experiment was repeated 20 times. To account for potential temporal effects, equal numbers of each of the three parasitoid species were tested each day, randomizing the order of testing between days.

Statistical Analysis

The durations of visits by parasitoids were analyzed by using Wilcoxon's signed rank tests and Mann–Whitney *U* test. The degree of avoidance was expressed as time allocated on leaf squares with treatments divided by total residence time on control and treatment leaf squares. The index of avoidance responses were analyzed by an ANOVA with chemical compounds, their concentrations and parasitoid species as main effects; the proportional data were arcsine transformed to stabilize the variance before this analysis. The amounts of

Fig. 1 Effect of seven-spot ladybird, *C. septempunctata*, and two-spot ladybird, *A. bipunctata*, chemical trails on time spent foraging by female aphid parasitoids, *A. ervi*, *A. eadyi*, and *P. volucre*, on broad bean, *V. faba*, leaf squares. Data are expressed as time spent on leaf squares treated with ladybird trails divided by total residence time on control and treated leaf squares in choice bioassays. Vertical lines indicate \pm S.E



trail chemical compounds were compared by Mann–Whitney *U* test. For the latter, the data were transformed to logarithms to stabilize the variance before analysis.

Results

In dual-choice leaf square bioassays conducted immediately after leaf exposure to *C. septempunctata* and *A. bipunctata* adults, parasitoids avoided the leaf squares treated with ladybird trails in all combinations of parasitoid and ladybird species (Wilcoxon’s signed rank test, $P < 0.05$; Fig. 1). The degree of avoidance of *C. septempunctata* trails was stronger than that of *A. bipunctata* trails for *A. eadyi* and *A. ervi* (Mann–Whitney *U* test, $P <$

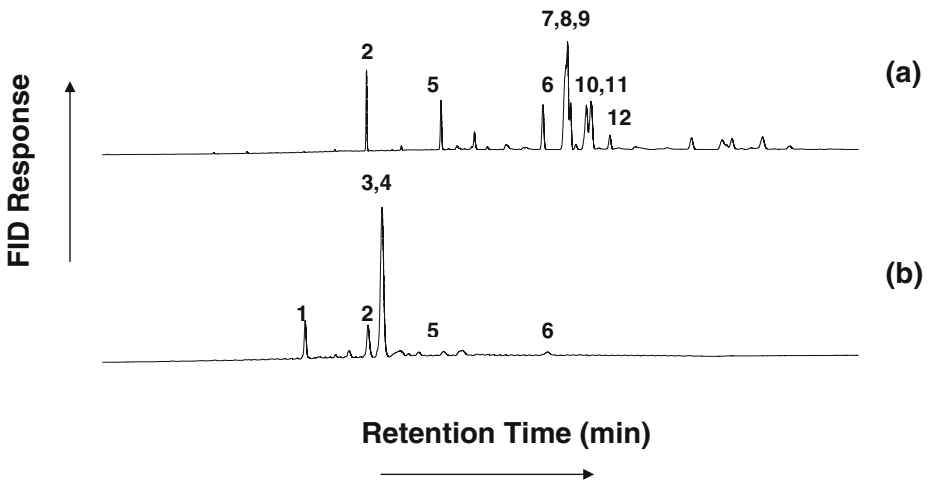


Fig. 2 Typical gas chromatograms of adult (a) seven-spot ladybird, *C. septempunctata*; (b) two-spot ladybird, *A. bipunctata*, footprint extracts. Peak numbers correlate to compounds listed in Table 1 identified by coupled GC–MS. Extracts were collected from individual *C. septempunctata* and *A. bipunctata* adults over 18 hr in a glass petri dish. Flame ionization detector responses are provided on the same scale for comparison

Table 1 Compounds identified from coupled GC–MS analysis of seven-spot Ladybird, *C. septempunctata*, and two-spot Ladybird, *A. bipunctata*, footprint extracts

Peak number (see Fig. 1)	Compound
1	<i>n</i> -Heneicosane ^{a,b}
2	<i>n</i> -Tricosane ^{b,c,d}
3	9-Methyltricosane ^{a,b}
4	7-Methyltricosane ^{a,b}
5	<i>n</i> -Pentacosane ^{b,c,d}
6	<i>n</i> -Heptacosane ^{b,c,d}
7	13-Methylheptacosane ^{a,d}
8	9-Methylheptacosane ^{a,d}
9	7-Methylheptacosane ^{a,d}
10	9-13-Dimethylheptacosane ^{a,d}
11	7,11-Dimethylheptacosane ^{a,d}
12	7,11-15-Trimethylheptacosane ^{a,d}

^a Tentative identification, based upon fragmentation patterns and MS data published elsewhere (Hemptinne et al., 2001).

^b Compound identified in adult two-spot ladybird, *A. bipunctata*.

^c Identification confirmed by peak enhancement on GC using authentic samples.

^d Compound identified in adult seven-spot ladybird, *C. septempunctata*.

0.05), but it was statistically similar for the trails of both ladybirds in the case of *P. volucre* (Fig. 1).

To identify the chemicals responsible for parasitoid avoidance, hexane extracts of footprint trails from adult *C. septempunctata* and *A. bipunctata* were analyzed by high-resolution GC and coupled GC–MS. Samples from both species were comprised almost entirely of aliphatic hydrocarbons (Fig. 2; Table 1). Components identified in both species were *n*-tricosane, *n*-pentacosane, and *n*-heptacosane. By comparison with authentic samples using GC, the levels of *n*-tricosane, *n*-pentacosane, and *n*-heptacosane in the footprint trails of *C. septempunctata* and *A. bipunctata* adults were determined. Larger amounts of *n*-pentacosane and *n*-heptacosane were found in the trails of *C. septempunctata* than in those of *A. bipunctata* (Mann–Whitney *U* test, $P < 0.05$), but no significant difference was found in the levels of *n*-tricosane (Table 2). Other major aliphatic hydrocarbons were identified tentatively as branched hydrocarbons by comparison with MS data from similar studies elsewhere (Table 1; Hemptinne et al., 2001; Nakashima et al., 2004). Compounds identified specifically for *C. septempunctata* included 13-, 9-, and 7-methylheptacosane; 9,13-, 7,11-

Table 2 Mean levels (Nanograms \pm S.E.) of three hydrocarbons found in chemical trail extracts of the Ladybirds *C. septempunctata* and *A. bipunctata* ($N = 6$)

Compound	Log amount \pm S.E.	
	<i>C. septempunctata</i>	<i>A. bipunctata</i>
<i>n</i> -Tricosane	2.77 \pm 0.06 a	2.73 \pm 0.14 a
<i>n</i> -Pentacosane	2.52 \pm 0.11 a	2.13 \pm 0.04 b
<i>n</i> -Heptacosane	2.69 \pm 0.10 a	2.40 \pm 0.06 b

Values followed by different letters in the same row are significantly different (Mann–Whitney *U* test, $P < 0.05$). Mean levels were determined per GC injection (4 μ l).

Table 3 Anova for effects of different hydrocarbons from Ladybird trails, hydrocarbon concentration, and parasitoid species on proportion of time spent foraging by female parasitoids on treated leaf squares

Factor	df	F	P
Hydrocarbon	2	8.1	<0.01
Concentration	3	5.9	<0.01
Parasitoid species	2	0.9	0.40
Hydrocarbon × concentration	6	0.7	0.67
Hydrocarbon × parasitoid species	4	2.6	0.036
Concentration × parasitoid species	6	0.4	0.87
Hydrocarbon × concentration × parasitoid species	12	0.8	0.66

dimethylheptacosane, and 7,11,15-trimethyl-heptacosane. Compounds identified specifically for *A. bipunctata* were *n*-heneicosane, and 9- and 7-methyltricosane.

An ANOVA of proportions of time spent by parasitoids on leaf squares treated with individual hydrocarbons from ladybird trails indicated significant effects of both hydrocarbon type and concentration (Table 3). The interaction between hydrocarbon type and parasitoid species was also significant (Table 3), indicating that parasitoids differed in their responses to different hydrocarbons.

The total residence times of *A. eadyi* on leaf squares treated with *n*-tricosane were shorter than those on control leaves for all treatment concentrations (Wilcoxon’s signed rank test, $P < 0.05$), but significant effects were only found at higher doses (0.4 and 4 ng) for *A. ervi* and only at the highest dose (4 ng) for *P. volucre* (Wilcoxon’s signed rank test, $P < 0.05$; Fig. 3). In the case of *n*-pentacosane, the residence times for all three parasitoid species were reduced by the hydrocarbon only at the two highest doses (0.4 and 4 ng; Wilcoxon’s signed rank test, $P < 0.05$; Fig. 3). There were no significant differences between residence times on control leaf squares and those treated with *n*-heptacosane, at any concentrations used, in the cases of the two *Aphidius* species, but *P. volucre* spent less time on treated leaf squares at the two highest doses (0.4 and 4 ng; Wilcoxon’s signed rank test, $P < 0.05$; Fig. 3).

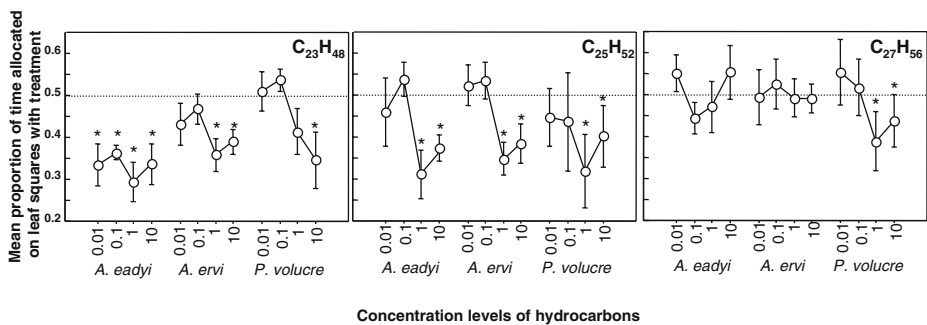


Fig. 3 Response of female aphid parasitoids, *A. ervi*, *A. eadyi*, and *P. volucre* to leaf squares treated with either *n*-tricosane ($C_{23}H_{48}$), *n*-pentacosane ($C_{25}H_{52}$), or *n*-heptacosane ($C_{27}H_{56}$). For each compound, concentrations of 10, 1, 0.1, and 0.01 $\mu\text{g/ml}$ were applied, equivalent to doses of 4, 0.4, 0.04, and 0.004 ng per leaf square. Data are expressed as time spent on leaf squares treated with ladybird trail hydrocarbons divided by total residence time on control and treated leaf squares. Vertical lines indicate \pm S.E. Asterisks indicate significant difference from control (Wilcoxon’s signed rank test, $P < 0.05$)

Discussion

To our knowledge, this is the first report that adult parasitoids vary in their avoidance of different species of intraguild predators, and that this variability potentially relates to differences in the amounts of specific chemical compounds in the predator trails. The two *Aphidius* species tested responded more strongly to trails of *C. septempunctata* than to trails of *A. bipunctata*, but there were no statistically significant differences in the responses of *P. volucre* to the trails of either of the two ladybird species. Components identified in both species were *n*-tricosane, *n*-pentacosane, and *n*-heptacosane, the presence of which in *C. septempunctata* had been previously demonstrated (Nakashima et al., 2004). All three parasitoid species showed avoidance responses to *n*-tricosane and *n*-pentacosane, as shown in a previous study (Nakashima et al., 2004), but only *P. volucre* responded to *n*-heptacosane. Other chemicals found specifically in *C. septempunctata* and *A. bipunctata* trails were not investigated for avoidance activity in this study, as previous work (Nakashima et al., 2004) showed that *n*-tricosane, *n*-pentacosane, and *n*-heptacosane accounted for parasitoid avoidance responses.

The differences in the levels of avoidance behavior shown toward the two different ladybird trails by both *Aphidius* species could be due to differences in the amounts of specific hydrocarbons present in the two trails (Table 2). *n*-Tricosane and *n*-pentacosane are known to be the main ladybird avoidance stimuli for *A. ervi*, and these two chemicals additively increase avoidance responses (Nakashima et al., 2004). This finding, together with the present results, suggests that larger amounts of *n*-pentacosane in the trails of *C. septempunctata* may induce the stronger avoidance responses recorded for *A. ervi* and *A. eadyi* to trails of this ladybird than to those of *A. bipunctata* (Fig. 1), the same chemical compounds stimulating the avoidance behavior in these closely related species. *P. volucre* showed no difference in the level of response to trails of the two ladybird species, but this parasitoid responded to all three hydrocarbons and so may have a more general sensitivity to hydrocarbons in ladybird chemical trails.

The presence of aliphatic hydrocarbons specific to *C. septempunctata* and *A. bipunctata* adults implies that these compounds have a function in the behavioral ecology of the ladybirds. Studies elsewhere have implied that such compounds may play a role in mediating intraspecific ladybird interactions (Hemptinne et al., 2001), specifically the oviposition deterrent response of adult *A. bipunctata* following detection of intraspecific larval footprints. Previous work on avoidance of *C. septempunctata* footprints by *A. ervi* (Nakashima et al., 2004) revealed virtually identical hydrocarbon profiles for adults and larvae. Thus, it can be expected that these compounds play a role in *C. septempunctata* oviposition deterrent behavior, and that a similar outcome can be expected for *A. bipunctata*. Further studies are required to confirm the role of these compounds.

As a specialist parasitoid of pea aphid, *A. eadyi* is likely to restrict its foraging to habitats containing legume plants (Pennacchio, 1989). Thus, *A. eadyi* would encounter a limited number of ladybird species, and principally *C. septempunctata*, which is a dominant species in legume crops (Ekbohm, 1994; Nakashima and Akashi, 2005). *A. ervi* forages commonly on legume plants, but also forages on other arable crops, including cereals, where *C. septempunctata* is the dominant aphidophagous ladybird (Wratten and Powell, 1991). Therefore, it may be adaptive for females of these two *Aphidius* species to optimize their sensitivity to the chemical trails of the ladybirds that they are most likely to encounter as intraguild predators, thereby maximizing the avoidance behavior that reduces the predation risk to their offspring. However, little is known about encounter rates in natural habitats, and such studies are needed to help elucidate the evolutionary development of intraguild interactions.

A. eadyi was the most sensitive of the three parasitoids to *n*-tricosane, responding to very low concentrations. Detection of early instar predators, which deposit much smaller amounts of trail chemicals than adults and mature larvae, could be important because ladybird larvae are likely to stay and complete development within an aphid patch (Dixon, 2000). Additionally, ladybird trails appear to have relatively short active periods, possibly due to absorption of the hydrocarbons into the plant cuticular lipid layer, or even evaporation/sublimation from the leaf surface over time (Nakashima et al., 2004). Increased sensitivity to one of the chemical components of ladybird trails may help *A. eadyi* to perceive trails that *A. ervi* and *P. volucre* cannot detect, giving this extreme specialist species a competitive advantage in the restricted range of habitats in which it forages. In contrast, *P. volucre* is a more generalist parasitoid at both the host species and host plant levels (Pennacchio, 1989). In this case, a more generalized sensitivity to trail chemicals may be advantageous, as parasitoids with broader host/habitat ranges would face a predation risk from a larger number of predator species.

The overall results showed that hydrocarbon types and their concentrations affected parasitoid avoidance responses, and the level of avoidance responses shown to the three chemical compounds differed among the parasitoid species (Fig. 3). *P. volucre* avoided all three hydrocarbons in contrast to the *Aphidius* species, which avoided only *n*-tricosane and *n*-pentacosane. Thus, the range of trail chemicals detected by the generalist *P. volucre* was wider than that detected by the two *Aphidius* species, which both have narrower host ranges. The specialist *A. eadyi*, which is only known to attack the pea aphid, *A. pisum*, was the most sensitive to *n*-tricosane, responding to this chemical at the lowest concentration tested.

The results of the present study suggest that oviposition preferences by aphid parasitoids in response to intraguild predators are common in *Aphidius* and *Praon* species. Nakashima and Senoo (2003) and Nakashima et al. (2004) suggested that both intraguild predation and its avoidance play important roles in determining top–down forces by predator guilds, and consequently affect herbivore populations in local areas. Although several reports have revealed that aphidophagous predators, such as coccinellids and chrysopids, are deterred from ovipositing when exposed to areas where heterospecific competitors are present (Ruzicka, 1998, 2001; Agarwala et al., 2003), there was little evidence for this behavior in parasitoids. We predict that intraguild predator avoidance by adult parasitoids should be widespread in other systems because immature parasitoids in/on hosts are always at risk from predators. Additionally, host/prey density is known to affect the degree of predator avoidance by parasitoids (Nakashima and Senoo, 2003) as well as intraguild predation (Lucas et al., 1998). Further research should determine how extrinsic (e.g., prey density) and intrinsic (e.g., experience and physiological state) factors affect the magnitude of intraguild predation and its avoidance to understand the role of these interactions in the dynamics of predator–parasitoid–herbivore systems.

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Keywords Osmeterial secretion · Lepidoptera · Papilionidae · Papilionini · *Chilasa* · *Papilio* · Aliphatic acid and ester · Monoterpene · Sesquiterpene

Introduction

The family Papilionidae includes over 500 species and is traditionally divided into three subfamilies: the Baroniinae (a single tribe, Baroniini), the Parnassiinae (tribes Zerynthiini and Parnassiini), and the Papilioninae (tribes Troidini, Graphiini, and Papilionini) (Munroe and Ehrlich, 1960; Scriber, 1995). The morphology, color, and patterning of papilionid larvae differ among species and instars. Some larvae appear to mimic bird droppings, whereas others are snake-like in appearance with false eyespots. Colorations, presumably cryptic, are typically green or brown, often with mottled or striped patterns. These visual traits are likely to be effective in reducing detection by predators (Takagi et al., 1995) though they may also serve as aposematic signals in unpalatable larvae (Järvi et al., 1981). Whatever their visual appearance, swallowtail larvae possess a characteristic two-pronged eversible gland (osmeterium) on the middorsal intersegmental surface between the head and the prothorax. When disturbed, they extrude this gland, from which an odoriferous liquid is secreted. Behavioral studies have shown that the osmeterial secretions are especially effective at deterring arthropod predators, including ants, spiders, mantids, wasps, and soldier bugs (Eisner and Meinwald, 1965; Honda, 1983a; Chow and Tsai, 1989; Takagi et al., 1995). In the field, Damman (1986) showed that mortality of *Eurytides marcellus* larvae due to ants and small spiders was substantially increased among larvae that had been experimentally prevented from extruding their osmeterial gland. Many insect predators, however, are able to ignore or circumvent the osmeterial defenses of swallowtails (Damman, 1986; Berenbaum et al., 1992; Takagi et al., 1995).

The chemical nature of the osmeterial secretion has been examined for very few species but in representatives of all swallowtail tribes. *Baronia brevicornis*, the only species in the tribe Baroniini, secretes two aliphatic acids as the osmeterial components in fifth instars (Eisner et al., 1970). Of approximately 14 species in the Zerynthiini (Hancock, 1983; Miller, 1987), two *Luehdorfia* and one *Sericinus* species produce monoterpene secretions in both the fourth and fifth instars (Honda, 1980a; Honda and Hayashi, 1995). The osmeteria of *S. montela* also contain aristolochic acids (Nishida, 1995). In the Parnassiini, with approximately 35 species, *Parnassius glacialis* produces aliphatic acids and esters in the osmeterial secretions from third and fourth (ultimate) instars (Honda and Hayashi, 1995). Although the Troidini includes about 130 species, only three have been examined for volatiles in osmeterial secretions. *Battus polydamas* secretes two sesquiterpenoids in abundance in fourth instars (Eisner et al., 1971), whereas *Atrophaneura alcinous* and *Pachliopta aristolochiae* produce sesquiterpenoids as dominant components in both fourth and fifth instars (Honda 1980a; Honda and Hayashi, 1995). In addition, osmeterial exudates from the caterpillars of several troidine species contain aristolochic acids (Nishida and Fukami, 1989; Nishida et al., 1993; Nishida, 1995; Sime et al., 2000). In the Graphiini, a tribe containing about 150 species, *E. marcellus* secretes two aliphatic acids as major osmeterial components in fifth instars (Eisner et al., 1970), whereas *Graphium sarpedon* and *G. doson* contain aliphatic acids and their esters as major components in both fourth and fifth instars (Honda, 1980a). The Papilionini is dominated by the large genus *Papilio*, consisting of about 200 species. About 20 of these have been investigated for the chemical nature of their larval osmeterial secretions. While most earlier studies were restricted to either the fourth or the fifth instars (e.g., Eisner et al., 1970; Lopez and Quesnel, 1970; Seligman and Doy, 1972), subsequent studies compared the secretions of both fourth and

fifth instars in a total of nine *Papilio* species. These confirmed that the fourth instars secrete terpenoids as the major constituents, while the fifth instars secrete aliphatic acids and their esters (Honda, 1980b, 1981; Burger et al., 1985; Young et al., 1986).

On the basis of whether or not the final larval ecdysis is accompanied by a shift in the dominant osmeterial chemistry, papilionid species can be divided into two groups. Whether their secretions are dominated by terpenoids or by aliphatic acids and esters, the species so far studied from most papilionid tribes belong to the “homogeneous” group (Honda, 1980a), in which there is no major change in chemistry. By contrast, those *Papilio* species for which sufficient information is available can be assigned to the “heterogeneous” group (Honda, 1980a). Whether or not the heterogeneous trait is characteristic of the tribe Papilionini, however, remains uncertain as such a small fraction of its species has been examined. Moreover, the larval host plants of those *Papilio* species for which we already know the osmeterial chemistry of both fourth and fifth instars belong either to the Rutaceae or the Apiaceae—families that share many features of their secondary chemistry (Hegnauer, 1971). To investigate the possibility that variation in the composition of osmeterial secretions might be related to host-plant chemistry, it is desirable to examine *Papilio* species with larval host plants in more distantly related families such as the Lauraceae and Magnoliaceae.

In this study, we describe the chemical composition of volatile substances in the osmeterial secretions from both fourth and fifth instars of eight species belonging to the tribe Papilionini. Six belong unambiguously to the genus *Papilio* (*P. demoleus*, *P. polytes*, *P. paris*, *P. macilentus*, *P. troilus*, and *P. glaucus*). *P. demoleus* and *P. paris* feed as larvae on rutaceous plants and are widely distributed from India to Southeast Asia and from West Asia to Southeast Asia and Australia, respectively. *P. polytes* and *P. macilentus*, Rutaceae feeders, occur from India to Southeast Asia and in China, the Korean Peninsula, and Japan, respectively. *P. troilus* inhabits the eastern regions of the United States and Canada, and feeds on lauraceous plants. *P. glaucus* is widely distributed in North America and is notable for its relatively wide spectrum of host plants, which includes the families Magnoliaceae and Lauraceae. Previous work revealed the dominance of two aliphatic acids in the osmeterial secretions of fifth instars of both *P. troilus* and *P. glaucus* (Eisner et al., 1970), but no data were presented for fourth instars.

We also included *Chilasa epycides* and *C. agestor*, Lauraceae-feeding swallowtails inhabiting Formosa, West China, and Burma. The dozen or so species in the genus *Chilasa* are unusual in their mimicry, as adults, of danaid butterflies that are known or suspected to sequester defensive compounds from their host plants. While the genus *Chilasa* is supported by Hancock (1983) and Igarashi (1984), other scholars include the group within the genus *Papilio* (Miller, 1987; Zakharov et al., 2004).

Methods and Materials

Insects

Larvae of *P. polytes* and *P. macilentus* originated from Yaeyama and Kanagawa populations, respectively, in Japan. They were reared on *Citrus* spp. and *Orixa japonica* (Rutaceae), respectively. Larvae of *P. troilus* and *P. glaucus* were derived from female adults collected in Ithaca, NY, USA, and reared on *Sassafras albidum* (Lauraceae) and *Liriodendron tulipifera* (Magnoliaceae), respectively. *P. demoleus*, *P. paris*, *C. epycides*, and *C. agestor* were all obtained from Taiwan. Larvae of *P. demoleus* and *P. paris* were raised on *Citrus* spp. and *Fagara ailanthoides* (Rutaceae), respectively, whereas

Cinnamomum camphora (Lauraceae) was used for rearing *Chilasa epycides* and *C. agestor* larvae. All larvae were reared at 25°C under a photoperiod of 16L:8D.

Collection of Secretions

Using the methods of Honda (1980b), we collected osmeterial secretions separately from fourth and fifth instars of each species on the third day after larval ecdysis by absorption into pieces of filter paper (5 × 5 mm, No. 2, Advantec, Tokyo, Japan). From these, they were each extracted by approximately 2 ml of purified (twice distilled) dichloromethane, and the resulting extracts were stored at -10°C until analysis. For each species except *C. agestor* and *P. glaucus*, 15–36 larvae were sampled, which permitted both qualitative and quantitative analysis. Chemical analyses were duplicated for these species, using different batches of larvae. Since only five or six larvae of *C. agestor* and *P. glaucus* were sampled, their secretions were subjected to qualitative analysis only.

In a study on the osmeterial chemistry of *P. demodocus*, Burger et al. (1985) found that individual fourth instars produced mixtures of terpenoids that were qualitatively similar to one another but varied greatly in quantitative content. The authors attributed this variation to different levels of irritation of the larvae during sampling: minor irritation resulted only in partial eversion of the forks, which were fully everted only on more persistent irritation. Such variability of osmeterial chemistry among individual larvae may have influenced the results of our study but was not explicitly measured. For those species with large enough numbers of larvae for quantitative analyses, we found no appreciable interbatch differences in the overall profiles of the secretions.

Chemical Analyses of Volatile Components

The secretion extracts were examined by gas chromatography (GC) and GC-mass spectrometry (GC-MS). GC analyses were conducted with a Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector at a temperature of 250°C, whereas GC-MS analyses were performed at 70 eV using a Shimadzu QP5000 mass spectrometer coupled with a Shimadzu GC-17A gas chromatograph. Most of the analyses were carried out with a Varian CP-Wax 58CB fused-silica capillary column (0.25 mm ID × 25 m for GC or 50 m for GC-MS, 0.20 μm film thickness), with a temperature program of 40°C (held initially for 2 min) to 220°C (held for 20 min) at 5°C/min. Some analyses, however, were conducted with a GL-Sciences OV-101 fused-silica capillary column (0.25 mm ID × 25 m, 0.20 μm film thickness), under the same analytical conditions described above (Honda, 1980b). Samples were injected splitless at 250°C using N₂ and He as the carrier gas (1 ml/min) for GC and GC-MS, respectively. Identification of the components was based on comparison of GC retention data and mass spectra with those of authentic compounds purchased commercially (Tokyo Chemical Industry) or with those of osmeterial components previously identified in other *Papilio* species (Honda, 1980b, 1981), unless otherwise noted. The percentage composition of each component was calculated by estimating its peak area as a percentage of the total peak area on gas chromatograms.

Results

Secretion Components from Fourth Instars

In the osmeterial extracts from fourth instars, 19, 7, 10, 24, 15, and 16 components with an area percentage of more than 0.1% were detected in *C. epycides*, *P. demoleus*, *P. polytes*, *P.*

Table 1 Volatile components of fourth instar osmeterial secretions of six papilionid butterflies

No.	Component	Composition (%) ^a					
		<i>Chilasa epycides</i>	<i>Papilio demoleus</i>	<i>Papilio polytes</i>	<i>Papilio paris</i>	<i>Papilio macilentus</i>	<i>Papilio troilus</i>
1	α-Pinene	6.1		1.0	8.6	22.3	5.4
2	Sabinene	0.1		4.9	0.2	0.6	19.5
3	β-Myrcene	1.9	0.2	1.1	2.4	24.6	9.1
4	Limonene	2.8		1.6	1.1	0.8	7.1
5	β-Phellandrene	7.1		0.4	1.8	3.3	19.0
6	(Z)-β-Ocimene	0.1				11.4	0.4
7	(E)-β-Ocimene				0.1	0.8	0.4
8	p-Mentha-1,4(8)-diene	0.5			0.1		8.3
9	C ₁₅ H ₂₄				1.3		
10	Methyl 3-hydroxy-n-butyrate	7.2	20.3	13.3	3.2		
11	C ₁₅ H ₂₄				23.7		
12	β-Elemene	0.7	28.8	2.4	15.6	2.1	
13	β-Caryophyllene	4.5	3.0	0.5	1.9		
14	C ₁₅ H ₂₄	0.6					
15	(E)-β-Farnesene			29.6	0.8	0.3	0.3
16	C ₁₅ H ₂₄						0.5
17	C ₁₅ H ₂₄				1.0		
18	C ₁₅ H ₂₄	17.7					
19	C ₁₅ H ₂₄					1.5	
20	C ₁₅ H ₂₄				1.5		
21	C ₁₅ H ₂₄	1.1			4.4	3.4	
22	(3Z,6E)-α-Farnesene					14.0	
23	(Z)-α-Bisabolene	14.6					14.1
24	C ₁₅ H ₂₄				4.8		
25	Germacrene-A	1.1	37.1	41.9		5.2	7.0
26	C ₁₅ H ₂₄						0.6
27	C ₁₅ H ₂₄						1.3
28	(E)-α-Bisabolene	1.1					0.2
29	Germacrene-B	0.6			3.8	0.2	
30	C ₁₅ H ₂₄ O				3.3		
31	C ₁₅ H ₂₄ O				11.2		
32	C ₁₅ H ₂₄ O		6.8				
33	Oxygenated sesquiterpenoid					5.6	
34	C ₁₅ H ₂₄ O				3.6		
35	C ₁₅ H ₂₄ O				0.8		
36	C ₁₅ H ₂₆ O				0.3		
37	C ₁₅ H ₂₄ O				0.3		
38	C ₁₅ H ₂₆ O						3.0
39	C ₁₅ H ₂₄ O	0.6					
40	C ₁₅ H ₂₄ O		1.1				
41	C ₁₅ H ₂₂ O	24.4					

^a Components less than 0.1% of the total are not listed. Major components exceeding 10% in each species are represented by bold figures.

paris, *P. macilentus*, and *P. troilus*, respectively (Table 1). In all species, the osmeterial secretions had a faint odor and most of the compounds detected were terpenoids.

Peaks 1–5, detected from five species, gave a molecular ion (M^+) at m/z 136 with fragmentation patterns typical of monoterpene hydrocarbons. Based on these mass spectra and GC retention data, peaks 1, 2, 3, 4, and 5 were identified as α -pinene, sabinene, β -myrcene, limonene, and β -phellandrene, respectively. Peaks 6 and 7 also had M^+ at m/z 136 and were determined to be (*Z*)- β -ocimene and (*E*)- β -ocimene, respectively. Peak 8 exhibited M^+ at m/z 136 [relative intensity (%): 59] and typical terpenoid fragment ions at m/z 121 (70), 107 (15), 93 (100), 91 (57), 79 (47), 65 (17), 53 (27), and 41 (61). This compound was readily identified as *p*-mentha-1,4(8)-diene.

Peak 10 gave several fragment ions, at m/z 117 (0.3), 103 (11), 87 (9), 74 (32), 59 (9), and 43 (100), but its M^+ never appeared. The fragmentation pattern and the GC retention datum of this compound conformed well to those of authentic methyl 3-hydroxy-*n*-butyrate.

Peaks 12 and 25, each present in five of the species tested, shared M^+ at m/z 204, the base peak at m/z 41, and fragmentation patterns characteristic of sesquiterpene hydrocarbons. The mass spectra and GC retention data of peaks 12 and 25 were in agreement with those of β -elemene and germacrene-A, respectively, which also occur in the osmeterial secretion of *Papilio protenor* (Honda, 1980b).

Peaks 13, 15, and 29, exhibiting M^+ at m/z 204, also appeared to be sesquiterpene hydrocarbons and readily corresponded to β -caryophyllene, (*E*)- β -farnesene, and germacrene-B, respectively.

Peak 22 was found only in *P. macilentus*. This component had M^+ at m/z 204 and fragment ions at m/z 189 (1), 161 (2), 147 (1), 133 (2), 119 (39), 107 (19), 93 (53), 79 (26), 69 (37), 55 (47), and 41 (100). The fragmentation pattern and GC retention datum were identical to those of an unidentified sesquiterpene hydrocarbon found in the osmeterial secretions from fourth instars of *P. protenor* (peak 10 in Honda, 1980b). In comparison with an authentic mixture sample of farnesenes, this component was recognized as (3*Z*,6*E*)- α -farnesene.

Peak 23 showed M^+ at m/z 204 (16) and characteristic fragment ions at m/z 189 (4), 175 (1), 161 (7), 147 (7), 133 (13), 119 (20), 109 (20), 93 (100), 79 (27), 67 (28), 55 (25), and 41(72). This component was identified as (*Z*)- α -bisabolene. Peak 28 was the (*E*)-isomer with a similar fragmentation pattern but a GC retention datum differing from that of peak 23.

Although structural elucidation of peaks 9, 11, 14, 16–21, 24, 26, and 27 was not achieved, all of them had M^+ at m/z 204 and fragmentation patterns characteristic of terpenoids, strongly suggesting that they were sesquiterpene hydrocarbons with the molecular formula of $C_{15}H_{24}$.

Table 2 Volatile components of fifth instar osmeterial secretions of six papilionid butterflies

Code	Component	Composition (%) ^a					
		<i>C. epycides</i>	<i>P. demoleus</i>	<i>P. polytes</i>	<i>P. paris</i>	<i>P. macilentus</i>	<i>P. troilus</i>
a	Methyl isobutyrate	37.3	28.9	4.6	12.5	72.0	49.5
b	Ethyl isobutyrate	1.2	0.1		0.6		0.1
c	Methyl 2-methylbutyrate	16.9	8.0	1.5	6.0	8.2	24.3
d	Ethyl 2-methylbutyrate	0.9	0.1		0.9		0.2
e	Acetic acid					0.2	
f	Isobutyric acid	13.9	33.0	51.0	49.3	12.7	1.4
g	2-Methylbutyric acid	24.7 ^b	30.0 ^b	42.9	23.3 ^b	6.9 ^b	15.5

^a Components below 0.1% of the total are not listed.

^b Combined percentage with other substance suggested as isovaleric acid.

Peaks **30–32**, **34**, **35**, **37**, **39**, and **40**, sharing M^+ at m/z 220, were all considered to be oxygenated sesquiterpenoids, for which the molecular formula of $C_{15}H_{24}O$ seems appropriate. Peak **33** gave a fragment ion at m/z 204 and the base peak at m/z 161, suggesting a sesquiterpene hydrocarbon. However, since the GC retention datum of this compound corresponded to those of oxygenated sesquiterpenoids rather than those of sesquiterpene hydrocarbons, it is likely an oxygenated sesquiterpenoid, the original M^+ of which did not appear in the mass spectrum. Peaks **36**, **38**, and **41** exhibited M^+ at m/z 222, 222, and 218, respectively, and were tentatively recognized as oxygenated sesquiterpenoids with molecular formulae of $C_{15}H_{26}O$, $C_{15}H_{26}O$, and $C_{15}H_{22}O$, respectively.

Secretion Components from Fifth Instars

In contrast to those of the fourth instars, the osmeterial secretions of fifth instars of all six species gave off intense and offensive odors. Seven compounds, all aliphatic acids or their esters, were identified in the extracts (Table 2).

Peak a showed M^+ at m/z 102 and was identified as methyl isobutyrate. From their mass spectra, peaks b–d were all assumed to be homologous esters. As the M^+ and base peaks were at m/z 116 and 43, 116 and 57, and 130 and 57, respectively, peaks b, c, and d were concluded to be ethyl isobutyrate, methyl 2-methylbutyrate, and ethyl 2-methylbutyrate, respectively.

Peak e was detected only in *P. macilentus*. This compound had M^+ at m/z 60, and the base peak at m/z 43, and was identified as acetic acid. Peak f, indicating M^+ at m/z 88, was recognized as isobutyric acid. Peak g, distributed in all species tested, was identified as 2-methylbutyric acid on the basis of its fragmentation pattern, with M^+ at m/z 102, and its GC retention datum. However, in *C. epycides*, *P. demoleus*, *P. paris*, and *P. macilentus*, the mass spectrum of this peak contained an ion at m/z 60, significantly higher in proportion than the value expected for 2-methylbutyric acid alone. As in previous studies (Honda, 1981), this result suggested that isovaleric acid, the base peak of which was at m/z 60, was present in small amounts.

Chemical Nature of Larval Secretion in *C. agestor* and *P. glaucus*

Although detailed chemical analyses were not carried out, the osmeterial secretions of fourth instars of *C. agestor* contained large amounts of methyl 3-hydroxy-*n*-butyrate and sesquiterpenoids, such as (*Z*)- α -bisabolene, β -caryophyllene, and β -elemene, as well as small amounts of monoterpenoids. The secretions of fourth instars of *P. glaucus* contained a relatively high proportion of monoterpenoids, such as α -pinene, *p*-mentha-1,4(8)-diene, and limonene, and also included (*Z*)- α -bisabolene and germacrene-A as dominant components. The secretions from fifth instars of both species contained isobutyric and 2-methylbutyric acids and their methyl esters as major constituents.

Discussion

Our findings that the osmeterial secretions from fourth instars of all eight species consist mainly of terpenoids, whereas those from the corresponding fifth instars comprise aliphatic acids and their esters, show that all these species alter the chemical profile of their osmeterial secretions at the final larval ecdysis. Although we investigated only two *Chilasa*

Table 3 Chemical characteristics of osmeterial secretions of papilionid larvae

Comp. type	Subfamily	Tribe	Species	Penultimate instar			Ultimate instar			Main host plant family	References
				MT	ST	A&E	MT	ST	A&E		
1	Papilioninae	Papilionini	<i>Papilio protenor</i>	++	++				Rutaceae	Honda (1980b)	
1	Papilioninae	Papilionini	<i>Papilio bianor</i>	++	++				Rutaceae	Honda (1981)	
1	Papilioninae	Papilionini	<i>Papilio maackii</i>	++	++				Rutaceae	Honda (1981)	
1	Papilioninae	Papilionini	<i>Papilio memnon</i>	+	++				Rutaceae	Honda (1981)	
1	Papilioninae	Papilionini	<i>Papilio helenus</i>	++	++				Rutaceae	Honda (1981)	
1	Papilioninae	Papilionini	<i>Papilio xuthus</i>	++	++	+			Rutaceae	Honda (1981)	
1	Papilioninae	Papilionini	<i>Papilio demodocus</i>	++	++				Rutaceae	Burger et al. (1985)	
1	Papilioninae	Papilionini	<i>Papilio macilentus</i>	++	++				Rutaceae	Present study	
1	Papilioninae	Papilionini	<i>Papilio polytes</i>	+	++				Rutaceae	Present study	
1	Papilioninae	Papilionini	<i>Papilio demoleus</i>		++				Rutaceae	Present study	
1	Papilioninae	Papilionini	<i>Papilio paris</i>	+	++				Rutaceae	Present study	
1	Papilioninae	Papilionini	<i>Papilio anchisiades</i>		++		+		Rutaceae	Young et al. (1986)	
1	Papilioninae	Papilionini	<i>Papilio machaon</i>	++	++				Apiaceae	Honda (1981)	
1	Papilioninae	Papilionini	<i>Papilio glaucus</i>	++	++				Magnoliaceae	Present study	
1	Papilioninae	Papilionini	<i>Papilio troilus</i>	++	++				Lauraceae	Present study	
1	Papilioninae	Papilionini	<i>Chilasa epycides</i>	+	++				Lauraceae	Present study	
1	Papilioninae	Papilionini	<i>Chilasa agestor</i>	+	++				Lauraceae	Present study	
2	Papilioninae	Troidini	<i>Atrophaneura atcinous</i>		++		++		Aristolochiaceae	Honda (1980a)	
2	Papilioninae	Troidini	<i>Pachioptia aristolochiae</i>		++		++		Aristolochiaceae	Honda and Hayashi (1995)	
2	Papilioninae	Graphiini	<i>Graphium sarpedon</i>			++			Lauraceae	Honda (1980a)	
2	Papilioninae	Graphiini	<i>Graphium japonicum</i>			++			Magnoliaceae	Honda (1980a)	
2	Parnassiinae	Zerynthini	<i>Luehdorfia japonica</i>	++			++		Aristolochiaceae	Honda (1980a)	
2	Parnassiinae	Zerynthini	<i>Luehdorfia puziloi</i>	++			++		Aristolochiaceae	Honda (1980a)	
2	Parnassiinae	Zerynthini	<i>Sericinus montela</i>	++			++		Aristolochiaceae	Honda and Hayashi (1995)	
2	Parnassiinae	Parnassiini	<i>Parnassius glacialis</i>			++		++	Papaveraceae	Honda and Hayashi (1995)	

1 = heterogeneous; 2 = homogeneous; MT = monoterpenoids; ST = sesquiterpenoids; A&E = aliphatic acids and esters; ++ = major components; + = minor components; blank =not detected.

species, it is likely that the genus *Chilasa*, as well as the genus *Papilio*, can also be assigned to the heterogeneous group in terms of the larval osmeterial secretion. Moreover, we found the same pattern in larvae of *P. glaucus*, which include the Magnoliaceae among their hosts, and in the larvae of *P. troilus* that, like those of the two *Chilasa* species, feed on plants in the family Lauraceae. Because larvae of previously studied *Papilio* species, all of which share the same pattern of shifting osmeterial chemistry, feed on the families Rutaceae or Apiaceae, it appears that the pattern is not related to host-plant affiliation. Combined with the earlier work on other *Papilio* species (Table 3), our results strongly suggest that the heterogeneous trait is characteristic of the tribe Papilionini as a whole. This distinguishes the Papilionini from other major swallowtail tribes, in which earlier work points to a lack of chemical change in osmeterial secretions between the penultimate and ultimate larval instar (Table 3).

As is common in the genus *Papilio*, larvae of four of the *Papilio* species (*P. macilentus*, *P. demoleus*, *P. paris*, and *P. polytes*) undergo a dramatic change in body coloration and pattern at the final larval ecdysis, coincident with the change in osmeterial chemistry. While the fourth instars are apparent mimics of brown and white bird droppings, the fifth instars resemble small green snakes (Igarashi, 1979). In *P. troilus* and *P. glaucus* (Igarashi, 1979), however, the change in appearance occurs at the fourth instar, thus preceding the shift in chemistry. This pattern is known also in *P. machaon*, where the shift from bird dropping to green and black stripes (Igarashi, 1979) likewise precedes the change in chemistry (Honda, 1981). Young et al. (1986) reported a shift in osmeterial chemistry from terpenoids to aliphatic acids in last-instars of *P. anchisiades* despite only minor changes in the mottled brown coloring of the larvae. A comparable pattern is seen in the two *Chilasa* species (Fig. 1). Therefore, it seems clear that there is no necessary ontogenetic linkage in papilionid larvae between the changes in osmeterial chemistry and those in larval appearance.



Fig. 1 Fourth (left) and fifth (right; protruding its osmeterium) instars of *Chilasa agestor*

The compositions of the fifth instar secretions revealed no surprises. All species were characterized by aliphatic acids and their esters. In particular, they shared methyl isobutyrate, methyl 2-methylbutyrate, isobutyric acid, and 2-methylbutyric acid as major components, in spite of considerable quantitative differences. These findings are consistent with those previously reported for other *Papilio* species (Honda, 1980b, 1981). The chemical profiles of secretions from fourth instars, by contrast, were characteristic of each species, especially in their content of sesquiterpenoids. Although most of the individual compounds have been reported earlier, (3*Z*,6*E*)- α -farnesene (**22**), (Z)- α -bisabolene (**23**), and (E)- α -bisabolene (**28**) have not been found previously in the osmeterial secretions of papilionid larvae. The osmeterial secretion from fourth instars of *P. macilentus* contained a large amount of (3*Z*,6*E*)- α -farnesene (**22**), earlier shown to be a host finding kairomone for fruit flies (Nigg et al., 1994) and the codling moth (Bengtsson et al., 2001). Because this sesquiterpene hydrocarbon turned out to be identical to an unidentified component from the secretion of *P. protenor* (Honda, 1980b), we confirm here that fourth instars of this species also secrete compound **22**. (Z)- α -Bisabolene (**23**), reported as the aggregation pheromone for green stinkbugs such as *Acrosternum hilare* and *Nezara viridula* (Aldrich et al., 1987, 1989), was a major constituent of the osmeterial secretions from *C. epycides* and *P. troilus*, alongside minor amounts of its (E)-isomer (**28**).

The larvae of at least some *Papilio* species are able to biosynthesize aliphatic acids from amino acids (Seligman and Doy, 1973) and terpenoids *de novo* from acetate precursors (Honda, 1983b, 1990). Also, the overall chemical profiles of the larval osmeterial secretions of at least some *Papilio* species remain unaffected even if the larvae are reared on different plants (Honda, 1983b). It seems likely, therefore, that the chemical composition of papilionine osmeterial secretions is largely heritable. Consistent with this hypothesis are the findings of K. Honda (unpublished data) that larvae of *P. protenor* from several different sites across the main Japanese island of Honshu share similar profiles of osmeterial chemistry. No data are yet available to permit comparison of more distantly related populations of papilionine swallowtails. However, larvae of the troidine species *Atrophaneura (Parides) alcinous* from Honshu show slight differences in sesquiterpenoid profiles from larvae in the Yaeyama islands (Okinawa Prefecture) (Honda, unpublished data). Thus far, the evidence suggests that the chemical profiles of osmeterial secretions are species-specific while subject to some degree of variation.

Preliminary phylogenetic comparisons of osmeterial chemistry within the Papilionini reveal few obvious trends. In some cases, closely related species (Zakharov et al., 2004) share similar osmeterial chemistry. Secretions from *P. demodocus* (Burger et al., 1985) and *P. demoleus* (this study), for example, both contain germacrene-A (**25**), β -elemene (**12**), and methyl 3-hydroxy-*n*-butyrate (**10**) as major components. On the other hand, secretions from *P. bianor* and *P. maackii* differ greatly from those of *P. paris*, their sister taxon (this study and Honda, 1981). Despite substantial interspecific variation in the terpenoid content of fourth-instar osmeterial secretions, these could be separated into a group rich in monoterpene hydrocarbons (nearly 50% of total peak area) and a group dominated by sesquiterpenoids (more than 70% of total peak area). In the monoterpene group were *P. macilentus* and *P. troilus*, whereas the sesquiterpene group consisted of *C. epycides*, *P. demoleus*, *P. polytes*, and *P. paris* (cf. Table 1). In previous studies, the secretions of most *Papilio* species were rich in sesquiterpenoids, whereas only *P. bianor* and *P. maackii* belonged to the monoterpene group (Honda, 1980b, 1981; Burger et al., 1985). Although *P. bianor* and *P. maackii* are sister species, they are more distantly related to *P. macilentus* and (especially) *P. troilus* (Zakharov et al., 2004). We discern no obvious relationship between overall terpenoid grouping and *Papilio* phylogeny.

Although the switch from terpenoids to aliphatic acids and esters at the final larval ecdysis in papilionine swallowtails is typically sharp and complete, exceptions are known. Young et al. (1986) found several terpenoids along with the dominant aliphatic acids and esters in the osmeterial secretions of fifth-instar *P. anchisiades*, whereas aliphatic acids and esters accompanied the typical terpenoids in secretions from fourth-instars of *P. xuthus* (Honda, 1981). Such examples suggest that there is no fundamental biochemical incompatibility between the two types of osmeterial secretion and that their typically sharp separation could be the result of selection rather than of developmental constraint.

The changes in osmeterial chemistry in papilionine larvae, like those in color and pattern, are likely to reflect shifting patterns of predation on larvae as they increase in size (Brower, 1984; Leslie and Berenbaum, 1990; Reavey, 1993). Young larvae are likely to be primarily attacked by small invertebrate predators, which are typically most successful when attacking prey similar in size or smaller than themselves (Stamp, 1986). Older larvae are likely to be more apparent to larger invertebrate predators and small vertebrates, for which they also represent greater food rewards (Reavey, 1993). Field studies on *P. polyxenes* (Feeny et al., 1985) and *P. xuthus* (Watanabe, 1981) have confirmed that young larvae suffer proportionately greater mortality due to small, invertebrate predators, whereas larger larvae are more vulnerable to larger predators such as wasps and birds. One might expect to find, therefore, that osmeterial secretions containing terpenoids are most effective as deterrents of smaller, invertebrate enemies, whereas those containing aliphatic acids and esters are most effective against larger invertebrates and perhaps small vertebrates.

Most experiments that demonstrate the effectiveness of osmeterial secretions in papilionine swallowtails have been conducted with last-instars, in which the secretions are primarily composed of aliphatic acids and esters (Table 3). These are capable of warding off attacks by a variety of invertebrate predators such as ants (Eisner and Meinwald, 1965; Honda, 1983a), mantids (Chow and Tsai, 1989), small spiders (Damman, 1986), and wasps (Takagi et al., 1995). The acid/ester profiles typically contain only a few compounds, often dominated by isobutyric acid and 2-methylbutyric acid (Table 2). These two acids, along with acetic acid, were among the five most potent of 17 individual compounds from osmeterial secretions tested by Honda (1983a) for their ability to deter ants from sucrose baits. Acetic and 2-methylbutyric acids, moreover, both evoked alarm behavior in the ant *Lasius niger* (Honda, 1983a). These secretions appear to act as powerful irritants, and their relatively uniform composition across the family seems to suggest selection for compounds of particular efficacy.

Although papilionine swallowtails are sometimes subject to severe bird predation (Hirose et al., 1980; Takagi et al., 1995), several studies have failed to show that their osmeteria have any significant deterrent effect against birds in a captive setting or suburban environment (Järvi et al., 1981; Honda, 1983a; Leslie and Berenbaum, 1990; Takagi et al., 1995). In natural habitats, though, papilionine females typically disperse their eggs over wide areas, and larvae are thinly distributed and less likely to trigger search image formation by foraging birds (cf. Brower, 1958; Takagi et al., 1995). Under these conditions, deployment of the osmeterium as part of a “threat display” might be more likely to deter the occasional and tentative initial attack by a foraging bird. Experiments by Järvi et al. (1981) and Leslie and Berenbaum (1990) have shown that the larvae of *P. cresphontes*, *P. machaon*, and *P. polyxenes* are distasteful to birds, with or without their osmeteria, when feeding on typical food plants. This unpalatability may result from sequestration of toxins from host plants in the Rutaceae and Apiaceae, and could be frequent among oligophagous swallowtails in the Papilionini (Leslie and Berenbaum, 1990). In such situations, foraging birds may learn by association to avoid the odor or taste of osmeterial secretions. Exploitation of these secretions

as aposematic cues by birds and perhaps other predators could provide strong selection for convergence in osmeterial chemistry. Larvae of palatable swallowtail species, such as *P. glaucus*, might gain protection through Batesian mimicry (Leslie and Berenbaum, 1990), whereas caterpillars of two or more protected species in the local community might benefit from Müllerian mimicry. If widespread, such chemical aposematism might provide a compelling explanation for why last-instar papilionine larvae, those most likely to be attractive targets for birds, share such similar osmeterial profiles of acids and esters.

The effectiveness of terpenoids, present in the secretions of earlier instars, has received little explicit study. Of several individual terpenoids included in Honda's (1983a) bioassays, most were weakly or moderately deterrent to ants. D-Limonene, however, was a strong deterrent to *Crematogaster matsumurai*, though not to *L. niger*, whereas caryophyllene oxide strongly deterred ants of both species. A convincing overall comparison of the efficacy of terpenoids and aliphatic acids/esters in osmeterial defense will require further research. However, a comparison of the two secretory profiles raises the possibility that the two types of secretion might function in different, although perhaps overlapping, ways. As shown in this report (Table 1), terpenoid profiles are typically diverse. These compounds are widely used by insects as attractants and deterrents in host recognition (Gershenson and Croteau, 1991; Harrewijn et al., 1995), and their function in osmeterial secretions may be as much to disrupt recognition as to provide a toxic defense. Blum (1981) has suggested that the chemical diversity typical of many arthropod defensive secretions may act as a chemical smokescreen, effectively concealing the emitter by producing undecipherable sensory input from the receptors of potential predators. Such a strategy could be especially effective against smaller invertebrate predators for which the chemical senses predominate in prey finding and capture. The lack of any apparent phylogenetic pattern among terpenoid osmeterial secretions, noted earlier, could result from evolutionary lability in terpenoid profiles. Chemical novelty is typically deterrent to phytophagous insects (Bernays and Chapman, 1987). If the same can be said of predatory insects, one might expect selection to favor the idiosyncratic production of novel metabolites in response to shifting suites of invertebrate enemies. It may be no coincidence that the plant families containing the majority of swallowtail host plants are unusually rich in essential oils that contain a wide variety of terpenoids (Hegnauer, 1983). Whether the osmeterial terpenoids are acquired from the plants or synthesized *de novo*, as seems more likely (Honda, 1983b, 1990), their deployment in defense would be consistent with an overall emphasis on crypsis in earlier instars.

The presence of aliphatic acids and esters in the osmeterial secretions of *Baronia*, *Parnassius*, and various species in the tribes Graphiini and Papilionini suggests that they represent a plesiomorphic trait in the family Papilionidae (Sime et al., 2000). It is striking that two tribes (Troidini and Zerynthiini) apparently lacking these compounds are rich in species that are known or suspected to sequester aristolochic acids and perhaps other toxins from their food plants in the Aristolochiaceae (Nishida, 1995; Sime et al., 2000). These compounds are present on the surfaces of eggs as well as in the cuticle and osmeteria of larvae, in pupae, and in the adults. Because they are strongly deterrent to birds and some invertebrates (Sime et al., 2000; Sime, 2002), it is tempting to speculate that they have replaced acids and esters in the osmeterial secretions of larvae in these tribes. Young (1973) has suggested that bodily unpalatability due to sequestered aristolochic acids is the primary defense of troidine larvae against birds and other vertebrates, while the osmeteria, containing both aristolochic acids and terpenoids, provide defense against invertebrates. The characteristic heterogeneity in osmeterial defense of larvae in the tribe Papilionini may represent an evolutionary compromise. Terpenoids, shared with the sister tribe Troidini (Miller, 1987), may be the primary defense against invertebrate enemies of the younger larvae while acids and esters

are deployed in the final instar as the more effective defense against larger invertebrates and perhaps vertebrates.

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Buchmann, 1981; Simpson and Neff, 1981; Buchmann, 1987; Simpson et al., 1990). One role suggested for the floral oil has been in the construction of nest cells that consist of several different materials depending on the species, including soil, sand, wood chips, or plant material (Vinson et al., 1996; Pereira et al., 1999; Jesus and Garófalo, 2000). Floral oil may be used to aid in the collection of these construction materials (see Vinson et al., 1996; Pereira et al., 1999), but it is not clear if the hardened cell wall also contains floral oil (see Vinson et al., 1996).

Furthermore, floral oil is used as a nest provision in many species, and Neff and Simpson (1981) suggested that the use of oil as a larval provision may be related to the high moisture content of the ground where these bees nest, oil being less likely to be leached from the nests by groundwater during the rainy season. In fact, we observed *C. aethiocesta* nesting in sand at the edge of estuaries that often flood (Vinson and Frankie, 1988). Neff and Simpson (1981) also suggested that their view of the importance of using oil in wet regions was supported by the fact that the oil-collecting habit has been lost in some species that nest in xeric environments.

However, some *Centris* species nest in twigs or above ground cavities (Frankie et al., 1988). Simpson et al. (1977) reported that the nest cells of *C. trigonoides*, a species that nests in cavities in twigs, contained pollen and lipid with no appreciable amounts of nectar (carbohydrate). Later, Neff and Simpson (1981) reported that nests of *C. maculifrons* from Peru contained oils as well as “appreciable” amounts of glucose and fructose, the later presumably from floral nectar sources. This led Simpson et al. (1990) to question the absence of nectar in the nest provisions in their studies of *Centris* nest resources. A similar report of provisioning with oil and nectar was reported for *C. vittata* (Pereira and Garófalo, 1996) and this mixture was suggested to make up the liquid provisions of *C. tarsata* (Aguar and Garófalo, 2004). Whereas both Vinson et al. (1996) and Jesus and Garófalo (2000) suggest that *C. analis* and *C. bicornuta* use nectar, there is no evidence that nectar is used, and it is not clear whether some floral oil is used to provision nests as well.

In the lowland dry forest of Costa Rica, we have been studying 14 species of *Centris*, all of which have been observed collecting floral oil from *Byrsonima crassifolia* (Malpighiaceae) (Vinson et al., 1993, 1997). Five of these species, in addition to *C. aethiocesta* (op. cit.), are ground nesters in the lowland dry forest, and their nests are subjected to wet season conditions (Vinson and Frankie, 1977; Coville et al., 1983, 1986; Vinson et al., 1987; Vinson and Frankie, 1988).

An analysis of the oil collected from *B. crassifolia* flowers yielded thin layer chromatography (TLC) spots consistent with glyceride-fatty acid standards (Vinson et al., 1997). Using gas chromatography (GC), we found that floral oil consist primarily of monoglycerides (60–70%) and diglycerides (15–20%), with less than 10% consisting of triglycerides, and less than 5% consisting of free fatty acids. Upon hydrolysis and methylation, the floral oil yielded six fatty acids ranging from C₁₆ to C₂₀ with the shortest chain fatty acid, myristic acid, accounting for 6%, and oleic acid making up the largest percentage at 30% of the total (Vinson et al., 1997).

When the liquid nest provisions of four of the available ground-nesting species (*C. adani*, *C. flavifrons*, *C. flavofasciata*, and *C. aethyctera*) were examined by TLC and GC, the results (Vinson et al., 1997) revealed an identical pattern of glycerides and fatty acids to the *B. crassifolia* floral oil. Only a trace of carbohydrate was detected. These results confirmed the earlier suggestion by Neff and Simpson (1981) that the nest liquid provisions of at least some of the ground nesters consisted of floral oil only.

However, we have also been working with several cavity or twig-nesting *Centris* that occur in the same dry forest. We observed that both the consistency and odor of the nest

provisions of four of these *Centris* spp. differed from those of the ground-nesting species, from the floral oils, and from the floral nectars we had collected. In particular, the nest liquid had a slight acrid or goat-like odor. This led us to examine the liquid contents of nests of four species (*C. analis*, *C. bicornuta*, *C. nitida*, and *C. vittata*). Here, we report the results of those examinations.

Methods and Materials

Study Site and General Study Preparation

We set out artificial wooden-nesting cavities (trap nests) in 1997 to 2000, and then sporadic trap nest placements since 2000, at the Hacienda Monteverde site, 8 km NNW of the town of Bagaces in the Tempisque region of Guanacaste Province (Frankie et al., 1993, 1998). Trap nests were first placed in January and then collected and replaced on a biweekly basis through June.

Between 1999 and 2002, trap nest sticks in the trap nest blocks (see Frankie et al., 1993 for a description of the trap nest blocks) that had just been completed, as evidenced by a fresh nest plug, were removed from the block. These sticks were maintained in a horizontal position (nests are constructed in a horizontal position) and returned to our field station. They were opened as described earlier for the ground-nesting species with some modification (Vinson et al., 1997). Thus, the sticks were split lengthwise, revealing a row of cells (Fig. 1). In most cases, the provisioned bee cells were also split lengthwise, revealing the contents. If this did not occur, we used a pair of forceps to cut a groove along the edge of

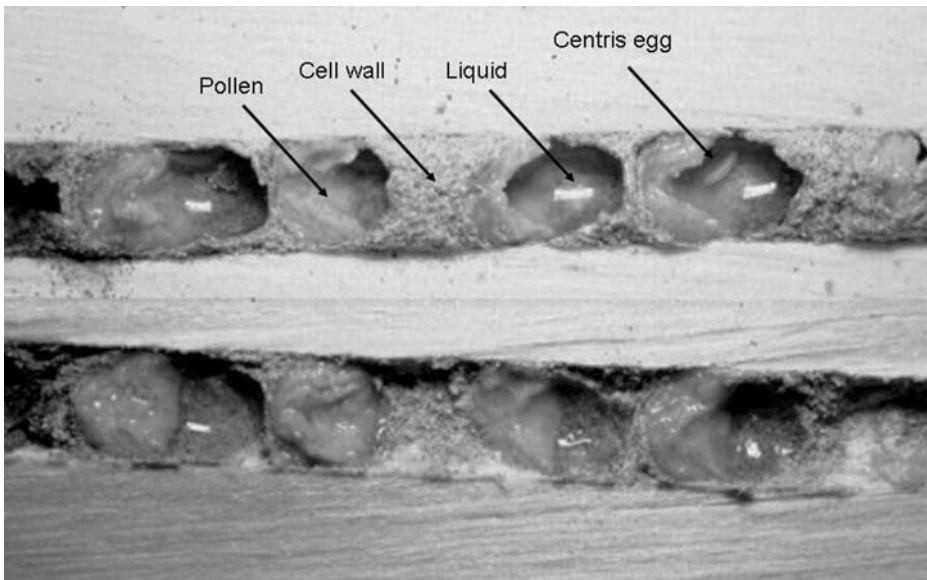


Fig. 1 Nest of *Centris bicornuta* in a pine stick split lengthwise showing four cells composed of the cell wall, pollen packed at the back end of the cell, overlaid with a liquid on which an egg is floated. The cell usually is three-fourths full of pollen and the liquid (nectar consisting of water and carbohydrate)

the exposed cell and then thrust the forceps through the wall and pried the side off. The opened cell was examined under a dissecting microscope. If a bee egg was still present and there was no evidence of parasites, the liquid contents were removed for analysis. The egg was removed and the liquid contents were collected in 10 μl calibrated 50 μl capillary tubes. Usually, more than one tube was needed to remove all the contents. The numbers of tubes, or fractions thereof, were recorded in microliters, and the tube contents were placed in 2 ml Wheaton vials (Alltech Associates, Deerfield, IL, USA), transported to Texas A&M University, and placed in the freezer until analysis. These samples were not refrigerated during the 3 d involved in transport.

Analysis

Nest contents had a slight acid, goat-like fragrance in the field and caused a slight burning sensation when sniffed, indicating the possible presence of short-chain acids. In preliminary studies, the liquid provisions were spotted on silica gel TLC plates (Merck Si60, F254), developed in hexane/ether (1:1), and the compounds were visualized with iodine vapor. The results of the TLC study, along with an indication that the contents were not oils because they were not greasy and drops on tweezers were sticky and could be cleaned with water, led us to partition the samples between distilled water and hexane. This was done to determine if any hexane-soluble compounds were present. Nest contents were soluble in water with the exception of a few pollen grains in some samples and a lot of pollen in the case of the nest contents of *C. vittata*. After partitioning between water and hexane, the hexane fractions were dried by passing through a short column of anhydrous sodium sulfate. The hexane extracts of the liquid provisions were analyzed for volatiles by GC on a 25 m \times 0.25 mm I.D. BP1 column (Scientific Glass Engineering, Austin, TX, USA), using 20 psig He carrier gas, with an initial temperature of 50°C for 1 min, to 250°C at 10°C/min. The extracts were found to contain almost no volatile materials separated from the solvent (see short-chain acid analysis below).

These results suggested that the liquid contents may consist of nectar. To determine if the liquid provisions consist of sugar, a carbohydrate analysis was undertaken. Thus, 5 μl of a standard glucose, fructose, and sucrose sample (50% wt/vol) were treated with 75 μl of Supersil (Alltech) silylating reagent and held at 70°C for 20 min. Gas chromatography conditions were the same as described above, except that the initial temperature was 110°C for 1 min, to 300°C at 15°C/min. Peaks at 8.71, 9.28, and 13.95 were used to quantify fructose, glucose, and sucrose, respectively. Bee nest liquid (5 μl) from the various nests was subjected to the same methods and conditions. For the purpose of this study, the sugars were quantified using glucose as the standard set at 1.

Because the presence of nectar was indicated, we applied 10 μl of the nest liquid from four *C. bicornuta* and two *C. analis* nests to preweighed microscope cover slips, desiccated for 3 d in a CaCl_2 desiccation chamber, and reweighed on a Mettler-Toledo GmbH[®] microbalance (Greifensee, Switzerland). The amount of sugar was expressed as percent of the original sample weight.

Tests with standard short-chain acids indicated that under the GC conditions for volatiles (above), they could not be seen. To test for their presence, butyl esters were prepared by reaction of 5 μl of the water-soluble nest provisions with 50 μl of 14% boron trifluoride in butanol at 65°C for 5 min. The reaction mixture was poured into 1 ml of water and extracted with 1 ml hexane, which was dried with anhydrous sodium sulfate. A standard was prepared by adding 5 μl each of C-2 through C-10 straight chain fatty acids to 500 μl of distilled water and treating 5 μl of the solution in the same manner as the water-soluble

nest contents. Two small butyl ester peaks from the bee nest samples were matched by their retention times with the butyl ester standards derived from butanoic and octanoic acids. However, a much larger peak, approaching 100% of the butyl ester sample, eluted between nonanoyl and decanoyl butyl esters and, therefore, could not be a simple straight chain butyl ester of a short-chain fatty acid. Mass spectrometry (MS) analysis showed the characteristic m/z 116 ion of *n*-butyl esters, but no parent ion was observed.

To identify the unknown butyl ester, we pooled a number of samples of *C. bicornuta* nest liquid. A sample of approximately 500 μg was purified for nuclear magnetic resonance (NMR) analysis by preparative GC, using a 3.66 m \times 4 mm I.D. 5% OV-101 on Chromosorb G column operated isothermally at 160°C, and a Brownlee–Silverstein liquid nitrogen cooled collection system (Brownlee and Silverstein, 1968). The purified sample was dissolved in 100 μl of CDCl_3 , and the analysis was performed using 3 mm Broad Band\Hydrogen or Hydrogen\Broad Band probes on a Bruker ARX 500 NMR instrument.

Possible Source of the Fatty Acids

To determine the possible source of the fatty acids, we concentrated on the nests of *C. bicornuta* because they were more common. First, we repeatedly collected the nest cell liquid as it was being provisioned by using a 50 μl capillary tube. By collecting the cell liquid as soon as the female bee left, she would continue to return with liquid, presumably until a certain cell liquid level was reached. As a result, the foraging bee foraged for and provided more nectar than is normally provisioned. This collection was continued until 1 ml or so was collected by repeatedly emptying the capillary tube into 2 ml Wheaton vials. Five hundred μl of hexane were added to the vial, which was sealed (to reduce the chance of oxidation, contamination, or enzyme action), and returned to Texas for analysis for fatty acids or their esters as described above. We repeated this collection from three different nests.

In addition to being collected with or being associated with the nectar, there are a number of other possible sources of the fatty acids; however, we only had the equipment and time to determine whether the female bee was adding these compounds directly to the provision. For this study, we collected females that had just completed provisioning a nest with the liquid, but prior to oviposition, which was indicated by two situations. First, inserting a thin wire into the nest, removing it, and then measuring the length of the contamination on the wire determined the depth of the provision. Second, conformation that oviposition had not occurred but was about to occur was evidenced by the presence of a slight ring at the point where the cell cap will be placed on the cell. This ring, inside the cell, is placed just prior to the female leaving and returning to oviposit and to begin to close the cell (Vinson and Frankie, unpublished data). The returning female was captured, placed in a vial on ice, then dissected a few hours later. Tissues removed were the crop and contents, head with associated glands, the hind gut, the poison and Dufours gland, the reproductive system and the abdomen devoid of digestive system, reproductive system, and accessory reproductive glands. These various tissues or parts were placed in hexane in 3 ml Wheaton vials until analysis for the presence of short-chain fatty acids (described above).

Results

For *C. bicornuta*, *C. analis*, and *C. nitida*, each cell is provisioned with pollen as a compact layer at the back of the cell followed by a provisioned layer of liquid. The liquid contents of

the cells of *C. bicornuta* averaged 88.6 μl ($N = 25$, range = 78–97 μl), and the cells of *C. analis* contained a similar amount of liquid (mean = 86.0 μl , $N = 12$, range = 76–95). We only had one cell of *C. nitida*, which contained 93 μl of liquid. This bee had become rare in our study area after 1996. In contrast, the contents of the cells of *C. vittata* consisted of a mixture of orange pollen with the liquid provisions. This mixture was very thick, which made the quantitative separation and measurement of the quantity of the liquid difficult. Thus, these data are not reported for this species.

The nest contents of the four wood- or twig cavity-nesting *Centris* species examined by TLC did not contain any mono-, di-, or triglycerides, or long-chain fatty acids detectable by TLC. Similar negative results were obtained with the hexane extract of the nest liquid, which left no detectable (weighable) residue upon evaporation. Evaporation of the water extract left a gum-like residue that was shown upon silylation to contain sugars. An evaluation of the carbohydrates in the liquid contents of the nests revealed that sucrose was present in small quantities (Table 1). Most of the liquid contents of the nest consisted of a solution of two monosaccharides, glucose and fructose, in a ratio of 1:2–3. No peaks other than glucose, fructose, or sucrose were revealed. The % total sugar in the liquid of five nests ranged from 41% to 57% (Ave. 47.2%).

Although no GC peaks above C-8 were found in the hexane extracts, peaks were found matching the retention times of butyl butanoate and butyl octanoate in approximately equal amounts in the derivatized samples. These were present as less than a tenth of 1% of the liquid sample. However, a larger peak, approximately 99.8% of the sample of butyl esters, had a retention time slightly shorter than that of butyl octanoate, indicating that it was not a butyl ester of a simple straight chain fatty acid. Because MS analysis revealed the characteristic m/z 116 ion of *n*-butyl esters but with no parent ion, visible, NMR analysis was used to identify this compound. The ^1H spectrum showed a methyl singlet at δ 2.21 and two sharp methylene triplets at δ 2.77 and δ 2.21, and a cross peak in the H, H COSY spectrum indicated that the methylene triplets were coupled to each other. The ^{13}C NMR spectrum had an additional carbonyl peak at about 201 ppm in addition to the expected ester peak at 173 ppm. From these data, the compound was identified as butyl levulinate (Fig. 2). Samples of levulinic acid and butyl levulinate were purchased (Aldrich) and found to be identical to the nest-derived materials.

An analysis of the liquid provisions collected just before oviposition failed to reveal any of the fatty acids or their esters. An analysis of the various possible sources of fatty acids or their esters from various tissues of the female bees also yielded negative results.

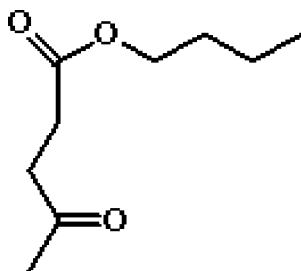
Table 1 Carbohydrates (Glucose Set at 1) found in the liquid contents of the nest cells of twig-nesting *Centris* spp. in guanacaste province, Costa Rica

Nesting species	Number of samples	Glucose (set at 1)	Fructose	Sucrose
<i>C. bicornuta</i>	4	1	2.70–2.81	T–0.0021
<i>C. analis</i>	3	1	2.43–3.97	T–0.0035
<i>C. nitida</i>	4	1	2.79–3.27	T–0.0028
<i>C. vittata</i> ^a	3	1	2.45–3.36	T–0.002

^a Only species where the pollen and liquid were thoroughly mixed.

T: trace.

Fig. 2 Butyl levulinate derived from the levulinic acid found in the nests of four twig-nesting *Centris* species in the dry forest of Costa Rica



Discussion

The liquid nest provisions of *C. bicornuta*, *C. analis*, *C. nitida*, and *C. vittata* did not contain any fatty acids above C_{14} characteristic of the triglycerides and fatty acids of the oil from *B. crassifolia* (Vinson et al., 1997). Although all four species appear to collect oil from *B. crassifolia*, the role of the oil remains unclear. The liquid nest provision did contain large quantities of carbohydrate, primarily glucose and fructose with a trace quantity of sucrose. The sugar concentration of 47.2% is slightly higher than a number of common nectar resources for these bees that are thought to be important for adult maintenance (Vinson et al., 1993). The average sugar concentration of the nectar of flowers of *Tabebuia rosea* (Bignoniaceae), *Caesalpinia eriostachys* (Caeciliaceae), *Dalbergia retusa*, *Gliricidia sepium*, *Lonchocarpus hondurensis*, and *Myrospermum frutescens* (Fabaceae) ranged from 20% to 42% (Frankie et al., 1983). The difference could be attributable to a number of factors, but the pollen is dry and may have soaked up some of the water, concentrating the sugars slightly.

In addition, trace amounts of several short-chain fatty acids were present, including levulinic acid. Although levulinic acid could be present as a monoglyceride, the monoglyceride would have shown up as a silylated product on GC analysis of the water/hexane extract. Furthermore, if it were a mono-, di-, or triglyceride product, it should have shown up as a GC peak when the hexane extract was examined before derivatization. Because BF_3 /butanol reagent can perform transesterification, it is possible that levulinic acid is present as a water-soluble polar ester. This seems unlikely and we believe that levulinic acid exists as the free acid. These acids probably serve as a preservative, because short-chain fatty acids, particularly levulinic acid, are known to be antimycotic (Radoev et al., 1961).

Both butanoic and octanoic acid have strong odors, whereas levulinic acid has little to no odor. Thus, the two short-chain saturated fatty acids may be responsible for the slight goat-like fragrance of the cell contents, but the unusual levulinic acid predominates and is more likely to play a role in retarding fungal growth.

Our results concerning the absence of floral oil in the provisions of *C. vittata* differ from the report of Pereira (1997), who reported that *C. vittata* provisioned the cell with pollen, nectar, and an oily substance that was deposited on the surface of the pollen and nectar “loaf” (a mixture of pollen and nectar that is a kneadable solid). We did not observe any nectar loaf, and no oil was found in the liquid when separated from the pollen. However, the provision of *C. vittata* differed from the provisions of all the other *Centris* species we have worked with in Costa Rica (Vinson et al., 1993, 1997) in that the pollen and liquid provisions were blended rather than being separate. The liquid in the other species was always placed on the pollen resource, resulting in two layers.

We were unable to find the source of the small amount of fatty acids in the provision collected before oviposition, suggesting that these compounds are not present in nectar

collected by the female bee. Also, the female bee does not appear to add these three fatty acids directly to the provision by regurgitation, or from gland secretions from the head, reproductive system, or abdomen. However, there are a number of other possibilities. Because we found these acids in the liquid provision several days after oviposition but from cells in which the egg had been removed, the possibilities include the production and release of these compounds by the female bee at or just following oviposition, the release of enzymes into the provision by the female, or something that leaches from the pollen. If an enzyme were involved, the 3 or 4 d exposure of the provisions to warm temperatures during transportation could have led to an increase in these compounds, increasing our ability to detect them.

Whereas the four *Centris* species reported here have been observed visiting and collecting oil from *B. crassifolia* (Frankie et al., 1989; Vinson et al., 1993), it is clear that any oil collected by the four *Centris* species is not being used as a nest provision. What the oil is being used for by these species is not clear. The results of this study support the suggestion (Neff and Simpson, 1981) that the habit of using oil provisions may be related to exposure of the nest cells to abundant water, because the cells of the small wooden cavity-nesting species would not be subjected to standing water, even in the wet season.

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Introduction

The key characteristic of ant societies is a reproductive division of labor, with morphologically specialized queens producing most or all the offspring, and workers performing other tasks such as foraging or caring for the brood (Wilson, 1971). In ca. 150 ectatommine and ponerine species, however, the specialized queen caste is rare or absent, and mated workers or “gamergates” take over the role of the queen in some or all of the colonies (Peeters, 1991). Because all individuals in these species are totipotent, i.e., able to mate and reproduce, there is potential for reproductive conflict (Monnin and Ratnieks, 2001; Ratnieks et al., 2006). One expression of this conflict is dominance behavior, which in most species is critical in establishing and maintaining a reproductive division of labor (Ito, 1993; Liebig et al., 1999; Monnin and Peeters, 1999). Dominance behavior, however, is potentially costly, because individuals that engage in dominance interactions generally work at a lower rate than labor workers (Cole, 1986; Gobin et al., 2003; Ito and Higashi, 1991; Monnin and Peeters, 1999; Monnin and Ratnieks, 1999).

Dominance interactions are usually most prominent immediately after colonies are orphaned, when the reproductive dominance hierarchy is first established (Gobin et al., 2001; Monnin and Peeters, 1999). Aggression typically occurs only between individuals of similar dominance status and reduces to a low level as soon as one or a few dominant individuals start to reproduce (Monnin and Peeters, 1999; Gobin et al., 2001; Cuvillier-Hot et al., 2004). This reduction in aggression may coincide with the development of a specific cuticular hydrocarbon profile in egg-laying individuals. Indeed, consistent chemical differences in the cuticular profiles between fertile and infertile individuals have now been shown in *Diacamma ceylonense* (Cuvillier-Hot et al., 2001), *Dinoponera quadriceps* (Monnin et al., 1998), *Harpegnathos saltator* (Liebig et al., 2000), *Myrmecia gulosa* (Dietemann et al., 2003), *Pachycondyla* cf. *inversa* (Tentschert et al., 2001; Heinze et al., 2002), and *Streblognathus peetersi* (Cuvillier-Hot et al., 2004). By secreting specific pheromones, it is thought that active egg layers signal their high fertility (Cuvillier-Hot et al., 2001) so that low-ranking individuals concede reproduction to high-ranking fecund individuals. In this way, the reproductive division of labor would be reinforced.

It is currently unknown whether a reproductive division of labor in queenless colonies can be regulated purely through chemical signaling, without the use of aggression. In some species, dominance behavior appears to be lacking altogether, even after colonies have been orphaned (*Gnamptogenys striatula*, Blatrix and Jaisson, 2000; *Pachycondyla berthoudi*, Peeters and Crewe, 1985, 1984; Sledge et al., 2001; *P. krugeri*, Wildman and Crewe, 1988; *Platythyrea lamellosa*, Villet et al., 1990; *P. schultzei*, Villet, 1991; *Rhytidoponera aurata*, Komene et al., 1999; *R.* sp. 12, Tay and Crozier, 2000; *Streblognathus aethiopicus*, Ware et al., 1990). This suggests that in these species, reproductive regulation is entirely pheromone-based.

The aim of the present study was to provide the first detailed test of the hypothesis that reproductive regulation is purely pheromone-based in a species that lacks dominance behavior. We used the facultatively queenless ant *G. striatula* (Blatrix and Jaisson, 2000) to examine the cuticular chemistry of fertile and infertile individuals with respect to different stages of ovarian development and mating status. As predicted, we showed that active egg layers have a unique cuticular hydrocarbon profile that, thus, can provide nestmates with reliable signals of fertility. In the interest of colony productivity, this allows reproduction to be regulated without the use of aggression.

Methods and Materials

Study Species

The ant *G. striatula* is a New World species, typically found in open habitats (Giraud et al., 2001) and humid forests (Lattke, 1995). Colonies are headed by either one or several queens, or more commonly, by several gamergates (up to 31; Blatrix and Jaisson, 2001). In our study population, 15 out of 23 colonies (65%) were queenless and headed by gamergates. Removing queens or gamergates from nests results in sexual calling and egg laying by some of the virgin workers inside the nest. The labor workers then transport nearby males to the nest, where the males will mate with any worker that assumes the sexual-calling position (Blatrix and Jaisson, 2000). The ability of workers to mate after loss of the queen or gamergates renders the colonies potentially everlasting.

Collection and Housing of Colonies

Colonies of *G. striatula* were collected in Santa Rosa National Park, Costa Rica, in October 1999 (four colonies) and October 2003 (six colonies). Colonies were transferred to the laboratory in Belgium, where they were housed in plaster of Paris nests with foraging arenas of $20 \times 10 \times 6$ cm or $20 \times 20 \times 6$ cm, depending on colony size, and glass covered nest cavities of $50 \times 40 \times 3$ mm to $150 \times 85 \times 3$ mm.

Collection of Individuals of Differing Reproductive Status

Five individuals were selected for each of the following categories from gamergate-right supply colonies and orphaned groups.

From gamergate-right colonies: G1—gamergates: mated workers, typically with well-developed ovaries, i.e., with many developing and mature oocytes, yellow bodies (an indicator of previous egg-laying; Billen, 1985; Fénelon and Billen, 1996), and the main or sole reproducers in the colony. The spermathecae of these individuals were opaque, indicating the presence of semen. G2—nonreproductive workers: workers with nonactive ovaries, i.e., without or with very few developing oocytes and without yellow bodies.

From orphaned colonies: O1—reproductive workers with fully developed ovaries: virgin workers with well-developed ovaries, i.e., with many developing and mature oocytes, and yellow bodies, an indication of high fertility and previous egg laying. A transparent spermatheca shows they were unmated. O2—reproductive workers with partly developed ovaries: virgin workers with numerous developing and mature oocytes but no yellow bodies. These intermediates were likely on the verge of oviposition or they might be ovipositing already (it is unclear how many ovipositions are required for the development of yellow bodies). O3—former reproductive workers: virgin workers with yellow bodies but only few developing oocytes. Video observation revealed that these individuals did not lay eggs and behaved like foragers. We therefore considered them former egg layers. O4—nonreproductive workers: virgin workers with nonactive ovaries, i.e., without or with very few developing oocytes and without yellow bodies.

To obtain workers of categories O1–O4, we created five orphaned groups of 20 virgin workers by isolating them from their natal gamergate colonies. When five eggs were present in the experimental chamber, each worker's cuticle was sampled for chemical analysis. Dissection of these workers showed they all had empty spermathecae. We also counted the number of ovarioles and the number of yolky and nonyolky oocytes, as oocytes

progress from nonyolky to yolky during maturation (Peeters, 1993; Gobin et al., 1998) and noted whether yellow bodies were present. To obtain five individuals for each O1–O4 category, we chose for each category five workers out of the dissected ants that had ovary characteristics that best fit the description. For categories O1 and O2, we retained the individuals with the highest number of yolky oocytes, and for categories O3 and O4 the individuals with the least or preferably no oocytes (and never having yolky oocytes). Samples from these individuals were analyzed for their cuticular chemicals.

Chemical Analysis

Cuticular chemicals were extracted by placing ants in individual vials containing 10 μl of hexane for 30 min. The chemical extracts were analyzed by gas chromatography (GC) in splitless mode [Agilent 6850 Series, Agilent Technologies, USA, equipped with a capillary column (Agilent HP-1; coated with methyl siloxane; length: 30 m; I.D.: 0.32 mm; film thickness: 0.25 μm ; Agilent Technologies) and flame ionization detector]. The injector and detector temperatures were 290°C and 300°C, respectively, with an inlet pressure of 6.74 psi and a constant flow of 1.1 ml/min. The oven was programmed from 90°C to 180°C at 20°C/min, then to 290°C at 5°C/min, and held for 30 min. We used helium as the carrier gas. Data were analyzed using *Chemstation* (Rev A.09.01, Agilent Technologies).

Gas chromatography/mass spectrometry analysis (GC-MS) was carried out 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 60°C to 250°C at 10°C/min and then from 250°C to 320°C at 5°C/min, and held for 7 min at 320°C. Helium was used as carrier gas, at a constant flow rate of 1.0 ml/min. Cuticular hydrocarbons (alkanes and mono-, di-, and trimethyl alkanes) were characterized by the use of standard MS databases and diagnostic ions and by determining Kovats indices by the method of Carlson et al. (1998). The position of the double bond in nonacosene and hentriacontene was not determined.

Statistical Analysis

Before doing multivariate analysis, data were standardized by calculating relative peak areas, i.e., peak areas of compounds divided by the total sum of all peak areas for one individual. This was performed to ensure that each individual was given the same weight in the analysis. The total sum of all peak areas for each individual was added as a control variable. We only considered compounds with relative peak areas >1% in at least two individuals. This reduced the number of compounds to 51. In two cases, MS revealed two different compounds with the same retention time in two individuals (peaks 18 and 25; Table 1). Because these compounds could not be distinguished with GC only, they were excluded in all statistical analyses.

Data were subjected to principal component analysis (PCA) to allow compounds that differed between groups to be graphically identified. To focus on the compounds with the greatest difference between groups, we only considered compounds that had factor loadings with an absolute value >0.75 on the first axis.

To assess the significance of the differences in cuticular profiles between the different groups, we used MANOVA on the first four factors of the PCA, which together explained 73% of the observed variation. All statistical calculations were performed using *Statistica* 6.0 (Statsoft, Inc.).

Table 1 Chemical compounds identified by GC/MS and their relative concentration for each group (group mean \pm standard deviation)

Peak	Compound name	Group					
		G1	G2	O1	O2	O3	O4
1	4-Methyl octacosane	5 \pm 5	2 \pm 2	2.5 \pm 0.7	0.6 \pm 0.8	0.15 \pm 0.03	0.06 \pm 0.03
2	Nonacosane	0.3 \pm 0.3	0.02 \pm 0.02	0.2 \pm 0.4	1.2 \pm 1.1	0.13 \pm 0.10	0.5 \pm 0.5
3	Nonacosane	2.0 \pm 0.9	7 \pm 3	1.9 \pm 0.6	1.8 \pm 0.7	7 \pm 3	4.1 \pm 1.6
4	11- and 13-Methyl nonacosane	0.6 \pm 0.2	1.6 \pm 0.6	0.24 \pm 0.19	0.32 \pm 0.10	2.6 \pm 1.1	2.4 \pm 0.7
5	3-Methyl nonacosane	13 \pm 7	2.6 \pm 1.0	10.6 \pm 1.4	9 \pm 4	3.1 \pm 0.6	2.0 \pm 0.5
6	Triacontane	0.8 \pm 0.3	3 \pm 2	0.8 \pm 0.3	1.5 \pm 1.0	4 \pm 2	2.5 \pm 1.0
7	3,15-Dimethyl nonacosane	1.9 \pm 1.0	0.08 \pm 0.09	2.3 \pm 0.6	2.6 \pm 0.6	0.16 \pm 0.05	0.10 \pm 0.07
8	12- and 18-Methyl triacontane	2.3 \pm 1.4	3.9 \pm 1.1	0.7 \pm 0.5	0.7 \pm 0.5	2.7 \pm 0.7	4.0 \pm 0.6
9	4-Methyl triacontane and 8, 12-dimethyl triacontane	3.0 \pm 0.9	1.2 \pm 1.5	2.8 \pm 0.3	2.1 \pm 0.9	0.91 \pm 0.19	0.52 \pm 0.11
10	6,10- and 6,12-Dimethyl triacontane	07 \pm 0.3	1.2 \pm 0.8	0.9 \pm 0.5	0.5 \pm 0.5	1.8 \pm 0.8	1.5 \pm 0.4
11	Hentriacontene	1.4 \pm 0.2	2.8 \pm 1.3	0.83 \pm 0.19	1.1 \pm 0.3	5 \pm 2	5.3 \pm 0.6
12	Hentriacontane	0.7 \pm 0.3	2.2 \pm 1.1	0.63 \pm 0.11	0.8 \pm 0.3	2.4 \pm 1.1	1.4 \pm 0.5
13	9-,11- and 13-Methyl hentriacontane	5 \pm 3	13 \pm 3	2 \pm 2	2.5 \pm 1.2	9.5 \pm 1.3	10.9 \pm 0.9
14	11,19-Dimethyl hentriacontane	2.2 \pm 1.7	0 \pm 0	1.7 \pm 1.0	1.7 \pm 1.0	0 \pm 0	0 \pm 0
15	7, <i>x</i> - and 9, <i>x</i> -Dimethyl hentriacontane	0 \pm 0	4.0 \pm 1.8	0.4 \pm 0.9	1 \pm 2	4.1 \pm 1.0	4.9 \pm 1.0
16	3-Methyl hentriacontane	8 \pm 4	5.9 \pm 0.7	6.0 \pm 0.9	5 \pm 2	5.1 \pm 1.3	5 \pm 0.5
17	3,11-, 3,13- and 3, 15-Dimethyl hentriacontane	13 \pm 5	1.1 \pm 0.3	14 \pm 2	16 \pm 5	1.2 \pm 0.5	0.9 \pm 0.4
18	10-, 12-, 14-, and 20-Methyl dotriacontane or 12, <i>x</i> -dimethyl dotriacontane	4.3 \pm 1.6 ²	8.3 \pm 1.8 ¹	4.1 \pm 0.8	4.3 \pm 1.1	6.8 \pm 1.3	8.3 \pm 1.3 ¹
19	4-Methyl dotriacontane	1.7 \pm 0.3	1.8 \pm 1.1	1.7 \pm 0.3	1.4 \pm 0.6	1.4 \pm 0.9	2.2 \pm 0.6
20	10,14- and 8,12-Dimethyl dotriacontane	1.6 \pm 1.1	5.2 \pm 1.4	1.9 \pm 0.3	1.8 \pm 1.1	4.7 \pm 1.0	5.5 \pm 1.2
20'	10,20-Dimethyl dotriacontane	0.5 \pm 0.2	1.5 \pm 0.5	0.33 \pm 0.10	0.3 \pm 0.2	1.7 \pm 0.6	1.7 \pm 0.3
21	4,10-, 4,12- and 4, 14-Dimethyl dotriacontane	3.3 \pm 1.5	1.0 \pm 0.3	4.5 \pm 1.2	5 \pm 2	0.7 \pm 0.3	0.8 \pm 0.3
22	9-,11- and 13-Methyl tritriacontane	2.6 \pm 1.2	6.8 \pm 1.9	1.9 \pm 0.2	2.0 \pm 1.0	5.3 \pm 1.7	5.9 \pm 1.0
23	9,21-, 9,23-, and 11, 23-Dimethyl tritriacontane	4 \pm 2	10.0 \pm 1.1	4.5 \pm 0.9	4 \pm 2	8 \pm 4	9.5 \pm 1.6
24	3,11-, 3,13-, and 3, 15-Dimethyl tritriacontane	6 \pm 2	0.36 \pm 0.09	7.6 \pm 1.3	8 \pm 3	0.5 \pm 0.3	0.30 \pm 0.01
25	12-,14-, and 20-Methyl tetratriacontane or 3,11,15-trimethyl tritriacontane	3 \pm 2 ²	1.6 \pm 0.7 ¹	5.3 \pm 0.7	5 \pm 2	2.1 \pm 0.8	2.4 \pm 0.6 ¹
26	10,14-Dimethyl tetratriacontane	0.6 \pm 0.5	1.9 \pm 0.4	0.91 \pm 0.15	0.9 \pm 0.5	1.0 \pm 0.5	1.8 \pm 0.4

Table 1 (continued)

Peak	Compound name	Group					
		G1	G2	O1	O2	O3	O4
27	4,x-Dimethyl tetraatriacontane	1.2 ± 0.3	0.9 ± 0.3	2.1 ± 0.5	1.9 ± 0.3	0.8 ± 0.3	0.9 ± 0.3
28	3,13- and 3,15-Dimethyl pentatriacontane	1.9 ± 0.8	0.18 ± 0.15	3.0 ± 0.4	3.2 ± 0.8	0.11 ± 0.13	0.09 ± 0.04
29	3,11,15-Trimethyl pentatriacontane	1.3 ± 0.8	0.4 ± 0.3	2.6 ± 0.4	2.6 ± 1.2	0.4 ± 0.2	0.40 ± 0.13
30	3,13- and 3,15-Dimethyl heptatriacontane	1.1 ± 0.7	0.03 ± 0.02	2.1 ± 0.5	2.0 ± 0.6	0.05 ± 0.07	0.05 ± 0.03
31	3,11,15-Trimethyl heptatriacontane	1.0 ± 0.8	0.05 ± 0.04	2.3 ± 0.4	2.0 ± 1.2	0.06 ± 0.07	0.005 ± 0.04

The peak numbers correspond with the numbers on the total ion chromatograms shown in Fig. 1. Peak 20', shoulder peak of peak 20. Group codes: from gamergate-right colonies: G1, gamergates; G2, nonreproductive workers; from orphaned colonies: O1 and O2, reproductive workers with fully and partly developed ovaries; O3, former reproductive workers; O4, nonreproductive workers. For peaks 18 and 25, the superscripts 1 and 2 indicate that it is the first resp. second of the two possible sets of compounds that occur.

Results

General Characteristics of Cuticular Chemical Profiles

The cuticular profiles contained a mixture of alkanes; alkenes; and mono-, di-, and trimethyl alkanes with chain lengths ranging from C₂₈ to C₃₇ (Fig. 1; Table 1). Several compounds occurred in high proportions in all individuals (nonacosane, 12- and 18-methyl triacontane, and 3-methyl hentriacontane, peaks 3, 8, and 16). Other compounds were specific to gamergates or nonreproductive workers (see below).

Statistical Analysis

Principal component analysis shows that differences in fertility explain most of the variation in cuticular hydrocarbon profiles, with the first axis of the PCA clearly differentiating active egg layers from nonreproductive workers (percentage of the variance explained: 44%; Fig. 2). Reproductive workers with fully and partly developed ovaries (O1 and O2) cluster together with gamergates (G1; Fig. 2). This shows that mating as such did not influence cuticular hydrocarbon profiles. Former reproductive workers (O3) cluster together with nonreproductive workers (O4 and G2; Fig. 2), and reproductive workers with fully developed ovaries (O1 and G1) cluster with reproductive workers with partly developed ovaries (O2). This demonstrates that chemical profiles accurately reflect current fertility independent of previous egg laying. A MANOVA on the first four factors of the PCA confirms that there were significant differences in the chemical profile of ants with high and low fertility (groups O1, O2, and G1 vs. groups O3, O4, and G2; $F_{4,23} = 57.3$; $P < 10^{-6}$) but that there were no significant differences detectable between mated (G1) and unmated egg layers (O1 and O2; $F_{4,23} = 1.42$; $P = 0.26$) nor between workers with (O1, O3, and G1) and without yellow bodies (O2, O4, and G2; $F_{4,23} = 0.78$; $P = 0.55$).

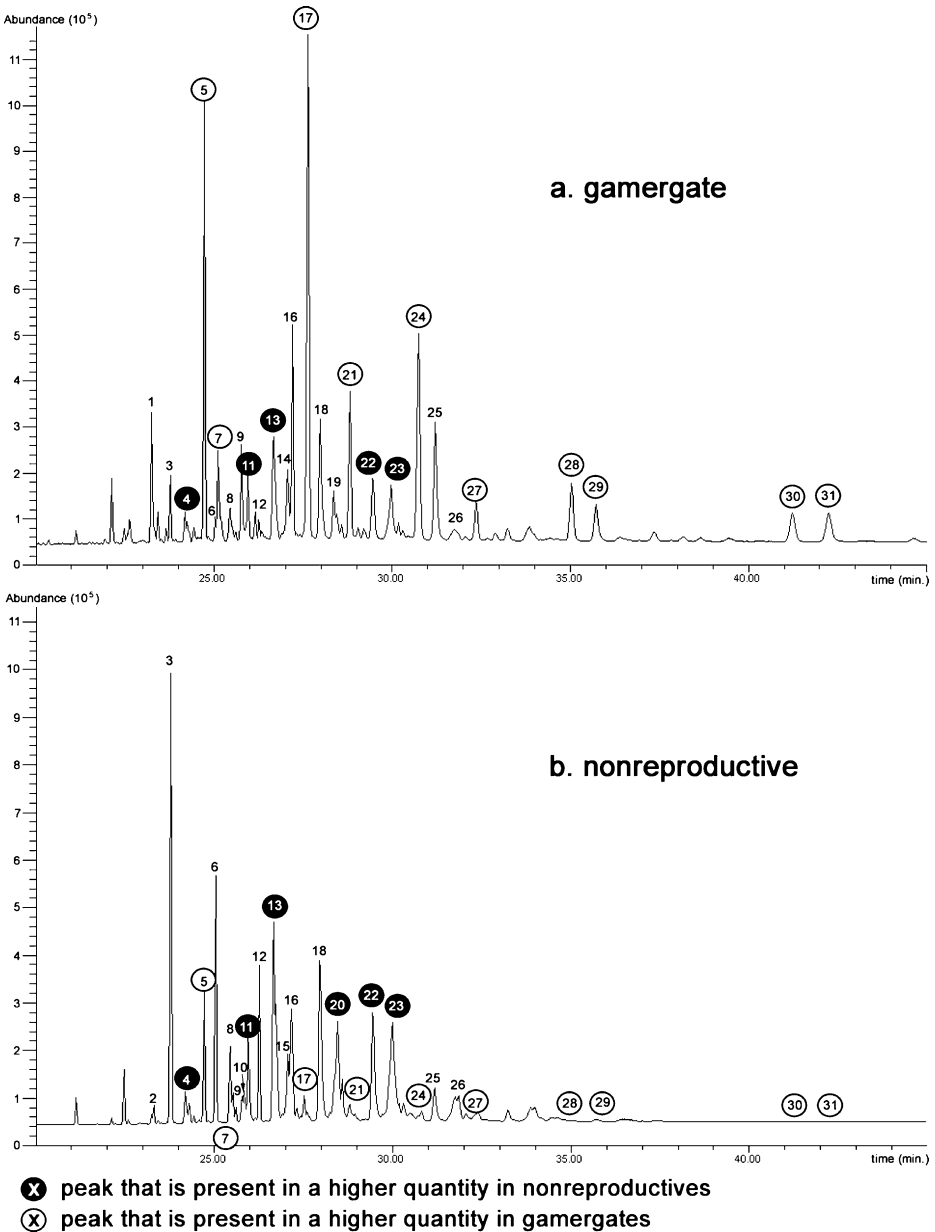
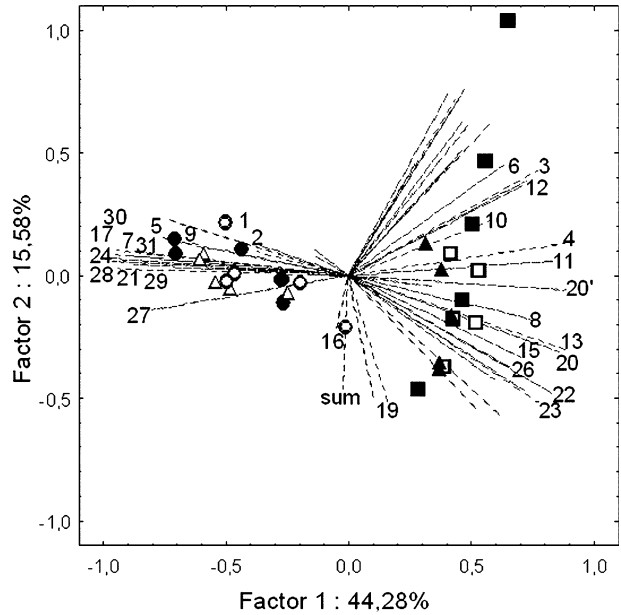


Fig. 1 Representative cuticular chemical profiles of a gamergate (a) and a worker with undeveloped ovaries (b). The peak numbers correspond to the identified compounds listed in Table 1.

Chemical Differences between Fertile and Infertile Individuals

To determine which compounds correlate most with differences in fertility, we can look at those compounds that show the strongest correlation with the first factor of the PCA (compounds with factor loadings with an absolute value >0.75; Fig. 2). In this way, it can

Fig. 2 Biplot showing the first two factors of a PCA. The symbols represent the factor scores of individuals belonging to different groups. The lines radiating from the origin indicate the factor loadings of the different compounds. The overlap between both plots shows the correlation between the two. Factor scores were reduced 10-fold to facilitate comparison with the factor loadings. Compound numbers correspond to those listed in Fig. 1 and Table 1. “Sum” is the total quantity of chemicals on the cuticle, i.e., the sum of all compounds. Symbols refer to the following groups: from gamergate-right colonies: (○) gamergates (G1) and (▲) nonreproductive workers (G2); from orphaned colonies: (Δ and ●) reproductive workers with fully and partly developed ovaries (O1 and O2), (■) former reproductive workers (O3), and (□) nonreproductive workers (O4).



be seen that there are 18 compounds that are strongly negatively correlated with factor 1, and occur in higher concentration on the cuticle of fertile individuals, i.e., gamergates and unmated egg-laying workers. These compounds comprise one monomethyl alkane: 3-methyl nonacosane (peak 5; Table 1); 15 dimethyl alkanes: 3,15-dimethyl nonacosane (peak 7), 3,11-, 3,13-, and 3,15-dimethyl hentriacontane (peak 17), 4,10-, 4,12-, and 4,14-dimethyl dotriacontane (peak 21), 3,11-, 3,13-, and 3,15-dimethyl tritriacontane (peak 24), 4,*x*-dimethyl tetratriacontane (peak 27), 3,13- and 3,15-dimethyl pentatriacontane (peak 28), and 3,13- and 3,15-dimethyl heptatriacontane (peak 30); and two trimethyl alkanes: 3,11,15-trimethyl pentatriacontane (peak 29) and 3,11,15-trimethylheptatriacontane (peak 31). In this group, peaks 17, 24, and 28 show the strongest negative correlation with factor 1 (factor loading <-0.95). The compounds 3,13- and 3,15-dimethyl pentatriacontane (peak 28), 3,13- and 3,15-dimethyl heptatriacontane (peak 30), and 3,11,15-trimethyl heptatriacontane (peak 31) are unique for active egg layers (Table 1).

Similarly, 14 compounds are strongly positively correlated with factor 1 (factor loadings >0.75), and occur in higher concentration on the cuticle of nonreproductive workers. These compounds comprise one alkene: hentriacontene (peak 11); eight monomethyl alkanes: 11- and 13-methyl nonacosane (peak 4), 9-, 11-, and 13-methyl hentriacontane (peak 13), 9-, 11-, and 13-methyl tritriacontane (peak 22); and five dimethyl alkanes: 10,14- and 8,12-dimethyl dotriacontane (peak 20) and 9,21-, 9,23-, and 11,23-dimethyl tritriacontane (peak 23). Peak 13 shows the strongest correlation with factor 1 (factor loading 0.89). None of the compounds included in the PCA were unique to nonreproductive workers. The total of all compounds (“sum”) was independent of the first principal component, and hence was independent of fertility (Fig. 2).

In summary, there were at least 32 compounds strongly correlated with reproductive status, 18 of which were more abundant on the cuticle of gamergates and reproductive workers, and 14 of which were more abundant in nonreproductive workers. In addition to these compounds, MS indicated that 10-, 12-, 14-, and 20-methyl dotriacontane (peak 18), and 12-, 14-, and 20-methyltettriacontane (peak 25) were unique to gamergates, and that 12,*x*-dimethyl dotriacontane (peak 18) and 3,11,15-trimethyl tritriacontane (peak 25) were unique for nonreproductive workers. Unfortunately, because the retention times of monomethyl dotriacontane and dimethyl dotriacontane (peak 18), and of monomethyl tettriacontane and trimethyl tritriacontane (peak 25), were identical, these differences could not be statistically confirmed in a larger sample by using GC.

Discussion

Our results show that there are pronounced differences in the cuticular chemistry of fertile and infertile individuals in queenless colonies of the ant *G. striatula*. Both qualitative and quantitative differences in over 32 hydrocarbons contributed to this result. Previously, cuticular hydrocarbons linked to fertility have also been found in ant species that form aggressive dominance hierarchies, for example, *D. ceylonense* (Cuvillier-Hot et al., 2001, 2002), *D. quadriceps* (Monnin et al., 1998), and *S. peetersi* (Cuvillier-Hot et al., 2004). These fertility signals are supposed to help in resolving intracolony conflicts by stimulating low-rankers to concede reproduction to more fecund high-ranking ones (Cuvillier-Hot et al., 2001). Our species, however, is unique in that it lacks any observable dominance behavior. Hence, our study is the first to show that chemical fertility cues may alone regulate reproduction. It remains to be elucidated which of the identified compounds act as the main signal. As several compounds are either up- or down-regulated depending on status, it is likely that more than one compound is involved in fertility regulation in *G. striatula*. In *P. inversa*, however, D'Ettore et al. (2004) showed that the ants are able to detect a single compound that is specifically associated with ovarian activity.

Overall, the chemicals characterizing fertile workers are hydrocarbons with longer chain lengths compared with the compounds found on the cuticle of infertile workers. A shift toward heavier hydrocarbons in ants with well-developed ovaries has also been found in other ponerine species, e.g., *P. inversa* (Heinze et al., 2002) and *H. saltator* (Liebig et al., 2000). The elongation reactions, following a switch to reproductive maturation, have been well studied in other insect taxa (Tillman-Wall et al., 1992; Blomquist et al., 1995, 1998; Tillman et al., 1999), and it is likely that a similar mechanism is acting in the species studied here, *G. striatula*. In two species, the dolichoderine ant *Linepithema humile* and the myrmecine ant *M. gulosa*, the shift has been shown to proceed in the opposite direction (de Biseau et al., 2004; Dietemann et al., 2003), with queens being characterized by shorter-chain hydrocarbons than workers. Mechanistically, the chemical differences between fertile and infertile individuals might be caused by the differential activity of oenocytes, which are involved in yolk production (Jensen and Borgesen, 2000) and are also the producers of cuticular hydrocarbons (Diehl, 1975; Fan et al., 2003).

A major conclusion of our results is that the chemical profiles accurately reflect the current fertility of an individual. This was shown by the fact that workers with yellow bodies in their ovaries, which are thought to be workers that have recently stopped laying eggs, had the same cuticular profiles as nonlaying workers. By contrast, it is clear that mating did not produce any detectable changes in cuticular chemistry, as gamergates and unmated workers with active ovaries had essentially the same chemical profiles. This also

appears to be true for other queenless ants (*D. ceylonense*, Cuvillier-Hot et al., 2002; *H. saltator*, Liebig et al., 2000) and it suggests that signaling fertility is generally more important than signaling mating status.

In summary, our results demonstrate that in the ant *G. striatula*, cuticular hydrocarbon profiles can provide nestmates with reliable information about each individual's fertility. We suggest that in this way, reproduction can be regulated without the use of aggression. The ability to regulate reproduction without the use of aggression has a large colony-level benefit, as costs associated with fighting, such as reduced colony efficiency and mortality, can be avoided. Nevertheless, most species go through an aggressive phase before chemical signals take over regulation of the reproductive conflict (among others Monnin and Peeters, 1999; Cuvillier-Hot et al., 2001, 2002, 2004; Gobin et al., 2001). *G. striatula* apparently lacks this aggressive phase, for a reason that remains unclear. Further studies are required to answer this.

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contents after disturbance. However, most of the volatiles released by bugs after disturbance came from Brindley's glands. The locomotor activity of fourth instars increased significantly after stimulation with the odors emitted by disturbed adults, as compared with larvae stimulated by the odor of undisturbed adults or by clean air. We also studied the directional behavioral response of fifth instars to the disturbance scent in a locomotion compensator. Larvae exposed to volatiles released by disturbed adults walked away from the direction of the odor. The results suggest that this blend or part of it functions as an alarm pheromone for *T. infestans*. We suggest that the metasternal glands of this species are involved both in the sexual and the alarm contexts, and that the Brindley's glands probably have both alarm and defensive roles.

Keywords Triatominae · Metasternal gland · Brindley's gland · Male aggregation pheromone · Alarm pheromone · Chemical communication · SPME · Chagas disease

Introduction

Triatomine bugs (Heteroptera: Reduviidae) are the natural vectors of the protozoan flagellate, *Trypanosoma cruzi*, the etiological agent of Chagas disease. This disease occurs from the southern United States to central Argentina, and it affects 16–18 million people (WHO, 1999; Dias et al., 2002). *Triatoma infestans* Klug 1834 is the main vector of Chagas disease in the southern countries of South America.

Most Reduviidae have several exocrine glands in the thorax and abdomen, i.e., the metasternal glands, Brindley's glands, dermal glands, ventral glands, and abdominal glands (Carayon et al., 1958; Staddon, 1983). Ventral and abdominal glands are apparently absent in the subfamily Triatominae, and only adult insects possess both metasternal and Brindley's glands (Schofield and Upton, 1978).

Brindley's glands produce chemicals that may have defense and alarm functions (Schofield, 1979; Ward, 1981). The saclike Brindley's glands are dorsally located; they extend into the lateral portion of the second abdominal segment and open onto the metathoracic epimeron (Kálin and Barrett, 1975; Staddon, 1983). When disturbed, adult triatomines emit a secretion from these glands (Games et al., 1974; Cruz López et al., 2001). Several reports show that in *Rhodnius prolixus* Stål 1859, and other species of Triatominae, this secretion or the blend emitted after disturbance is composed of isobutyric acid (Pattenden and Staddon, 1972; Games et al., 1974; Schofield, 1979) or a mixture of isobutyric acid and other minor compounds, including diverse fatty acids and unidentified esters (Rojas et al., 2002; Guerenstein and Guerin, 2004). Based on the behavioral response of triatomines to synthetic isobutyric acid, Ward (1981) suggested that the compounds emitted by Brindley's glands act as an alarm pheromone. It is unclear whether isobutyric acid and other compounds emitted by Brindley's glands constitute an imaginal alarm signal, or whether they also play a role affecting the behavior of immature instars. Moreover, the behavioral responses evoked when isobutyric acid is released by conspecific insects have not been characterized.

The metasternal glands are located in the ventral metathorax and consist of a small reservoir and an unbranched or branched secretory tubule. The opening of each gland is located laterally to the sternal apophyseal pit. 3-Methyl-2-hexanone is produced by the metasternal glands of *Dipetalogaster maxima* (Uhler, 1894) (Rossiter and Staddon, 1983), and detected when bugs of this species are disturbed (Guerenstein and Guerin, 2004). The compositions of the secretions of these glands in other triatomines are unknown (Cruz López et al., 1995).

Behavioral data indicate that copulating pairs of *R. prolixus* (Baldwin et al., 1971) and *T. infestans* (Manrique and Lazzari, 1995) emit a volatile signal that promotes the aggregation of males around the couple and apparently mediates polyandry. The chemical identity of this signal is unknown. Fontán et al., (2002) described a series of compounds, including aldehydes, from copulating *T. infestans*, and they indicated that these compounds could represent the sexual pheromone of that species. Rojas et al., (2002) partially identified the contents of Brindley's glands in *R. prolixus* and suggested that those compounds would affect sexual behavior. However, the behavioral data did not clearly support this hypothesis.

In this report, we demonstrate that the metasternal glands of *T. infestans* are sources of volatile compounds, and we identify several of the substances emitted. We suggest that the contents of the metasternal glands of *T. infestans* are emitted during copulation, and we examine whether or not the metasternal gland contents are concurrently emitted with the products of Brindley's glands during the mechanical disturbance of adults. Moreover, we attempt to clarify the identity of volatiles released by disturbed adult *T. infestans* and volatiles emanating from a preparation of excised Brindley's glands. Along with results on the chemical identities, we present behavioral experiments that suggest their biological roles.

Methods and Materials

Insects

T. infestans was reared in the laboratory at $28 \pm 1^\circ\text{C}$ and fed on live chickens. Insects used for identification of gland and disturbance odors came from a colony collected from Northern Minas Gerais state, Brazil. This colony has been maintained for 20 yr at the Laboratory of Triatomines and Epidemiology of Chagas disease. Insects were sorted by sex as fifth instars (Espínola, 1966) and maintained apart until the experiments were performed. Emerged adults were fed weekly and kept in plastic containers, which had a piece of folded filter paper as substrate. Insects used for chemical sampling were virgin and prone to copulate (Manrique and Lazzari, 1994). For behavioral assays, we used fourth and fifth instars that had been starved for 1–2 wk postecdysis.

Volatile Sampling: Solid Phase Microextraction

Solid phase microextraction (SPME) (Arthur and Pawliszyn, 1990) was used to sample odors in the headspaces of vials containing adult bugs or preparations of excised metasternal or Brindley's glands. Among several fiber coatings tested, the CAR/PDMS stationary phase (75 μm film thickness) (Supelco, Bellefonte, PA, USA) was used throughout this study, because it proved especially effective for collecting most of the compounds present in the headspace.

Gas Chromatography Coupled to Mass Spectrometry

Volatiles were analyzed on a Shimadzu 17A gas chromatograph (GC), linked to a mass selective detector (Shimadzu 5050 A). The interface temperature was 250°C , and the ionization energy was 70 eV. The mass spectrometer was programmed to scan for masses of 40 up to 300 m/z . The capillary column used was a SupelcoWax-10; 30 m length, 0.25 mm i.d., 0.25 μm film thickness. The SPME fibers were inserted in the heated split/splitless injector (240°C) for 1 min to desorb the compounds. The split ratio was set to 1:4. Helium

(30 cm/sec linear velocity) was used as the carrier gas. The temperature program for the column oven was 50°C for 1 min, heating to 75°C at 10°C/min, and finally to 240°C at 15°C/min.

Preliminary identification of the volatiles studied here were based on the analysis of their mass spectra, and comparisons of the experimental data with a standard mass spectrum library (NIST). In most cases, the structures proposed were confirmed through injections of synthetic standards.

Sampling Procedure

Analyses of Volatiles Released by Metasternal and Brindley's Glands

Adult *T. infestans* were dissected several days after feeding to avoid contamination with feces (another potential source of volatiles). Before dissection, insects were kept at -18°C for 10 min to avoid disturbances during subsequent manipulations. They were then fastened with plasticine (Massa de modelar base amido, Faber-Castell, Sao Carlos, Sao Paulo, Brazil), leaving their thorax and abdomen exposed. These preparations were placed into an ice-cold phosphate buffer solution to minimize the evaporation of substances. Immediately after mounting the insects, we used a microsurgical knife to remove a pair of either metasternal or Brindley's glands together with an attached piece of cuticle surrounding the gland opening (ca. 0.25 mm²). Preparations were placed into a glass vial (2 ml), after which the vial was closed with a Teflon®/silicone-lined cap and kept in an ice bath until the end of the dissections that followed. For the analysis of the metasternal gland volatiles, each sample was prepared by using eight glands from at least four insects of the same sex. Three samples were analyzed for each sex. For the analysis of the Brindley's gland volatiles, each sample was prepared from 10 glands from at least five insects of the same sex. Three samples of these glands were analyzed for each sex. Control samples of cuticular pieces with internal tissues from other segments of the insects were prepared and similarly analyzed. Before adsorbing the volatiles by SPME, the preparation was sonicated (Thorton Inpec Eletrônica, Vinhedo, Sao Paulo, Brazil, 40 kHz, 100 W) for 3 min and then heated at 50°C for 10 min. The fiber was inserted through the Teflon®/silicone septum into the closed gland-containing vial, and sampling proceeded at 50°C for 30 min. Volatiles were desorbed from the SPME fiber in the injection port of the GC under the chromatographic conditions described above.

Odors Emitted by Copulating Couples

A *T. infestans* female and a male were gently introduced into a 10-ml vial, by using a piece of filter paper to avoid pressing the insects with forceps (see below). The vial was closed with a Teflon®/silicone-lined cap. After copulation had begun, volatiles present in the headspace were sampled for 10 min with the SPME fiber. Volatiles were desorbed inside the GC injection port immediately after sampling the headspace. Five assays were performed at 25 ± 1°C and 53–60% RH. Each pair of insects was used twice to sample the odors emitted: (1) before copulation and (2) during copulation. The pair was then discarded.

Because no volatiles were detected by gas chromatography-mass spectrometry (GC-MS) analysis before and during copulation using the full mass scan mode, we decided to search for key compounds from metasternal and Brindley's glands of adult *T. infestans*, by using the more sensitive single ion monitoring GC-MS analysis mode (SIM). 3-Pentanone (with characteristic ion fragments at *m/z* 29, 57, and 86) was chosen as the diagnostic compound for the metasternal gland, as it was the most abundant compound found in these glands

(see Results). Isobutyric acid (with characteristic ion fragments at m/z 27, 41, 43, 55, and 88) was used as the diagnostic compound for the Brindley's glands, as it was the most abundant compound found in these glands (see Results). For the single ion monitoring experiment, each pair of insects was used only once (during copulation) and then discarded.

*Analyses of Volatiles Released by Disturbed *T. infestans**

To study the volatiles released by disturbed insects, two individuals per assay were placed in a 10-ml vial containing a piece of filter paper (3×1.5 cm) as substrate. The insects were each disturbed by pressing one of their legs with forceps, placing them into the vials, and subsequently agitating them for 15 sec. After the insects had been introduced, the vials were closed with caps equipped with Teflon[®]/silicone-lined septa. Volatiles were collected for 10 min ($25 \pm 1^\circ\text{C}$, 53–60% RH) with the SPME fiber. Two control series were performed: vials with filter paper only ($N = 5$ assays), and vials with undisturbed insects ($N = 5$ assays, 2 bugs per assay). In the latter case, insects were allowed to climb onto a piece of filter paper, which was subsequently introduced into the vial to avoid disturbing the insects. In all cases (control and treatment), volatiles were desorbed by immediately inserting the fiber in the GC injection port after sampling the headspaces of the vials.

A total of 17 treatment assays were conducted for females ($N = 3$ assays, two bugs per assay), males ($N = 3$ assays, two bugs per assay), and heterosexual pairs ($N = 11$ assays, two bugs per assay). As no qualitative differences were detected between males and females, the identification of compounds from disturbed insects was made by pooling the results from all samples.

Quantifying the Odors Detected in Triatomine Samples

To quantify some of the substances detected from metasternal glands or from copulating pairs, we performed a series of quantification trials with synthetic substances. Known quantities of the latter compounds were added to equivalent vials and exposed to the same environmental and handling conditions as were used in gland or copulation assays. Afterwards, SPME samplings were performed for constructing calibration curves.

*Behavioral Responses of *T. infestans* Larvae to Scent Emitted by Disturbed Adults*

Assays of Locomotor Activity

We studied whether volatile substances emitted by disturbed adults evoked changes in the level of locomotor activity of ca. 45-d-old, fourth instars of *T. infestans*. In each assay, one insect was placed in an experimental arena (Fig. 1), either in the presence or in the absence of volatiles emitted by disturbed adults. The arena was an acrylic box ($13.5 \times 9.5 \times 4$ cm height) divided into two horizontal compartments (each 2 cm high), the upper one with a removable cover with a series of lines painted on the outside. This compartment had Teflon[®] tape wrapped around its inner perimeter to eliminate corners and thereby diminish the thigmotactic responses of bugs, i.e., the spontaneous tendency to maximize contact of the body with the substrate. The lower compartment had a stimulus delivery tube connected by an open screw cap (with a 1.5-cm hole in its center) and fixed in the center of the inferior face to allow the volatiles to diffuse from the delivery tube into the lower compartment. Uniformly spaced holes (1.5 mm diam) in the base of the upper compartment and in the substrate (a piece of filter paper) allowed the diffusion of the volatile stimulus

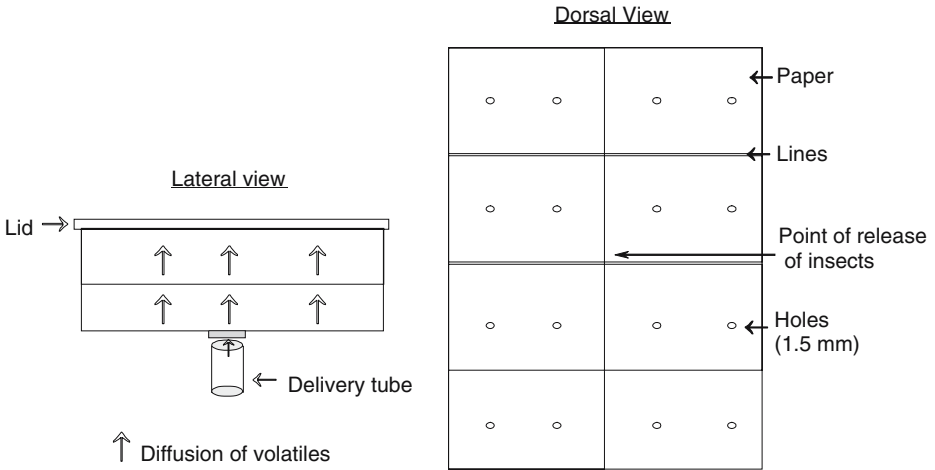


Fig. 1 Experimental arena used for measuring the walking behavior (locomotor activity) of *Triatoma infestans* larvae stimulated with volatiles released by disturbed *T. infestans* adults

from the lower to the upper compartment. Insect behavioral responses (i.e., locomotor activity) were quantified by counting activity bouts, which occurred every time a bug crossed one of the lines in the upper face of the box. The number of activity bouts was counted for a period of 5 min.

There were three treatments in this experiment: (1) larvae stimulated by volatiles coming from disturbed adults (disturbed); (2) larvae stimulated by volatiles coming from undisturbed adults (undisturbed); and (3) larvae without stimulation (control) ($N = 20$ for each treatment). In the first treatment, two adult bugs were placed into the delivery tube and disturbed by pressing their legs with forceps, and then tapping the tube for 20 sec. Volatiles were allowed to diffuse for 5 min to permit their passage from the tube to the lower and upper compartments. Subsequently, one larva was placed in the center of the upper compartment of the arena and after 2 min of acclimatization, its behavior was filmed with an infrared-sensitive camera and recorded on videotape. In the second series, adults were allowed to climb onto a piece of filter paper before being placed into the delivery tube (to avoid disturbance); the experiment then proceeded as in the first series. In the third series, a clean stimulus delivery chamber was exposed in order to determine the baseline activity level. In all cases, the room temperature was maintained at $25 \pm 2^\circ\text{C}$, and the experiments were performed in complete darkness. Differences between treatments were analyzed by one-way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison tests (Zar, 1984).

Assays of Orientation Response

For the analysis of the orientation responses of fifth instars of *T. infestans* towards odors from disturbed adults, behavioral assays were performed by using a locomotion compensator (Barrozo et al., 2003). It consisted of a hollow Styrofoam sphere (97 mm diam, 2.85 g weight) that floated on a vertical air stream generated by an air pump. The bugs were starved (1 wk) and tethered by attaching the thorax and abdomen with two-sided sticky tape to a freely rotating stiff steel wire, centered at the apex of the sphere. Once

tethered in the apparatus in a normal walking posture, the animals started spontaneous locomotion when brought into contact with the sphere, by displacing it with their legs. Insects could rotate freely without modifying their distances to the source. Larvae tested were placed and kept on the apex of the sphere for 2 min to acclimatize before the beginning of each test. Movements of the insects were recorded for 3 min with an infrared-sensitive camera. Two glass containers (20 ml), sealed with plastic caps and located at opposite sides of the sphere, were used for the delivery of stimuli. Each cap was attached by a Teflon[®]/silicone tubing (20 cm) to a glass tube (0.65 cm diam, 8.5 cm length). The outlet of each tube was positioned 5 cm away from the insect. The behavior of each insect was recorded for 3 min in the absence or presence of the stimulus. To test for response asymmetry, a control series was performed in which both stimulus containers were empty ($N = 20$). Afterwards, stimulated insects were tested, i.e., one container was empty, whereas the other contained odors coming from disturbed adults ($N = 22$). In this series, one adult insect was disturbed as described above.

To quantify the behavioral responses of the larvae, we established a line that divided the top view of the sphere into two equal areas: the stimulus (i.e., odor from disturbed adults) was presented perpendicularly to that line. We determined the time that bugs spent walking towards these two areas. Three different categories of behavior were considered: walking towards the source; walking away from the source; and no walking (i.e., stationary behavior). We computed an orientation index as the difference between the time that the bugs spent walking towards one vial (i.e., either the vial containing the volatiles from disturbed animals or the clean vial in the test or control series, respectively) and the time that the bugs spent walking to the opposite side, divided by the total experimental time (3 min). The position of the stimulus was randomly alternated between assays. The resulting differences between treatments were analyzed with the Mann–Whitney test (Zar, 1984). The behaviors of control animals were also compared by means of a one-sample nonparametric test (Wilcoxon signed rank test) to determine whether those behaviors differed from those expected by chance (i.e., 50% of the time walking towards each of the sides).

Results

Secretion from Metasternal Glands

The secretion from the metasternal glands contained several compounds, some of which were highly volatile aliphatic ketones and alcohols (Fig. 2, Table 1). No compounds from metasternal glands were detected in the analyses of control samples, i.e., cuticular pieces and other internal tissues. The six substances identified from the glands were consistently found in all samples of both sexes obtained from metasternal glands. 3-Pentanone was the main component in all samples. The quantities of 3-pentanone present in the samples apparently varied between 10 and 100 μg per adult. Similarly, the quantities of 2-methyl-1-butanol and 3-pentanol varied between 1 and 10 μg per insect, and 3-hexanol apparently varied between 0.1 and 1 μg per insect.

We were not able to establish the identity of the second largest peak. The molecular ion is apparently 143, indicating a nitrogen-containing compound (Fig. 3). The even fragments m/z 128, 114, and 86 might result from nitrogen-containing fragments formed by cleavages of methyl-, ethyl-, and butyl groups, respectively. Fragment m/z 101 could have been formed by a molecular rearrangement losing a common m/z 42 mass unit (i.e., propene), whereas the m/z 57 and 41 fragments could be butyl- and propenyl cations, respectively. The short elution time

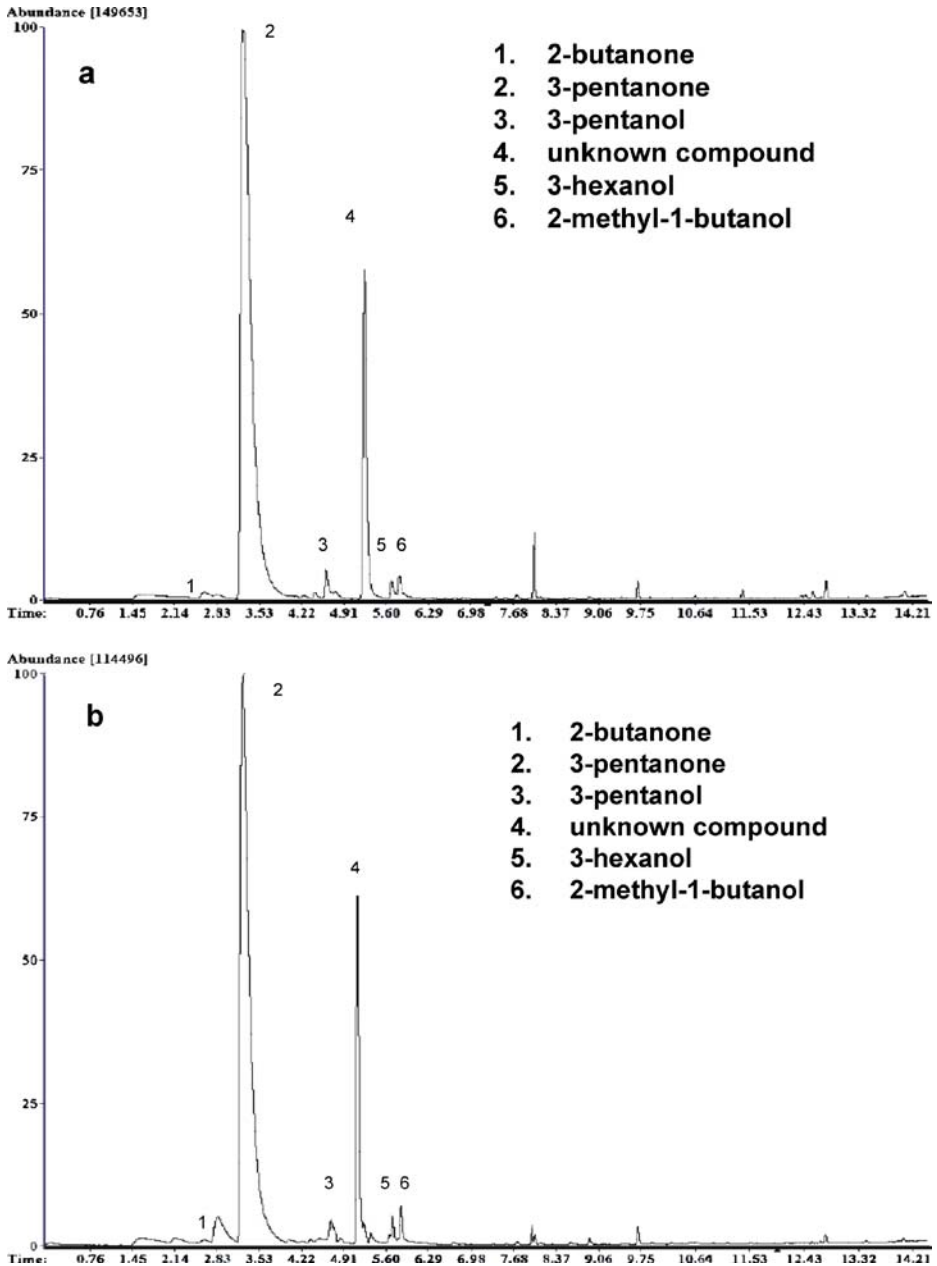


Fig. 2 Gas chromatograms of the volatile compounds present in the metasternal glands of *T. infestans* females (a) and males (b)

Table 1 Volatile compounds in the headspace of the metasternal glands of female and male *Triatoma infestans*

Peak	Compound ^a	Retention time of commercial standard	Females		Males	
			Mean retention time (min)	% of total sample	Mean retention time (min)	% of total sample
1	2-Butanone	2.60	2.61	0.5	2.61	0.4
2	3-Pentanone	3.24	3.24	76.5	3.24	84.7
3	3-Pentanol	4.66	4.60	1.7	4.65	1.5
4	Unknown	—	5.23	16.3	5.17	10.8
5	3-Hexanol	5.69	5.67	0.7	5.68	0.7
6	2-Methyl-1-butanol	5.82	5.81	4.3	5.82	1.9

^aThe mass spectrum of each gland compound was identical with that of the corresponding synthetic reference compound.

from the GC column makes it unlikely that the unknown compound has a molecular weight greater than 143. The hypothetical molecular formulas $C_9H_{21}N$ and $C_8H_{17}NO$ were considered and a few analogs of saturated amines and amides were synthesized. These compounds had very different mass spectra and eluted much later than the unknown compound.

Odors Emitted by Copulating Pairs of *T. infestans*

Volatile compounds were not detected before or during copulation by GC-MS analysis under full mass scan conditions. Nevertheless, SIM analysis mode revealed that 3-

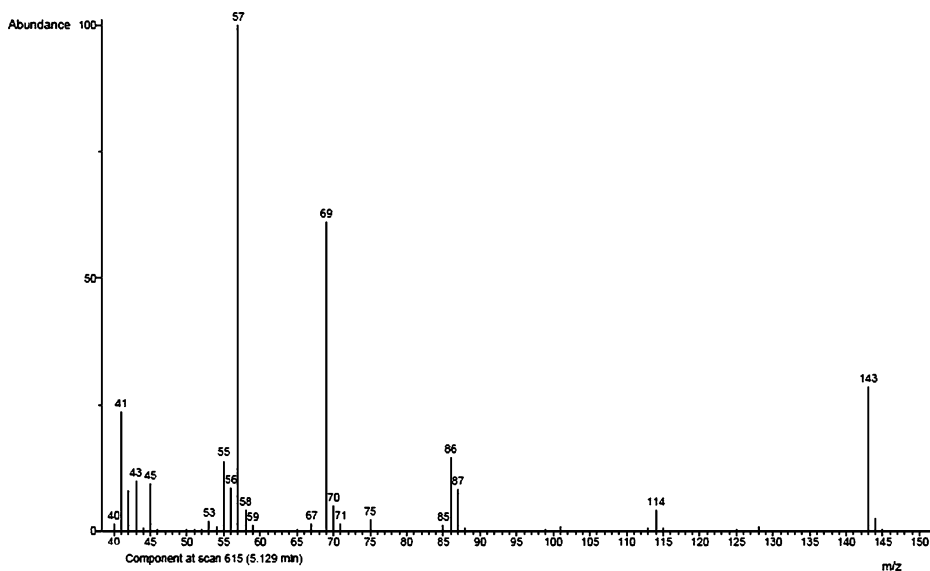


Fig. 3 Mass spectrum of the unidentified compound from *T. infestans* metasternal glands that eluted at approximately 5.20 min

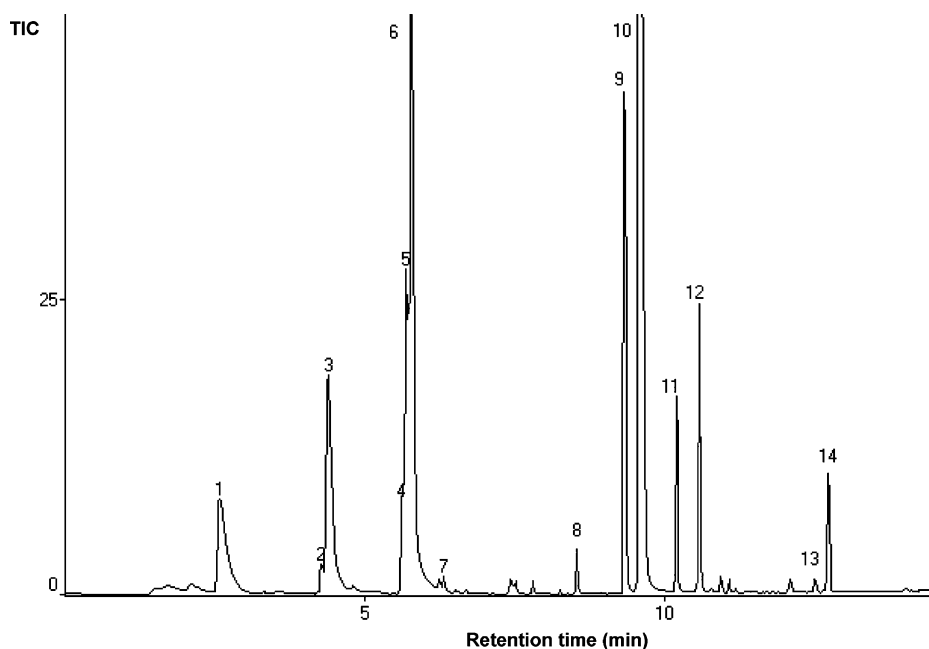


Fig. 4 Total ion chromatogram of the volatile compounds present in the headspace of Brindley's glands (pool of 10 glands) of *T. infestans* adults (see Table 2 for an explanation of the peak numbers)

pentanone was never found in control assays, but was in the headspace over one-third of the samples from copulating couples. A single small peak of isobutyric acid in one of these experiments was also detected, and it was much smaller than the corresponding 3-pentanone peak. Under our experimental conditions, the minimum detectable amount of isobutyric acid was 100 pg.

Table 2 Volatile compounds in the headspace of Brindley's glands of adult *Triatoma infestans*

Peak	Compound	Mean retention time (min)	% of total sample ^a
1	2-Butanone	2.59	5.7
2	Isobutyl propanoate ^b	4.29	0.8
3	Isobutyl alcohol	4.41	9.6
4	Unidentified ester ^b	5.63	1.7
5	Unidentified ester ^b	5.71	9.6
6	2-Methylbutyl and 3-methylbutyl alcohol	5.81	21.1
7	3-Methyl-3-buten-1-ol ^b	6.33	0.3
8	Acetic acid	8.55	0.5
9	Propionic acid	9.36	6.8
10	Isobutyric acid	9.61	35.3
11	Butyric acid	10.23	2.2
12	2-Methylbutyric acid	10.61	4.1
13	Phenylethyl isobutyrate	12.29	0.4
14	Phenylethyl alcohol	12.76	1.8

^a Mean values ($N = 4$) from two female and two male samples (10 glands pooled/sample) calculated from the area percent of each peak.

^b Compounds not identified by injection of synthetic standards.

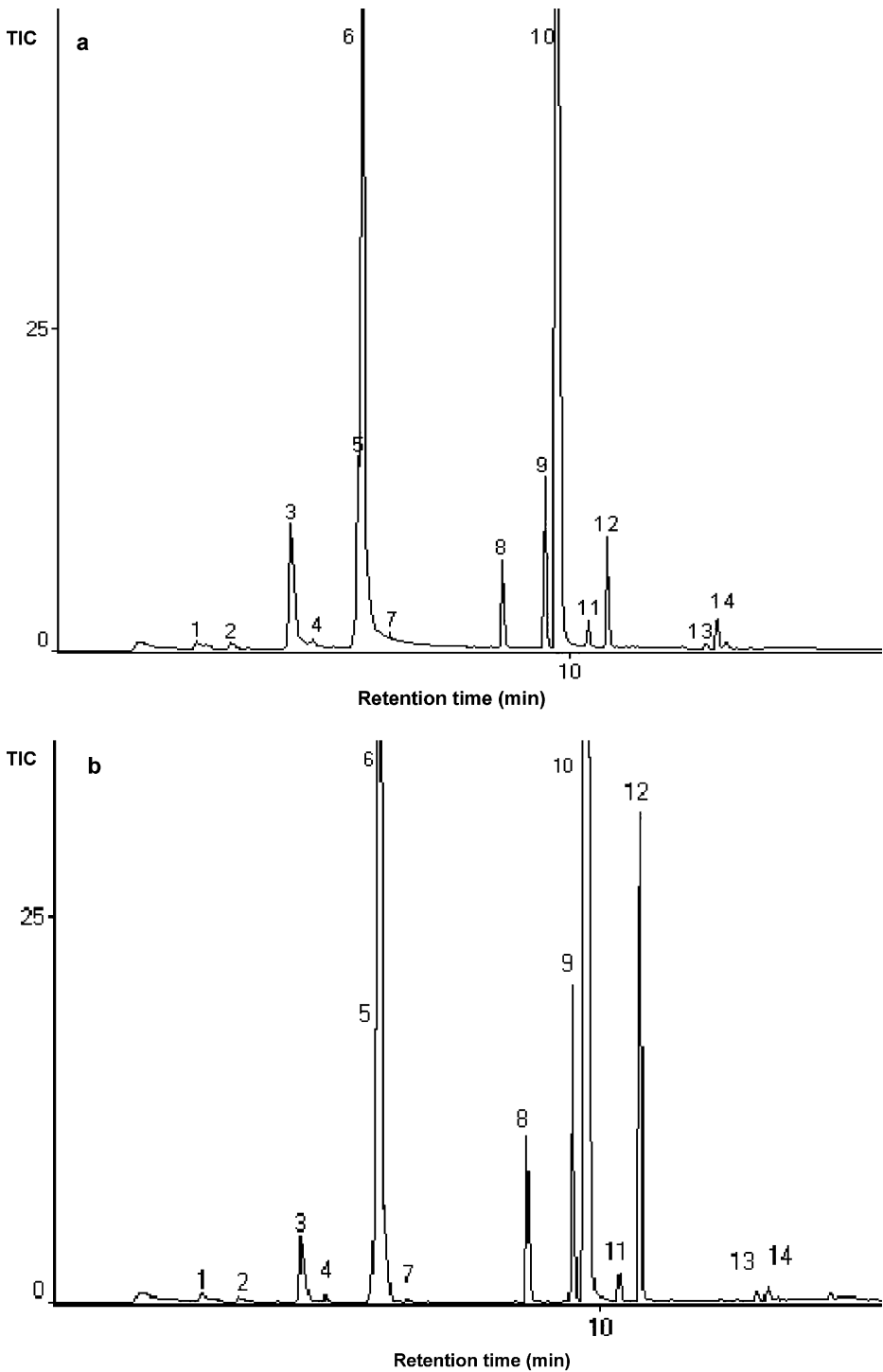


Fig. 5 Total ion chromatograms of the volatile compounds present in the headspace of mechanically disturbed *T. infestans* adult females (a) and males (b) (see Table 3 for an explanation of the peak numbers)

Secretion from Brindley's Glands

Compounds found in the headspace of vials containing excised Brindley's glands were primarily short chain acids, alcohols, esters, and a ketone (Fig. 4, Table 2). Isobutyric acid was the main component of this blend; 2-butanone and 2-methylbutyric acid are newly described from these glands. Two aromatic compounds (phenylethyl alcohol and phenylethyl isobutyrate) were also present at low levels. We did not find any qualitative differences between the sexes in the composition of the volatile blend (Table 2). Volatiles collected from pieces of cuticle and internal tissues (not shown) did not contain any of the compounds found in the headspace of glands, except for a peak related to traces of acetic acid (peak 8, Fig. 4).

Volatiles Released by Disturbed *T. infestans*

Compounds found in the headspace of disturbed *T. infestans* adults included short chain acids, alcohols, esters, and ketones (Fig. 5, Table 3). We did not find any qualitative differences between the sexes in the composition of the volatile blend. No compounds were detected in the control series with undisturbed insects or in the control vials with filter paper. Isobutyric acid (peak 10) was the main component of the mixture, comprising approx. 50% of the total peak area of all compounds in either sex (Table 3). 2-Butanone (peak 1), 3-pentanone (peak 2), 2-methylbutyl alcohol (peak 6), 3-methyl-3-buten-1-ol (peak 7), and 2-methylbutyric acid (peak 12) are reported here for the first time as volatiles emitted by *T. infestans*. Some of the compounds, possibly esters, released by disturbed bugs could not be identified.

Table 3 Volatile compounds in the headspace of disturbed female and male *Triatoma infestans*

Peak	Compound	Females		Males	
		Mean retention time (min)	% of total sample ^a	Mean retention time (min)	% of total sample ^a
1	2-Butanone	2.72	0.5	2.69	0.3
2	3-Pentanone	3.37	0.5	3.39	0.2
3	Isobutyl alcohol	4.54	7.0	4.53	2.6
4	Isobutyl isobutyrate	4.98	0.5	4.98	4.2
5	Amyl isobutyrate or isoamyl isobutyrate or 2-methylbutyl isobutyrate (unresolved peak) ^b	5.87	5.2	5.86	4.3
6	2-Methylbutyl and 3-methylbutyl alcohols	5.96	30.5	5.94	26.4
7	3-Methyl-3-buten-1-ol ^b	6.47	0.2	6.47	0.1
8	Acetic acid	8.69	1.5	8.67	2.1
9	Propionic acid	9.50	4.2	9.49	3.7
10	Isobutyric acid	9.72	46.1	9.71	50.4
11	Butyric acid	10.36	0.5	10.35	0.3
12	2-Methylbutyric acid	10.73	2.0	10.72	4.6
13	Phenylethyl isobutyrate	12.66	0.4	12.65	0.2
14	Phenylethyl alcohol	12.87	0.9	12.86	0.6

^a Mean values ($N = 6$) from three female and three male samples (two insects/sample) calculated from the area percent of each peak.

^b Compounds not identified by injection of synthetic standards.

Behavioral Responses of *T. infestans* Larvae to the Scent Emitted by Disturbed Adults

Assays of Locomotor Activity

When we analyzed the larval responses of *T. infestans* to volatiles from disturbed adults, we found that the locomotor activity of the bugs was affected by the treatment (ANOVA: $F = 19.12$, $df = 2,57$, $P < 0.001$). The activity level of bugs stimulated by the odor of disturbed adults was higher than the activity level of bugs exposed to the odor of undisturbed adults (Tukey–Kramer, $q = 4.06$, $df = 2,57$, $P = 0.016$) or of a clean vial (Tukey–Kramer, $q = 8.74$, $df = 2,57$, $P < 0.001$) (Fig. 6). Even though we did not detect volatile substances in the headspaces of undisturbed adults, the larvae also appeared to show significantly higher activity levels when adults were present than in clean-vial assays (Tukey–Kramer, $q = 4.68$, $df = 2,57$, $P = 0.047$).

Assays of Orientation Response

The behaviors of nonstimulated *T. infestans* larvae in the locomotion compensator did not show differences from the response expected by chance (Wilcoxon signed rank test: $W = 14$, $P = 0.8124$). This indicated that control animals spent an equivalent amount of time walking in both halves of the sphere. When larvae were exposed to volatiles released by

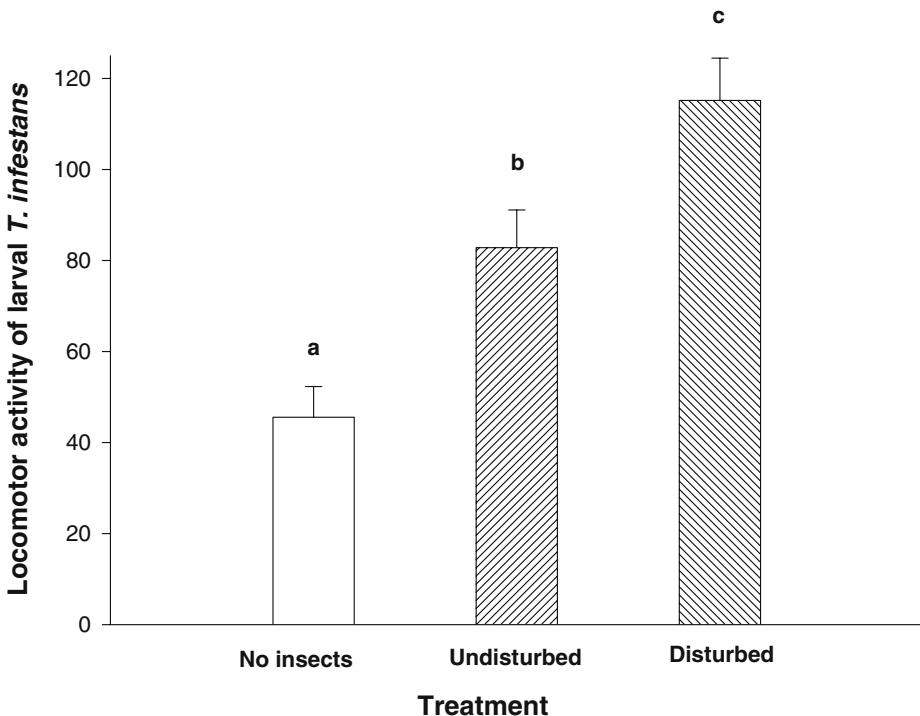


Fig. 6 Locomotor activity of *T. infestans* larvae stimulated with clean air, air from undisturbed *T. infestans* adults (ANOVA, $F = 19.12$, $df = 2,57$, $P < 0.001$). Pairwise comparisons were significant for all three pairs [(a, b) $P = 0.047$, no insects vs. undisturbed insects; (a, c) $P < 0.001$, no insects vs. disturbed insects; (b, c) $P = 0.016$, undisturbed insects vs. disturbed insects, $N = 20$]

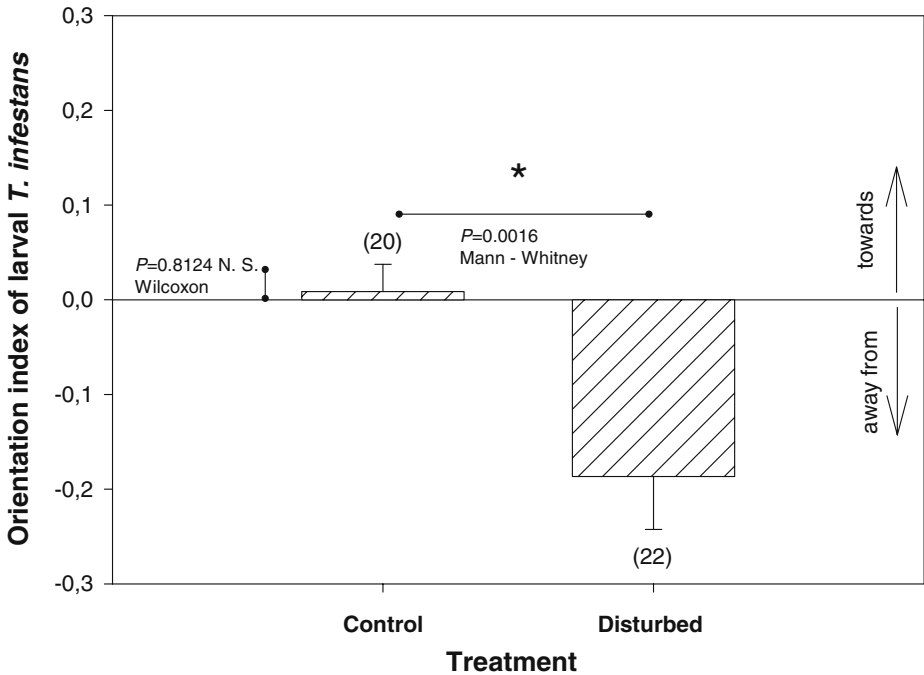


Fig. 7 Orientation response of *T. infestans* larvae exposed to volatiles from disturbed *T. infestans* adults on a locomotor compensator. Control series ($N = 20$): clean air vs. clean air. Test series ($N = 22$): odors from disturbed adults vs. clean air

disturbed adults, they walked away from the stimulus (Mann–Whitney, $U = 94$, $P = 0.0016$) (Fig. 7).

Discussion

We evaluated the origins and functions of chemicals produced by exocrine glands of *T. infestans*, and observed that these insects produce blends of odors that promote both nondirectional and directional responses, probably for avoidance of predators. We propose that the metasternal glands are the sources of a number of volatile substances, of which at least the main component of the secretion is apparently emitted during copulation.

Our data show that the metasternal gland of *T. infestans* is the source of a scent, whose main constituent is 3-pentanone. This compound has been reported as a male-emitted aggregation pheromone of at least two species of Coleoptera (Giblin-Davis et al., 1996; Rochat et al., 2000). Other detected compounds include the corresponding alcohol, 3-pentanol, and other aliphatic alcohols. No differences between males and females were found in the composition of the scent produced by these glands.

T. infestans males aggregate around mating pairs and this behavior supports the existence of a male aggregation pheromone in this species (Manrique and Lazzari, 1995). We detected 3-pentanone in one-third of the copulating pairs of *T. infestans*, and suggest that this emission may have a role in the communication of *T. infestans* in the context of sexual behavior. Fontán et al. (2002) reported the release of isobutyric acid in quantities of

about 250 ng by one pair of *T. infestans* during copulation, but we found this compound only in one of 15 copulation assays. In that case, the peak of isobutyric acid was small and only just above our detection threshold of approximately 100 pg of this substance. The results thus showed a more prominent emission of metasternal gland substances during the copulation of *T. infestans*. Aliphatic acids, alcohols, and aldehydes had also been reported as being released during copulation by *T. infestans* (Fontán et al., 2002), but we did not detect any aldehydes in the secretion from metasternal glands or Brindley's glands.

In some insect species, the same volatile substances are involved in different behavioral contexts (e.g., for defensive purposes and for sexual attraction; Blum, 1996). It has been suggested that the glands producing those compounds have evolved from nonsexual to sexual functions. Several vertebrates also emit isobutyric acid and related compounds produced in the Triatominae, so such compounds may also function as host-related kairomones (Guerenstein and Guerin, 2001). Therefore, increased activity after stimulation with a single synthetic compound, e.g., a short aliphatic acid or another vertebrate-related compound, may not necessarily be related to a sexual context.

The profile of compounds present in excised Brindley's glands matched that emitted by mechanically disturbed adult insects, suggesting that most (but not all) of the volatiles released by bugs during the disturbance came from these glands. No differences were found between males and females in the composition of the scent produced by these glands.

We found that the blend produced by Brindley's glands included most of the compounds described in previous reports. This might indicate that the previously conflicting results regarding the compositions of those secretions might have arisen as a result of differences in methodology. Therefore, we propose that the blend produced by Brindley's glands is complex, based primarily on isobutyric acid but includes other fatty acids, a ketone, several aliphatic alcohols, esters, and an aromatic alcohol and ester.

During disturbance, adult *T. infestans* of both sexes released a complex mixture of at least 14 different compounds (Table 3). 3-Pentanone, one of the compounds released after mechanical disturbance, was the main component of the metasternal glands of this species (Table 1), but it was not detected among the volatiles collected from excised Brindley's glands (Table 2). It remains unclear whether 3-pentanone and/or other compounds secreted by the metasternal glands of *T. infestans* are relevant in the alarm or defensive contexts.

In *T. infestans*, Cruz López et al., (1995) reported an unidentified chromatographic peak likely containing an unresolved mixture of amyl and isoamyl alcohols in samples from disturbed insects and from Brindley's glands. This might be related to our resolved peak 6, corresponding to 2-methylbutyl and 3-methylbutyl (isoamyl) alcohols. According to our results, amyl alcohol (*n*-pentan-1-ol) was not part of that peak, because its retention time in our chromatographic conditions differed from those of the other two alcohols (data not shown). In addition, the spectrum observed for that peak appeared to be determined by the binary mixture reported here. Moreover, isobutyric acid (peak 10) was not the only acidic component of the mixture (Cruz López et al., 1995; Guerenstein, 1999). In agreement with Hack et al., (1980) and Juárez and Brenner (1981), we found acetic (peak 8), propionic (peak 9) and butyric (peak 11) acids among volatile compounds emitted by disturbed insects. We also detected alcohols and esters in the mixture, as reported by Cruz López et al., (1995) and Guerenstein (1999). In this sense, the experimental protocol used here, allowing the detection of a wider spectrum of emitted substances, appeared to be more sensitive than those previously used for studies on triatomine secretions.

Differences in reports of the composition of volatiles emitted by disturbed insects and/or by excised Brindley's glands might be attributable to different methodologies used, e.g., volatile sampling techniques or chromatographic conditions. The use of different colonies

could also account for these discrepancies, suggesting that insects from diverse populations emit different blends of compounds. However, the fact that the profile of volatiles described here includes most of the compounds previously reported weakens the latter hypothesis.

Ward (1981) suggested that *T. infestans* releases isobutyric acid at high concentrations for defense and also for communication as an alarm pheromone. However, this suggestion was solely based on the quantification of avoidance responses from adult bugs to different concentrations of synthetic isobutyric acid. Kälén and Barrett (1975) found that stimulation with pure isobutyric acid dissolved aggregations of adult *R. prolixus*, but this was not observed when the animals were stimulated by volatiles released by disturbed adults. The same authors observed no response when fifth instars were stimulated with pure isobutyric acid, thus arguing that only adults might be alerted by this signal. In the present work, we quantified the effect of the naturally occurring blend of volatiles released by mechanically disturbed *T. infestans* adults on the behavior of larvae. Our experiments showed that odors coming from disturbed adults evoked a significant increase in the locomotor activity of larvae. Although we could not detect any compound from undisturbed adults using SPME (using full scan mode or SIM), the increase in locomotor activity of larvae to undisturbed adults was evident and significant. This suggests that undisturbed adult bugs also emit volatile substances that promote increased larval activity. The identity and function of these odors in the biology of *T. infestans* remains unclear.

The responses observed in the experiments that tested odors from disturbed bugs using the locomotor compensator revealed a directional escape behavior that would lead the insects away from the source (i.e., a tactic mechanism; Kennedy, 1977). The dual effect of the blend to (1) increase the activity of neighboring conspecifics, and (2) evoke repellency to lead the insects away from the source, provides additional evidence for the role of the blend of odors as an alarm pheromone for *T. infestans*. It is likely that these substances also have a defensive function, particularly the short chain fatty acids, because of their corrosive and probably toxic nature (Kälén and Barrett, 1975; Kanehisa and Kawazu, 1982; Hilker and Schulz, 1994). Isobutyric acid topically applied to *R. prolixus* larvae can cause what previous authors described as “paralysis” (Kälén and Barrett, 1975). It has also been shown that isobutyric and 2-methylbutyric acids function to deter predators in the caterpillar of *Papilio troilus* (Eisner, 2004).

It is worth emphasizing that, during disturbance, two pairs of exocrine glands (Brindley’s and metasternal glands) discharge their contents. It is likely that substances from different glands have different roles in the biology of these insects. Behavioral experiments to evaluate the responses of larvae stimulated by the scents emanating from disturbed adults with occluded metasternal or Brindley’s glands should improve our understanding of the exact functions of these glands. Future studies should also analyze the functions of the compounds produced by metasternal and Brindley’s glands and determine which odors, if any, are responsible for evoking the aggregation response of males around copulating pairs. Alternative potential sources of chemical signals in glandular areas described by Barth (1980) and Weirauch (2003, 2004) should also be considered in those studies.

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Introduction

Usnic acid is synthesized by a mycobiont (Bjerke and Dahl, 2002; Cocchietto et al., 2002) of several epiphytic lichens (Shibamoto and Wei, 1984), and has been found in species from a wide range of phylogenetically distant genera (Ingólfssdóttir, 2002). Lichens undergoing stressful climatic conditions [temperature, humidity, ultraviolet and photosynthetically active radiation (PAR), oxidative stress] synthesize large amounts of secondary metabolites, including usnic acid (BeGora and Fahselt, 2001; Caviglia et al., 2001; Bjerke et al., 2002; Toledo Marante et al., 2003). It may accumulate in the thallus, reaching up to 6% of dry mass (Proksa et al., 1996). The rhizines of lichens can penetrate tissues, and usnic acid may enter into vascular plants (Ascaso et al., 1980). Moreover, it can be lixiviated from the lichen thallus by rainwater on the plant surface or into the soil and taken up by leaves and roots, respectively (Dawson et al., 1984). Usnic acid is able to pass through the plasmalemma (Vavasseur et al., 1991). The physiological effects differ considerably, depending on the type of plant treatment (leaf, xylem, root, or intracellular feeding) (Vavasseur et al., 1991). Usnic acid has a pH-dependent toxic effect; its interaction with individual metabolic processes may be modified by the pH in the cell compartments (Bačkor et al., 1998; Gardner and Müller, 1981). Several studies support the allelopathic activity of usnic acid against different organisms (Gardner and Müller, 1981; Carbonnier, 1986; Lauterwein et al., 1995; Proksa et al., 1996; Ingólfssdóttir, 2002). Two natural enantiomers of usnic acid (“+” and “-”) differ in their biological activity and probably their mechanisms of action. In natural conditions, in some thalli more (+)- than (-)-enantiomer is produced (Huneck and Yoshimura, 1996; Kinoshita et al., 1997). A number of reports have documented the biological effects of both enantiomers. Romagni et al. (2000) tested both for phytotoxicity; (-)-usnic acid proved significantly more potent. The (+)-enantiomer has proved more effective as an antimicrobial agent (Ghione et al., 1988; Lauterwein et al., 1995), although no specific mode of action has been determined. The antimitotic effects of (+)-usnic acid on cultured plant cells and its ability to inhibit seed germination is known (Cardarelli et al., 1997). Long-term feeding of a micromolar concentration of (+)-usnic acid to roots retarded plant growth, altered morphological features, and reduced the accumulation of biomass and some elements (Lechowski et al., 2006). A (+)-usnic acid concentration greater than 40 μM led to death; the duration of survival depended on concentration in the medium. Maize and sunflower plantlets treated by root feeding with (+)-usnic acid for only 24 hr decreased photosynthesis and transpiration rates (Lascève and Gaugain, 1990).

Limiting studies on the long-term action of usnic acid have demonstrated phytotoxic effects on vascular plants similar to effects observed in nature. The literature provides some results on global long-term effect of allelochemicals synthesized by lichens on the physiology of some trees (Orús et al., 1981), but few systematic studies under controlled conditions have been reported. To date, all experiments on the effect of this allelochemical on plant metabolism have dealt with crops (lettuce, maize, spinach, tobacco, tomato) (Inoué et al., 1987; Lascève and Gaugain, 1990; Cardarelli et al., 1997; Romagni et al., 2000) or other plant species (*Commelina communis*, *Funaria hygrometrica*) (Gardner and Müller, 1981; Vavasseur et al., 1991) that will never be encountered in nature. Nonetheless, the results obtained by this route may be applicable to communities rich in lichens.

This article provides data on the effects of long-term treatment with (+)-usnic acid on a range of metabolic processes observable in plants. We used tomato plants because they are a genetically uniform material, their physiological processes are well known, and they are easy to cultivate under controlled conditions. Ecologically relevant physiological studies are needed on plants growing in lichen-rich vs. lichen-poor communities.

Methods and Materials

Plant Material

The experiments used tomato plants (*Lycopersicon esculentum* Mill. cv. Perkoz F₁). Within 10 d of seed germination (DAG), plants were transplanted to a chemostatic system for hydroponic cultivation previously described in detail (flow rate, aeration, and temperature of medium) by Bialczyk et al., 1994). Plants were cultivated in Hoagland media (pH 6.9 ± 0.5) containing 0–30 μM of (+)-usnic acid (Aldrich). The solubility of usnic acid depends on pH (Dawson et al., 1984), so the medium pH was monitored, kept constant, and corrected when necessary during cultivation. To maintain a constant concentration, the medium in the growth chamber (20 l, 25 plants) was changed every second day. During plant growth, usnic acid concentration of the media was assayed by HPLC analysis as described by Caviglia et al. (2001). Plants were cultivated for 21 d (from 10 to 31 DAG) in a glasshouse under a natural photoperiod, with maximum PAR at about 1000 μmol/m² sec and 70–75% relative humidity. Plant growth conditions and the method of preparing the media with (+)-usnic acid were previously described (Lechowski et al., 2006).

Chlorophylls and Carotenoids

Chlorophylls (chl) and carotenoids (car) were extracted from leaves with 80% aqueous solution of acetone (v/v). Quantitative determinations were carried out as described by Wellburn (1994). Results were expressed as pigment content per dry mass (DM) unit.

Photodegradation of Chlorophyll

High irradiation was applied to a detached leaf that was placed on damp filter paper and screened by black light-resistant foil, with the remaining 1 cm² exposed fragment subjected to irradiation. Radiation was provided by two 1000-W projector lamps (Tungsram) and concentrated by two independent optical systems adjusted so that the light spots (1 cm²) formed at the plane of the leaf surface were superimposed. Leaves were irradiated for 1–6 hr at constant PAR of 5000 μmol/m² sec, measured with a spectroradiometer (Li-1800; Li-Cor, USA). Infrared radiation emitted by the lamps was eliminated by passing through heat-eliminating cutoff filters (5 cm well of 3% aqueous solution of CuSO₄, and 1.5 mm BG-38 glass filter; Schott, Jena, Germany). The leaf surface temperature during irradiation was 25 ± 1°C as measured with a constantan–copper thermocouple. After irradiation, chlorophyll was extracted, and its concentration was determined as described above.

Gas Exchange

Gas exchange (photosynthesis, respiration, and transpiration) of fully expanded second leaves was monitored in situ with an open gas exchange system [model LCA 2 (ADC Ltd., Hoddesdon, UK) supplied with a PLC-B Parkinson Leaf Chamber] at 25°C, air flow of 300 ml/min, and CO₂ concentration in the air flow at 350 ± 8.2 μmol/mol with or without PAR of 1000 μmol/m² sec. Respiration was measured in the dark and transpiration in both the light and dark.

Water Use Efficiency

The water use efficiency (WUE) coefficient was calculated as the ratio of the net CO₂ assimilation rate and transpiration rate (Rawson et al., 1977).

Hill Reaction

The PS II activity of chloroplasts isolated from the leaf was expressed as the electron transport rate determined using 2,6-dichlorophenol indophenol (DCPIP) as the electron acceptor. Chloroplasts from leaves of the control plants were isolated in the cold as described by (Synková et al., 1997). The concentration of chlorophyll in the supernatant was determined as described above. The Hill reaction was followed in the assay medium having a final volume of 2 ml, with 50 μmol DCPIP, 40 μg chlorophyll, and 6 μl (+)-usnic acid in acetone, and 6 μl acetone without (+)-usnic acid for the control. The final concentration of usnic acid in the tested mixture ranged from 0 to 40 μM. Samples were irradiated with 1000 μmol/m² sec PAR for 3 min at 25°C. DCPIP photoreduction was assayed spectrophotometrically (Cary UV–Vis Varian Bio 50 spectrophotometer) at 590 nm.

Stomata Resistances and Density

The diffusive resistance of stomata (r_s) was measured for water vapor on both surfaces of the leaves with an automatic porometer (Delta-T, A4, UK). The density of stomata on both surfaces (number of stomata per unit area) of the second leaves was estimated by microscopic examination of cellulose acetate imprints. Leaf disks 0.5 cm diam were pressed onto acetone-soaked cellulose acetate sheets supported on a glass slide. The epidermal imprint was made halfway between the base and tip of the leaves, and halfway between the midrib and edge of the leaflets. After several minutes, leaf disks were gently removed after the cellulose acetate sheet had dried. Stomata density per unit area of leaf was counted under a microscope with a micrometric scale.

Root Hydraulic Conductance

These experiments used roots of plants cultivated for 21 d in media with different concentrations of (+)-usnic acid (A), or roots of control plants transplanted for 5 hr to media with different concentrations of (+)-usnic acid (B). Root hydraulic conductance (RHC) [L_p , ml H₂O/MPa hr g root fresh mass (FM)] was calculated with Fiscus's formula (Fiscus, 1975): $L_p = J_v / [\alpha(P_x - P_e)]$, where J_v is the water flux (ml/hr), α is the reflection coefficient, and P_x and P_e are the pressure (MPa) of the xylem exudate and external solution, respectively. For calculation of L_p , α was set to 1. The J_v rate was calculated from the quotient of the collected volume of xylem exudate and the collecting time. For measurements of J_v , plant shoots were removed 3 cm above the root. The phloem was removed from 2 cm of the stem length. Next, the stem was wiped off with a damp paper towel to avoid contamination with phloem exudate and wounded cells. After 20 min the xylem exudate was collected with a Pasteur pipette for 30 min (storage on ice) and subsequently stored at -20°C. J_v and P_x were determined in the light phase of the diurnal cycle. At the end of the experiments, roots were completely drained on filter paper and FM was determined.

Osmotic and Water Potential

The osmotic potentials (ψ_o) of the xylem exudate, the nutrient solutions, and the cell sap of the leaf or roots were determined with a dew-point microvoltmeter. Cell sap of the leaf or roots was squeezed onto filter paper for ψ_o measurements. Water potential (ψ_w) was measured in leaf disks (7 mm diam) or root pieces (30 mg FM). Plant materials for determining ψ_o or ψ_w were placed in the sample chambers of a Wescor C-52 thermocouple psychrometer for 1 hr and then equilibrated with a dew-point microvoltmeter (model HR-

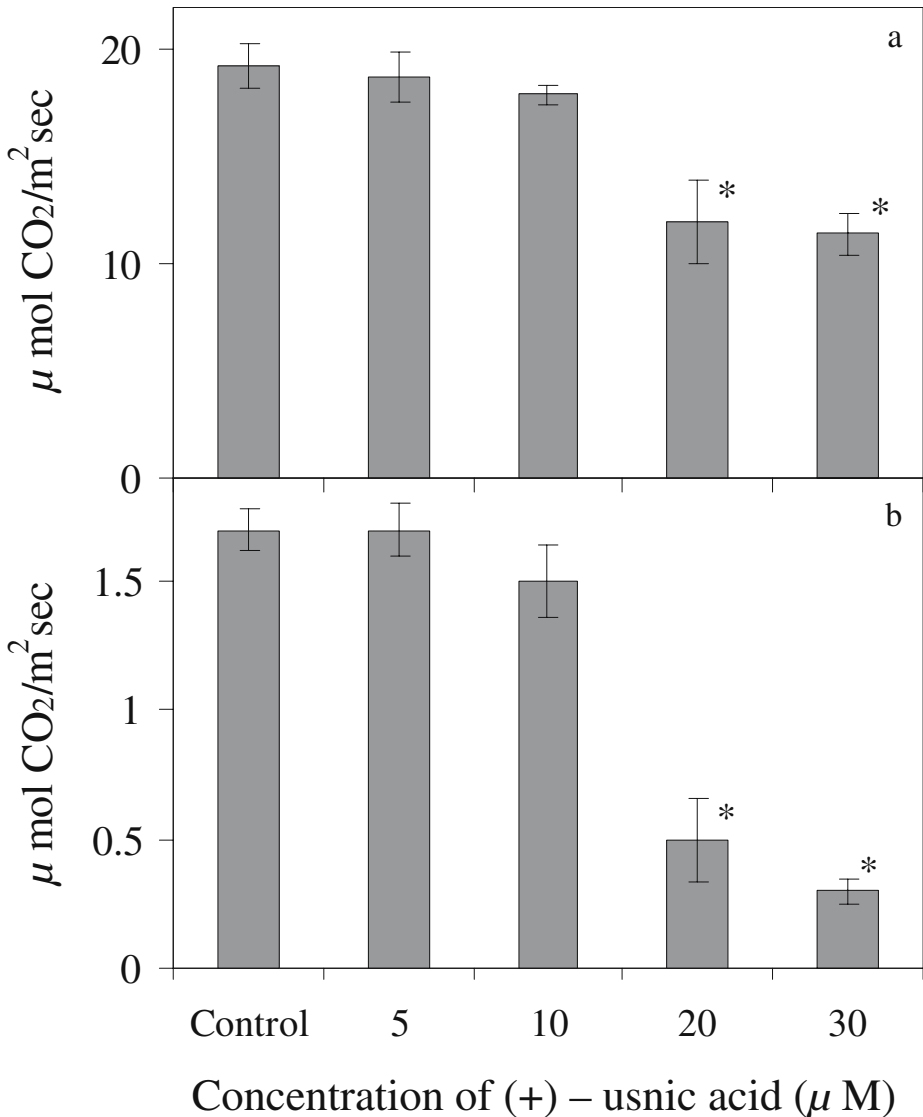


Fig. 1 Photosynthesis (a) and respiration (b) rates of leaves of tomato plants cultivated 21 d (10–31 DAG) in media with different concentrations of (+)-usnic acid. Values are means of five replicates. Bars represent \pm SE. *Significant difference from the control at $P < 0.05$

33T, Wescor, USA) operated in the dew-point hygrometric mode, and recorded when the instrument readings were stable. A solution of 55 mM NaCl of known water potential (-2.5 MPa at 25°C) was routinely used to verify the calibration of the individual chamber.

Statistical Analyses

The reported data are averages of five replicates. The standard errors (SE) of the means were determined. All obtained results were subjected to one-way ANOVA, and the means were tested for significant differences ($P < 0.05$) with Tukey's test.

Results

Gas Exchange

Tomato plants cultivated long-term in media containing (+)-usnic acid had lower photosynthetic activity (Fig. 1a). The degree of that decrease depended on the (+)-usnic acid concentration in the growth media. At ≤ 10 μM concentration, the reduction was within the margin of statistical error ($<7\%$). The rate photosynthesis in media containing 20 or 30 μM (+)-usnic acid declined 38% or 41% vs. control, respectively. Respiration was inhibited by usnic acid more than photosynthesis was. The respiration rate was reduced by 70% and 80% vs. control in plants cultivated in media with 20 and 30 μM usnic acid, respectively (Fig. 1b).

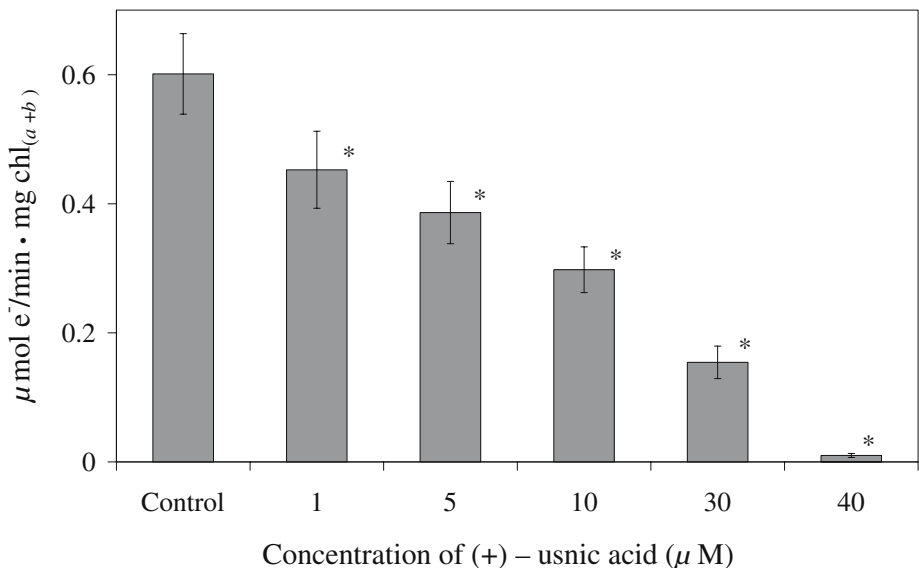


Fig. 2 Hill reaction activity of chloroplasts isolated from tomato leaves and incubated with different concentrations of (+)-usnic acid. Values are means of five replicates. Bars represent \pm SE. *Significant difference from the control at $P < 0.05$

Table 1 Chlorophyll and carotenoid content in leaves of tomato plants cultivated 21 d (10–31 DAG) in media containing different concentrations of (+)-usnic acid^a

Concentration of usnic acid (μM)	Photosynthetic pigments (mg/g dry mass of leaves)				Chl _a /Chl _b	Chl _{tot} /Car
	Chl _{tot}	Chl _a	Chl _b	Car		
0 (control)	19.54 ± 0.83	15.20 ± 0.69	4.34 ± 0.31	4.51 ± 0.17	3.50 ± 0.24	4.33 ± 0.21
5	15.60 ± 1.04*	11.77 ± 0.75*	3.83 ± 0.05	3.41 ± 0.21*	3.07 ± 0.15	4.57 ± 0.14
10	15.26 ± 0.52*	11.43 ± 0.37*	3.83 ± 0.18	3.15 ± 0.19*	2.98 ± 0.10*	4.84 ± 0.23
20	14.31 ± 0.40*	10.62 ± 0.40*	3.69 ± 0.43*	3.08 ± 0.05*	2.88 ± 0.15*	4.65 ± 0.35
30	13.65 ± 0.76*	10.12 ± 0.35*	3.53 ± 0.34*	2.93 ± 0.06*	2.87 ± 0.16*	4.66 ± 0.23

^a Values are means of five replicates ±SE.

*Significant difference from the control at *P* < 0.05.

Hill Reaction

The Hill reaction activity of isolated chloroplasts depended on the concentration of (+)-usnic acid in the reaction mixture (Fig. 2). With 1 μM (+)-usnic acid it fell by 25% vs. control. Increases in concentration gradually reduced it further until complete inhibition of the Hill reaction occurred in the 40 μM treatment.

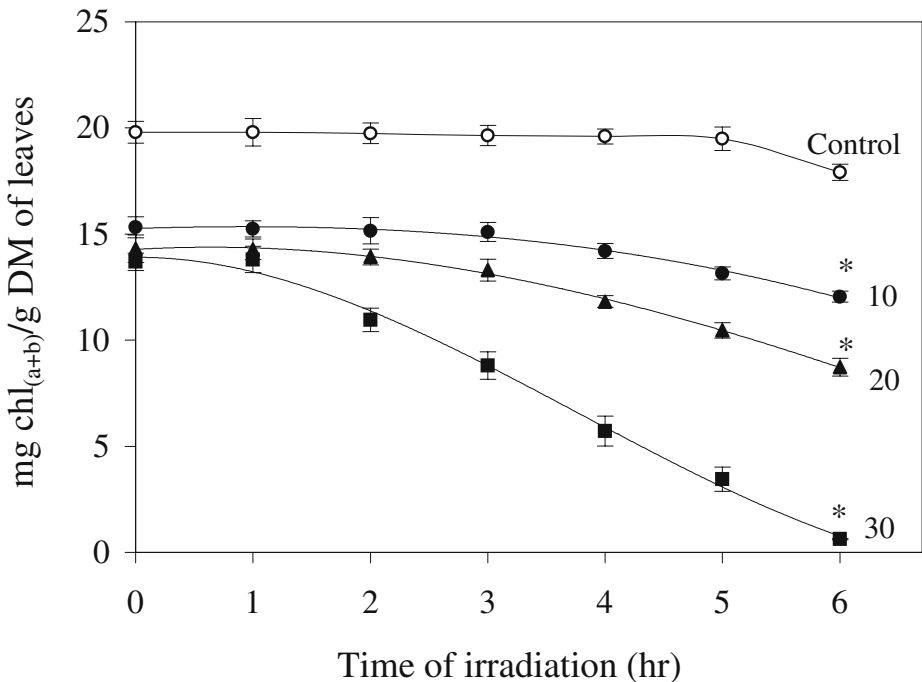


Fig. 3 Kinetics of chlorophyll photodegradation in tomato leaves irradiated with high-intensity PAR (5000 μmol/m² sec). Numbers at the ends of the curves represent concentrations of (+)-usnic acid (μM) in the growth medium. Values are means of five replicates. Bars represent ± SE. *Significant difference from the control at *P* < 0.05

Chlorophyll and Carotenoid Content

Results of the chlorophyll and carotenoid assays in tomato leaves are presented in Table 1. Generally, long-term cultivation of plants in media containing (+)-usnic acid led to reduction of photosynthetic pigment content. In leaves of plants cultivated in media with 30 μM (+)-usnic acid, chl_{tot} , chl_a , chl_b , and car content decreased vs. control by 30%, 33%, 18%, and 35%, respectively. The depressive effect of 30 μM (+)-usnic acid concentration on chl_a accumulation was 1.67 times stronger than its effect on chl_b accumulation; the $\text{chl}_a/\text{chl}_b$ ratio fell from 3.5 (control) to 2.87. Under these cultivation conditions, the $\text{chl}_{\text{tot}}/\text{car}$ ratio increased from 4.33 (control) to 4.66. This result indicates that usnic acid had a greater depressive effect on carotenoid than on chlorophyll accumulation.

Photodegradation of Chlorophyll

As presented in Fig. 3, photodegradation of chlorophyll was dependent on irradiation time. The lag phase and chlorophyll degradation phase were reduced in leaves of plants cultivated in media with 30 μM (+)-usnic acid; the lag phase was only 1 hr, and chlorophyll content declined more or less linearly to the sixth hour (chlorophyll content declined to less than 5% of the initial value). Leaves of the control plants were more resistant to high-intensity PAR. The lag phase in the control leaves was 5 hr; after that time the chlorophyll photodegradation phase began, and after 6 hr irradiation, the chlorophyll content decreased by 10% of the initial value. Chlorophyll content of detached leaves left under growth irradiance did not change significantly over the duration of the experiment (data not shown).

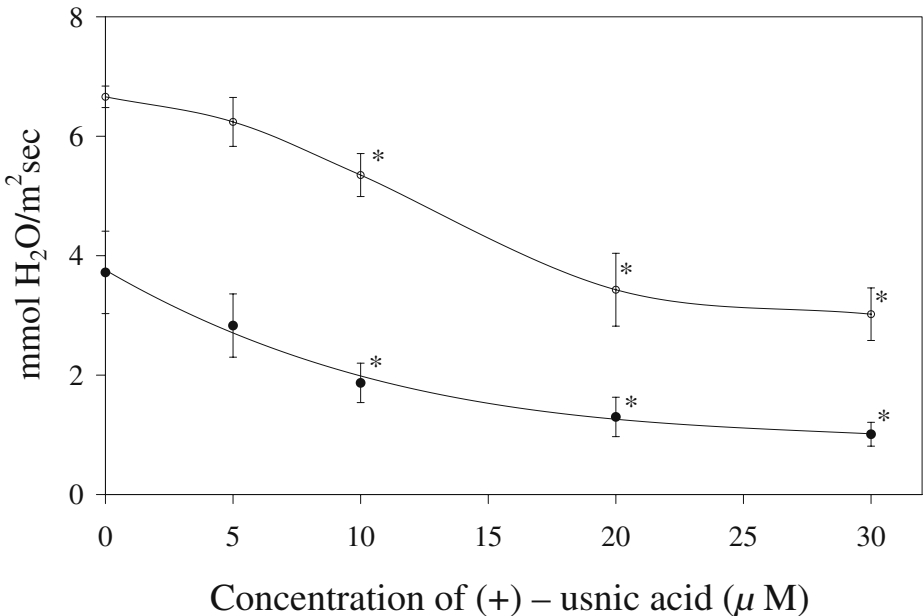


Fig. 4 Transpiration rate in light (open symbols) and dark (close symbols) of leaves of tomato plants cultivated 21 d (10–31 DAG) in media with different concentrations of (+)-usnic acid. Values are means of five replicates. Bars represent \pm SE. *Significant difference from the control at $P < 0.05$

Table 2 WUE coefficients of tomato plants cultivated 21 d (10–31 DAG) in media containing different concentrations of (+)-usnic acid^a

Concentration of usnic acid (μM)	WUE ($\text{mmol CO}_2/\text{mol H}_2\text{O}$)
0 (control)	2.97 ± 0.23
5	3.08 ± 0.28
10	3.51 ± 0.20
20	$3.86 \pm 0.14^*$
30	$3.98 \pm 0.22^*$

^a Values are means of five replicates \pm SE.

*Significant difference from the control at $P < 0.05$.

Transpiration and Water Use Efficiency

The rate of leaf blade transpiration gradually dropped as (+)-usnic acid concentration in the growth media increased (Fig. 4). Compared with the control, the transpiration rate of plants cultivated in media containing $30 \mu\text{M}$ (+)-usnic acid was 2.1-fold lower in the light and 3.7-fold lower in the dark. The calculated WUE coefficients (Table 2) correlated positively with the (+)-usnic acid concentration in the medium during plant cultivation. Plants cultivated in media with $30 \mu\text{M}$ (+)-usnic acid had a WUE coefficient 1.5 times higher than in the control.

Stomatal Characteristics

Plants cultivated for 21 d in media containing $\leq 10 \mu\text{M}$ (+)-usnic acid did not differ significantly from the control in the diffusive resistance of their stomata (Fig. 5). Increasing

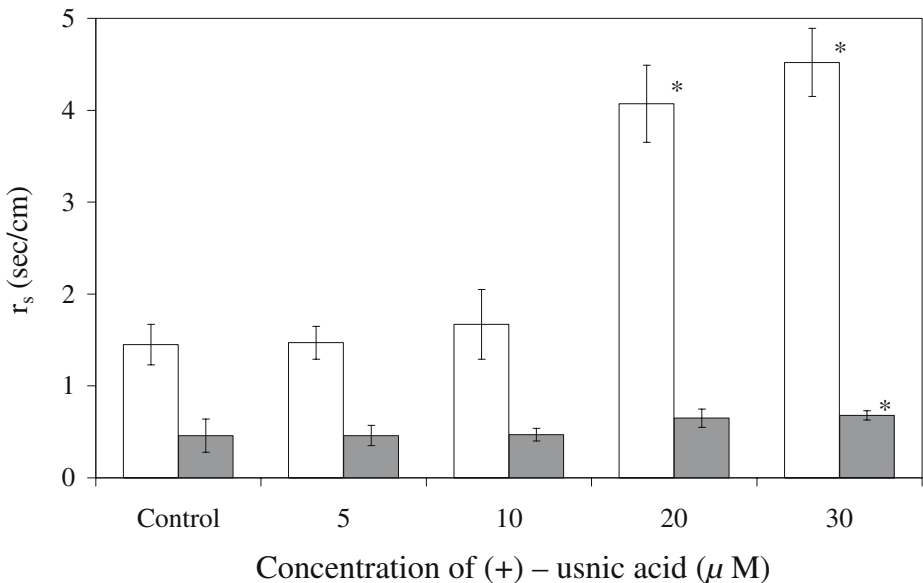


Fig. 5 Stomatal diffusive resistance (r_s) for the water vapor on upper (white column) and lower (gray column) surfaces of the leaves of tomato plants cultivated for 21 d (10–31 DAG) in media with different concentrations of (+)-usnic acid. Values are means of five replicates. Bars represent \pm SE. *Significant difference from the control at $P < 0.05$

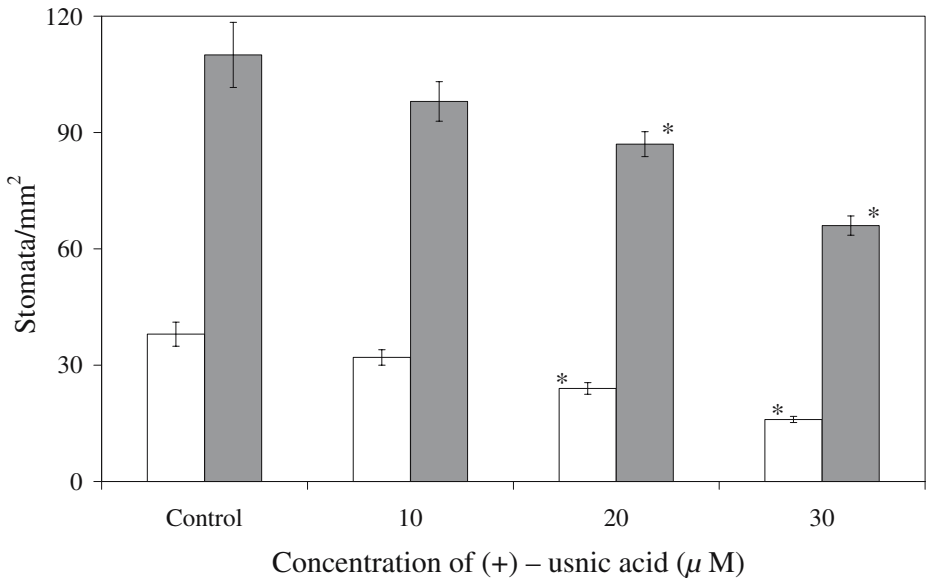


Fig. 6 Stomata densities on upper (white column) and lower (gray column) surface of leaves of tomato plants cultivated for 21 days (10–31 DAG) in media with different concentrations of (+)-usnic acid. Values are means of five replicates. Bars represent \pm SE. *Significant difference from the control at $P < 0.05$

its concentration in the growth media caused a rise in r_s and in the r_s ratio of the upper/lower surfaces of leaves. In plants cultivated in media with 30 μM (+)-usnic acid, the r_s of the upper surface of leaves increased by 3.1-fold, and 1.5-fold for the lower surface, and the r_s ratio of the upper/lower surfaces of leaves increased from 3.1 in the control to 6.6. The increase in r_s in leaves of plants cultivated in media with (+)-usnic acid might be a result of the decline in the density of stomata per surface unit as its concentration in the growth media increased (Fig. 6). The stomatal densities of plants cultivated in media containing 30 μM (+)-usnic acid were 60% and 40% lower than the control on the upper and lower surfaces, respectively, and the ratio of stomata frequency on the upper/lower surfaces decreased from 0.34 (control) to 0.21. As the concentration of (+)-usnic acid in the growth media rose, root hydraulic conductance decreased (Table 3); by limiting water transport to the leaf, this could affect r_s and consequently lower the transpiration rate.

Table 3 Effect of (+)-usnic acid concentration on hydraulic conductance of tomato roots^a

Concentration of usnic acid (μM)	A	B
0 (control)	25.67 \pm 1.2	25.67 \pm 1.2
5	15.07 \pm 0.8*	21.05 \pm 1.0
10	8.21 \pm 0.4*	16.43 \pm 1.1*
20	4.12 \pm 0.2*	11.88 \pm 0.8*
30	2.08 \pm 0.1*	8.47 \pm 0.5*

A: Plants cultivated for 21 d (10–31 DAG) in media with different concentrations of (+)-usnic acid; B: control plants incubated for 5 hr in media with different concentrations of (+)-usnic acid.

Values are means of five replicates \pm SE. *Significant difference from the control at $P < 0.05$.

^a ml $\text{H}_2\text{O}/\text{MPa hr g}$ fresh mass.

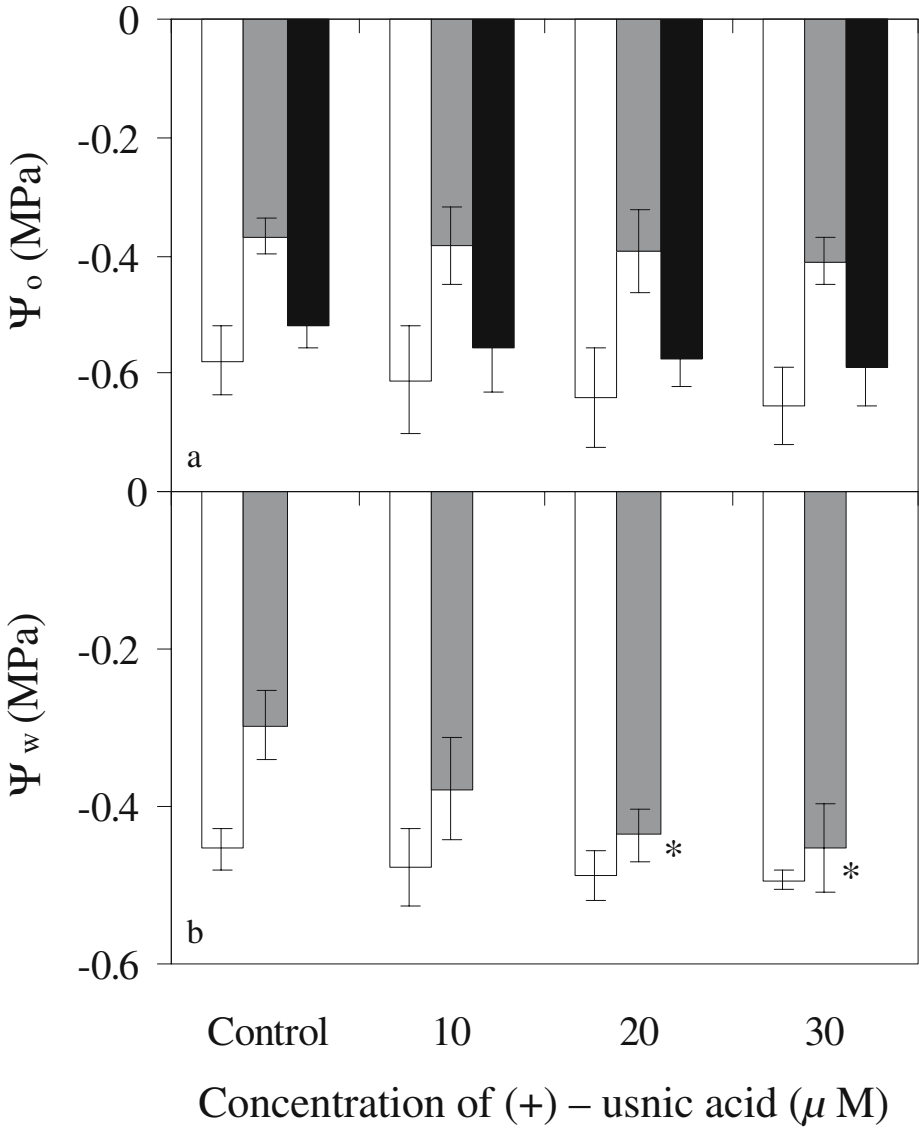


Fig. 7 Osmotic potential ψ_o (a) and water potential ψ_w (b) of tomato plants cultivated for 21 d (10–31 DAG) in media with different concentrations of (+)-usnic acid; leaves (white column), roots (gray column), and xylem exudate (black column). Values are means of five replicates. Bars represent \pm SE. *Significant difference from the control at $P < 0.05$

Osmotic and Water Potential

In roots, xylem exudate, and leaves, ψ_o did not decline significantly as the (+)-usnic acid concentration in the growth media increased (Fig. 7a). The maximum difference between ψ_o determined for the organs of control plants and of plants cultivated in media with 30 μ M (+)-usnic acid was 13%. In contrast to ψ_o , ψ_w decreased from control levels by 52% in roots and 9% in leaves of plants cultivated in media with 30 μ M (+)-usnic acid (Fig. 7b).

Root Hydraulic Conductance

RHC decreased in both the long-term (21 d) and short-term (5 hr) treatments with (+)-usnic acid. In both cases, the fall in RHC was drastic, and the results were proportional to the concentration of (+)-usnic acid in the media (Table 3). In the treatment with 30 μM (+)-usnic acid, the RHC value fell from control levels by 12.3-fold for long-term and 3-fold for short-term exposure.

Discussion

Usnic acid exerts a multidirectional toxic effect on metabolic processes in plants (Cardarelli et al., 1997; Romagni et al., 2000; Cocchietto et al., 2002; Lechowski et al., 2006), which is likely associated with its action on key enzymes (Vavasseur et al., 1991). The lower content of photosynthetic pigments in the leaves treated with usnic acid (Table 1) may be the result of depressed synthesis and/or increased susceptibility to degradative processes. Romagni et al. (2000) found that (–)-usnic acid irreversibly inhibited protoporphyrinogen oxidase IX activity, which catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX in the chlorophyll biosynthesis pathway. It is also a strong inhibitor of enzymes of the carotenoid biosynthesis pathway: phytoene desaturase, which converts phytoene to carotenoids (Romagni et al., 2004), and/or 4-hydroxyphenylpyruvate dioxygenase-catalyzed synthesis of plastoquinone, which activates phytoene desaturase (Endo et al., 1994). Photodegradation of chlorophyll by irradiation with our very-high-intensity PAR was significantly accelerated in plants cultivated in media with (+)-usnic acid, via shortening of both the lag and the photooxidation phases (Fig. 3). The duration of the lag phase of chlorophyll photodegradation reflects the action of all the protective mechanisms responsible for photostabilization of the photosynthetic apparatus, which ensure the undisturbed functioning of the electron transport chain (Lechowski and Bialczyk, 1990; Osmond, 1994). The effects of the interaction of these two stresses, (+)-usnic acid and high irradiance, suggest that usnic acid can predispose chlorophyll to photoinhibitory damage by: (1) inhibiting electron flow in thylacoid membranes by chelating Mn^{2+} ions and disturbing the electron flow from the splitting of water to PS II (Inoué et al., 1987; Cardarelli et al., 1997); (2) binding to the electron transport component at the oxidation side of PS II (Inoué et al., 1987); (3) reducing the content of carotenoids, which quench excess excitation energy from chlorophyll in its triplet state and release it in a nonradiative way (Müller et al., 2001); and (4) causing the formation of a highly reactive singlet oxygen, resulting in destabilization of thylacoid membranes (Romagni et al., 2004).

The leaves of plants fed (+)-usnic acid had reduced water content and more dry matter in the leaf blade, largely the result of an increase in the share of cell wall structural elements (Lechowski et al., 2006). The decrease in CO_2 diffusion to carboxylation centers (Fig. 5) and the water loss (Fig. 4) in plants treated with (+)-usnic acid may be attributable to increased r_s resulting from narrowing of the stomatal aperture and also to the lower density of stomata on the leaf (Fig. 6). Stomata closure induced by (+)-usnic acid may take the form of a direct disturbance of guard cell physiology when (+)-usnic acid is applied to the leaf surface (Lascève and Gaugain, 1990), or a secondary effect on water transport when plants are fed via roots. In both cases, the guard cells lose turgor and the stomatal aperture narrows. The direct effect of usnic acid on the guard cells that induce stomatal closure may be attributable to one or more of the following: (1) chelation of K^+ ions or ionophoresis towards K^+ ions (Carbonnier, 1986), which are basic components of the osmotic potential;

(2) increased membrane permeability (Bačkor et al., 1997) and leakage of electrolytes from cells under light (Romagni et al., 2004); and (3) reduction of the ATP pool when photosynthetic electron transport is disrupted (Orús et al., 1981; Inoué et al., 1987; Cardarelli et al., 1997), respiration is diminished (Fig. 1b) and oxidative phosphorylation is decoupled (Abo-Khatwa et al., 1996; Vavasseur et al., 1991).

Both long- and short-term feeding with (+)-usnic acid via roots dramatically reduced RHC (Table 3), the water transpiration rate (Fig. 4), and turgor (Lechowski et al., 2006). The mechanism of this interaction is not yet fully explained. One reason might be strong reduction of root growth (Lechowski et al., 2006) and the consequent decrease in water absorption surface. As illustrated in the present experiments, the decline in RHC (Table 3) does not result from the lack of a water potential gradient on the water transport route (Fig. 7), but more likely from changes in cell membrane permeability (Bačkor et al., 1997; Romagni et al., 2004). As illustrated by the calculated WUE coefficients (Table 2), restriction of the effective supply of water by usnic acid, and reduction of transpiration, led to more efficient use of water. This result agrees with earlier data from (Lascève and Gaugain, 1990). The mode of action of usnic acid remains unknown, and the explanations put forward remain speculative.

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Anthraquinones and anthrones also have antimicrobial and antiviral activity (Teuscher and Lindequist, 1994, and references therein).

A galerucine species intensively investigated with respect to the presence of anthraquinones is the tansy leaf beetle, *Galeruca tanacetii* L. (Coleoptera: Chrysomelidae), which deposits eggs in clutches of about 65 eggs in autumn on dry plant material. These clutches overwinter on the plants until larvae hatch in spring (April–May) (Obermaier et al., 2006, and references therein). Despite the presence of anthraquinones and anthrones, freshly laid eggs of *G. tanacetii* are frequently parasitized by *Oomyzus galerucivorus* (Hymenoptera: Eulophidae).

The host plants of *G. tanacetii* (mainly yarrow and tansy) do not contain anthraquinones and anthrones (Hilker and Schulz, 1991). Thus, these polyketides must be produced in the beetles. When studying the biosynthesis of these defensive components in *G. tanacetii*, we hypothesized that: (1) *G. tanacetii* is able to produce anthraquinones and its precursors by its own specific enzymes, and/or (2) endosymbionts associated with the beetle produce these components.

This study addressed the question whether anthraquinones and anthrones are actively produced within the eggs. Two motifs drove our study: (1) On one hand, knowledge of the site of anthraquinone biosynthesis in *G. tanacetii* may help elucidate whether the producer is *G. tanacetii* itself or a symbiotic microorganism. If anthraquinones are actively produced inside the eggs, mRNA coding for polyketide synthases involved in the biosynthesis of 1,8-dihydroxylated anthraquinones, for example, may specifically be searched for at this site. (2) On the other hand, we are not aware of any previous investigation that has analyzed whether the defensive products change during egg development. Knowledge of changes in quantities of such products during egg development will provide clues to the activity of the embryo and its potential to contribute to egg defense. Thus, we determined the quantities of anthraquinones and anthrones shortly after egg deposition and shortly prior to larval hatching. An increase in anthraquinone content would indicate an active anthraquinone biosynthesis within the eggs. Metabolization of the substances by the embryo would be indicated by a decrease in total anthraquinone and anthrone contents.

Insect eggs are known to respond to microbial attack via induction of several enzymes (Gorman et al., 2004). To elucidate whether parasitization of *G. tanacetii* eggs by the wasp *O. galerucivorus* affects the anthraquinone content and induces/suppresses anthraquinone production, parasitized eggs were also included in our measurements.

Methods and Materials

Insects

Egg clutches of *G. tanacetii* were collected at the end of October (autumn) and the end of March (spring) in the nature conservation area “Hohe Wann”, Lower Franconia, Germany. Each clutch was divided into two parts: one was directly frozen and stored at -80°C , and the other was kept in glass vials at 25°C , 18:6 hr (L:D) cycles. When parasitoids emerged from the latter, the clutch was labeled “parasitized”. When only beetle larvae emerged, the clutch was denoted “unparasitized”. Thirty unparasitized and 17 parasitized egg clutches were analyzed.

Sample Preparation

The anthraquinone-free extrachorion (Meiners et al., 1997) was removed from the eggs, and 20 eggs of a clutch were used per sample. While anthraquinones found in microorganisms

and marine animals are present as free substances, plants contain anthraquinones mainly bound to glycosides (Teuscher and Lindequist, 1994). Because we cannot exclude bindings of anthraquinones in *G. tanacetii*, the egg extract was subjected to acidic hydrolysis in order to measure the total anthraquinone and anthrone quantities. Twenty eggs of an egg clutch were homogenized in 100 μ l 1 N HCL in acetone, and this mixture was incubated at room temperature for 24 hr. This method had been shown to completely hydrolyze glycosidically bound anthraquinones (Derksen et al., 2003). To measure the nonconjugated, free anthraquinones, 20 eggs of an egg clutch were homogenized in 100 μ l acetone with 1% acetic acid and also incubated at room temperature for 24 hr.

All samples were centrifuged at 13,000 rpm for 1 min. The supernatant was applied onto an SI column (Isolute[®]Spe columns: 100 mg, 3 ml; IST, Mid Glamorgan, UK), equilibrated with 100 μ l acetone prior to application. A first elution was made with 250 μ l acetone. Before the second elution step with 250 μ l acetone, 25 μ l of the internal standard (2-hydroxy-methyl-anthraquinone; 0.1 mg/ml; Aldrich, Steinheim, Germany) were applied to the column. Two final elution steps were made with 250 μ l methanol each. Twenty μ l of the eluate were evaporated with N₂. The dried sample was resolved in 20 μ l BSTFA (BSTFA + TMCS, 99:1; Supelco, Bellefonte, PA, USA) and incubated for 1 hr at 80°C.

GC-MS Analysis

One μ l per sample was analyzed by coupled gas chromatography-mass spectroscopy (GC-MS) (Fisons model 8060 GC coupled to an MD 800 quadrupole mass spectrometer) (Thermo Finnigan, Egelsbach, Germany) under the following conditions: injector temperature, 240°C; column, DB-1 fused silica, 30 m \times 0.32 mm i.d.; film thickness, 0.25 μ m (J & W Scientific, Folsom, CA, USA); carrier gas, helium with inlet pressure 10 kPa; temperature program, 100°C for 4 min, increase in temperature with a rate of 10°C/min to 280°C; solvent delay, 10 min. Measurements were conducted in the single ion mode (SIM) with the following *m/z* values of silanized anthraquinones (chrysazin: *m/z* 369, chrysophanol: *m/z* 383), anthrones [dithranol: *m/z* 442 (all from Aldrich, Steinheim, Germany), chrysarobin: *m/z* 456 (gift from Alfred Köpf, Zürich, Switzerland)], and the internal standard (2-hydroxy-methyl-anthraquinone: *m/z* 279; Aldrich, Steinheim, Germany). For quantification of the anthraquinones and anthrones, relative peak areas were calculated as a quotient of the peak area of the substance and of the internal standard. Calibration curves were obtained by injection of standard substances under the conditions described above.

Table 1a Comparison of anthraquinone and anthrone contents in unparasitized eggs

Eggs ^a	Free AQ ^b				AQ total (hydrolyzed) ^b			
	Chrysarobin	Dithranol	Chrysophanol	Chrysazin	Chrysarobin	Dithranol	Chrysophanol	Chrysazin
Before	508.64	0.42	220.12	5.99	899.19	37.65	441.94	14.06
winter	(\pm 219.65)	(\pm 0.30)	(\pm 170.96)	(\pm 3.99)	(\pm 281.83)	(\pm 31.65)	(\pm 213.91)	(\pm 9.25)
After	337.74	0.32	213.04	4.74	897.78	13.06	309.71	7.38
winter	(\pm 183.87)	(\pm 0.36)	(\pm 59.18)	(\pm 2.08)	(\pm 378.24)	(\pm 7.61)	(\pm 86.27)	(\pm 2.37)
P ^c	0.014	0.165	0.237	0.604	0.950	0.003	0.033	0.054

^a Eggs of *Galeruca tanacetii* collected before ($N = 15$) and after winter ($N = 15$).

^b All amounts are expressed in ng/egg; mean (\pm SD).

^c Mann-Whitney *U* test.

Table 1b Comparison of anthraquinone and anthrone contents in parasitized eggs

Eggs ^a	Free AQ ^b				AQ total (hydrolyzed) ^b			
	Chrysarobin	Dithranol	Chrysophanol	Chrysazin	Chrysarobin	Dithranol	Chrysophanol	Chrysazin
Before winter	318.16 (±100.81)	0.4 (±0.29)	159.84 (±65.09)	6.49 (±2.69)	587.58 (±194.14)	20.99 (±10.41)	447.91 (±206.94)	15.01 (±8.59)
After winter	159.01 (±104.51)	0.16 (±0.11)	150.06 (±66.96)	5.15 (±2.80)	953.76 (±1355.91)	11.72 (±12.57)	312.03 (±97.42)	10.30 (±6.41)
<i>P</i> ^c	0.006	0.025	0.770	0.329	0.696	0.064	0.097	0.205

^aEggs of *Galeruca tanacetii* collected before ($N = 7$) and after winter ($N = 10$).

^bAll amounts are given in ng/egg; mean (±SD).

^cMann-Whitney *U* test.

Data Calculation and Statistics

All data were calculated on the basis of the quantity of anthraquinone and anthrone per single egg. Anthraquinone and anthrone quantities were statistically compared by using the Mann-Whitney *U* test (Statistica 4.5 scientific software, StatSoft, Hamburg, Germany).

Results and Discussion

Eggs of *G. tanacetii* contained chrysarobin and chrysophanol as major components, whereas dithranol and chrysazin were detected only in small amounts or traces. The mean weight of a single egg (without extrachorion) was 425 µg (SD ± 101 µg, $N = 19$). The % weight of total anthrones and anthraquinones per freshly laid unparasitized egg was highest for chrysarobin (approx. 0.21%), followed by chrysophanol (approx. 0.1%), dithranol (approx. 0.009%), and chrysazin (approx. 0.003%). The total amounts of these components were much higher than the amounts of free substances, indicating that they tended to bind, likely to cryoprotective components (Wang and Kang, 2005) (Table 1a, b).

Significant seasonal differences were detected when comparing anthrone and anthraquinone quantities of freshly laid eggs and eggs close to larval hatching:

- In unparasitized eggs (Table 1a), the amounts of free chrysarobin decreased during the winter diapause. Total amounts (free and bound) of the anthrone dithranol (−65%) and

Table 1c Statistical comparison (*P* levels)^a of anthraquinone and anthrone contents between unparasitized and parasitized eggs

	Compound	Free AQ	AQ total
Before winter	Chrysarobin	0.032	0.026
	Dithranol	0.698	0.418
	Chrysophanol	0.972	0.972
	Chrysazin	0.503	0.805
After winter	Chrysarobin	0.003	0.134
	Dithranol	0.096	0.267
	Chrysophanol	0.013	0.956
	Chrysazin	0.912	0.267

^aMann-Whitney *U* test; significant differences are in bold; amounts are given in Table 1a, b.

the anthraquinone chrysophanol (−30%) decreased significantly. The total chrysazin content tended to be much lower (−50%) after winter ($P = 0.054$).

- In parasitized eggs (Table 1b), the quantities of the free anthrones (chrysarobin and dithranol) decreased significantly during the winter period. In contrast, total anthrone and anthraquinone contents of parasitized clutches did not significantly change during winter.

If these components bind to carbohydrates or other components, they would be detected after the acidic hydrolysis (Derksen et al. 2003). In contrast to plants, no insoluble cell material with anthraquinone binding affinity is known. If the anthrones were transformed to dianthrones by oxidative coupling (Teuscher and Lindequist, 1994), the dimers or their fragments should have been detectable. However, no such components were found. If anthrones and anthraquinones were transformed and precipitated (as discussed for carminic acid in *Dactylopius coccus*; Hernández-Hernández et al., 2003), we also should have been able to find the dark precipitates in the *G. tanacetii* eggs; however, nothing similar to this was detected. Thus, the results indicate that the embryo or endosymbionts within the eggs degrade the anthrones and anthraquinones.

When comparing unparasitized and parasitized eggs, the anthraquinone and anthrone contents of the samples collected before winter (i.e., shortly after parasitization) differed from those collected after winter (Table 1c). The total amounts of chrysarobin were lower in parasitized eggs than in unparasitized ones only before winter. The amounts of free chrysophanol were significantly lower only after winter in parasitized eggs. However, the amounts of free chrysarobin were lower in parasitized eggs than in unparasitized ones both shortly before and after winter.

On one hand, the lower amounts of anthrones and anthraquinones in parasitized eggs might point to the involvement of these components in immediate immune responses against invaders. On the other hand, the difference between parasitized and unparasitized eggs might be attributable to the metabolization of these components by the developing parasitoid living in the host egg. However, the fact that the parasitoid is excreting polyketides with its feces within the host egg indicates that these components may pass the parasitoid gut at least in part unchanged (Meiners et al., 1997).

In summary, our study provided no evidence for the production of anthraquinones within eggs, thereby excluding anthraquinone-producing activity of the embryo. If endosymbionts are involved in anthraquinone biosynthesis and if they are vertically transferred by eggs, they are inactive during the host egg stage. Parasitization of the eggs also does not induce the production of anthraquinones and anthrones. Instead, a decrease in these components during embryogenesis was detected both in parasitized and unparasitized eggs, indicating metabolic transformation of the components by the *G. tanacetii* embryo and the parasitoid larva, respectively. Therefore, our data suggest that anthraquinones and anthrones are transferred into the eggs by the mother, and that the site of biogenesis of these polyketides is not within the eggs.

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Intact lima bean plants attract the predatory mite, *P. persimilis* when they have been exposed to volatiles emitted from conspecific plants infested by its prey, *T. urticae* (Dicke et al., 1990). Furthermore, in this tritrophic system, the exposed intact lima bean plants increase: (1) their direct defenses against spider mites (Arimura et al., 2000), and (2) the production of herbivore-induced volatiles that attract *P. persimilis* (indirect defense) (Choh et al., 2004). These enhanced defenses are called “priming,” which has intensively been studied in plant–pathogen interactions (Conrath et al., 2002). In addition, we recently reported that intact lima bean plants increased the secretion of extrafloral nectar (EFN) when they were exposed to VIP, and that this consequently increased the residence time of *P. persimilis* on the exposed plants (Choh et al., 2006; Kost and Heil, 2006). We also found that unexposed lima bean plants increase EFN production when they are infested by the herbivorous mite, *T. urticae* (herbivore-induced EFN; Choh et al., 2006). We hypothesize that such an herbivore-induced EFN production was also primed by previous exposure to VIP. We tested the hypothesis by changing the duration of the infestation.

Methods and Materials

Plants and Mites

Lima bean plants were grown in soil in a greenhouse at $25 \pm 2^\circ\text{C}$, 60–70% r.h. under a photoperiod of 16:8 hr (L:D). We used plants grown for 4–6 d after germination.

T. urticae were obtained from the Laboratory of Ecological Information, Graduate School of Agriculture, Kyoto University, and were reared on lima bean plants in a climate-controlled room ($25 \pm 2^\circ\text{C}$, 60–70% r.h., 16:8 hr L:D).

Exposure of Plants to Volatiles

For exposure of plants to VIP, we used six acrylic $60 \times 60 \times 60$ cm cages with two 30×30 cm windows on opposite sides of the cage. Three cages were used for the experiment, and another three for control. The windows were covered with $225 \mu\text{m}$ nylon gauze, and airflow within the cage was below detection level. The cage had a 30×30 cm sliding door at the front. As odor sources, we used 8 plants that had been infested with 60 adult *T. urticae* females per plant for 1 d. The number of the odor source plants was determined on the basis of our preliminary experiments to ensure plant exposure to sufficient amounts of volatiles. Eight uninfested plants were used as control odor sources. Two uninfested plants were placed into a cage with the odor source plants (see Choh et al., 2006 for details of the setup) and exposed to either VIP or uninfested plant volatiles (UPV) for 10 d in a climate-controlled room ($25 \pm 2^\circ\text{C}$, 60–70% r.h., 16:8 hr L:D). All plants were placed in plastic containers (12 cm diam, 9 cm high) filled with water to prevent the migration of *T. urticae* from infested plants to uninfested plants. The distance between the exposed plants and the odor source plants was 25 cm. Both infested and uninfested plants had 2–3 unfolded trifoliolate leaves 10 d after exposure. We used newly cleaned cages for each experimental replicate. Under these experimental conditions, we visually inspected the exposed plants for signs of infestation and found that no spider mites invaded the exposed plants.

Measurement of EFN

After exposure of plants to VIP and UPV for 10 d, most of the EFN that had already been secreted was removed from VIP- and UPV-exposed plants with $5 \mu\text{l}$ capillaries (Ringcaps[®],

Hirschmann Laborgeräte GmbH, Germany). Measuring the length of nectar in the capillary enables the direct and precise quantification of the collected nectar volume. One of two exposed plants in a cage was used for the *T. urticae* infestation experiment, and the other was used as an intact (uninfested) control. We placed 30 *T. urticae* on the two primary leaves (total 60 *T. urticae* per plant) of the VIP- or UPV-exposed plants. As a control, no *T. urticae* were placed on VIP- and UPV-exposed plants. Thus, EFN quantities were measured in four sets of plants: (1) plants exposed to VIP and infested later, (2) plants exposed to VIP and kept uninfested later, (3) plants exposed to UPV and infested later, (4) plants exposed to UPV and kept uninfested later.

The amounts of newly secreted EFN were measured 2 d after infestation with *T. urticae* (herbivore-induced EFN). After removal of most of the induced EFN, we kept the plants for another 2 d under the same conditions, and then the secreted EFN was measured on the fourth day of infestation. Similarly, EFN secretion on intact plants (constitutively produced EFN) was measured 2 and 4 d after exposure to plant volatiles. We used 20 plants per treatment. The collection was always carried out between 10 and 12 A.M. in case the secretion of nectar showed a diel pattern (Raine et al., 2002). The experiments were conducted in a climate-controlled room (25 ± 2°C, 60–70% r.h., 16:8 hr L:D.). The amounts of secreted EFN per plant were analyzed by using a *t*-test and a Tukey-Kramer’s test.

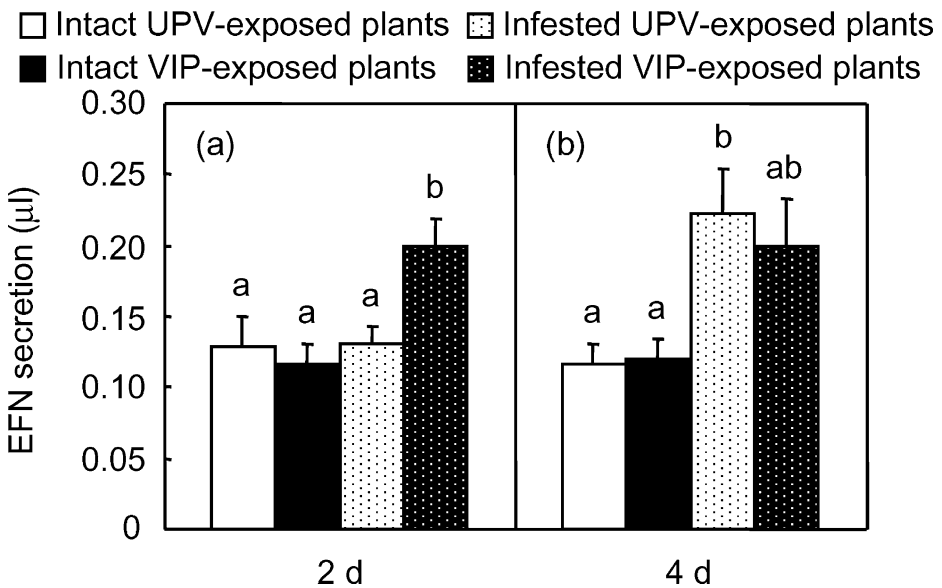


Fig. 1 Quantities of extrafloral nectar (EFN) (mean ± SE) secreted by lima bean plants obtained from following treatments: intact UPV-exposed plants, intact VIP-exposed plants, infested UPV-exposed plants, and infested VIP-exposed plants. UPV = uninfested plant volatiles; VIP = volatiles from infested plants. The duration of plant volatile exposure was 10 d. Immediately after exposure, test plants were infested with *T. urticae*, and controls were kept uninfested (intact). Quantities of EFN were measured (a) 2 d and (b) 4 d after cessation of plant volatile exposure. Different letters above bars indicate significant differences among treatments within each time period by Tukey-Kramer’s test ($P < 0.05$).

Results and Discussion

EFN secretion of intact lima bean plants was increased when measured directly after a 10-d exposure to volatiles from infested plants (intact UPV-exposed plants: $0.928 \pm 0.053 \mu\text{l}$; intact VIP-exposed plants: $1.38 \pm 0.113 \mu\text{l}$; $P < 0.001$, *t*-test). These findings corroborated those of our previous study (Choh et al., 2006). When measuring EFN quantities 2 and 4 d after cessation of a 10 d exposure to plant volatiles, no differences were observed between intact VIP- and UPV-exposed plants ($P > 0.05$, Tukey-Kramer's test; Fig. 1a and b). Results indicate that increased EFN secretion of intact plants in response to exposure to VIP (Choh et al., 2006) did not continue once exposure was terminated. Compared with intact UPV-exposed plants, UPV-exposed plants infested by *T. urticae* did not significantly increase EFN secretion 2 d after infestation ($P > 0.05$, Tukey-Kramer's test; Fig. 1a). However, UPV-exposed plants infested with *T. urticae* secreted significantly more EFN during days 3 and 4 of infestation when compared with intact UPV-exposed plants ($P < 0.05$, Tukey-Kramer's test; Fig. 1b).

VIP-exposed plants infested for 2 d by *T. urticae* secreted significantly more EFN than the other three treatments ($P < 0.05$, Tukey-Kramer's test; Fig. 1a). Although VIP-exposed plants infested by *T. urticae* for the following 2 d (third and fourth days of damage) secreted more EFN than VIP- and UPV-exposed uninfested plants, these differences were not statistically significant ($P > 0.05$, Tukey-Kramer's test; Fig. 1b). Similarly, EFN secretion did not differ significantly between VIP- and UPV-exposed plants that were infested by *T. urticae* for the following 2 d (i.e., 4 d after damage) ($P > 0.05$, Tukey-Kramer's test; Fig. 1b).

The enhanced EFN secretion observed in the first 2 d may be adaptive to the plants, as previous studies have already demonstrated that increased EFN secretion by lima bean plants can be used for indirect defense (Kost and Heil, 2005; Choh et al., 2006). Together with our previous study (Choh et al., 2004), we showed priming effects of plant–plant interaction in lima bean plants: VIP exposure not only resulted in increased VIP emission (Choh et al., 2004), but also in increased herbivore-induced EFN secretion ($P < 0.05$, Tukey-Kramer's test; Fig. 1a). This protects against attack by herbivores, thus benefiting lima bean plants in nature (Heil, 2004).

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revealed important ecological roles of ascidian crude extracts and isolated metabolites (reviewed in Pawlik, 1993; Paul et al., 2006). Although the ecological and applied importance of tunicate secondary metabolites is well recognized, little is known about the dynamics of their production. The few existing studies have focused on the intraspecimen location, at both the cellular and organism levels (Pawlik, 1993; Paul et al., 2006).

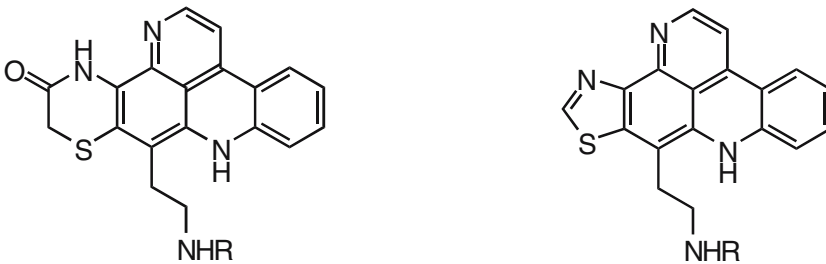
Temperate seas present strong seasonal fluctuations in environmental parameters that are often reflected in the life-cycle patterns of marine invertebrates (reviewed in Coma et al., 2000). Therefore, temporal changes in the production of secondary metabolites can be expected as a result of trade-offs with other biological functions or as a response to biotic interactions, both of which vary seasonally. So far, however, no study has attempted to determine the temporal variation of co-occurring secondary metabolites in an ascidian.

Colonies of the ascidian *Cystodytes* spp. generally lack epibionts and present scarce signs of predation (López-Legentil et al., 2006). In the Mediterranean, (López-Legentil et al., 2005a) described two chemotypes in the nominal species *C. dellechiajei*, one of which presented the sulfur-containing pyridoacridines shermilamine B (**1**), kuanoniamine D (**2**), and their deacetylated forms, deacetylshermilamine B (**3**) and deacetylkuanoniamine D (**4**). All of them were isolated from a purple morph (Fig. 1). Some of these substances are cytotoxic (Bowden, 2000). In addition, the crude extract of this morph showed anti-predatory properties against both puffer and damselfish, but not against sea urchins (López-Legentil et al., 2006). The main goal of this study was to establish whether there was a temporal variation in the production of these four pyridoacridine alkaloids.

Methods and Materials

Sampling

We monitored a population of the purple morph of *Cystodytes* in L'Escala (NE Spain; 42° 06'52"N, 3°10'07"E). From July 2004 to February 2006, five different colonies per month were randomly collected by scuba diving. They were frozen alive, freeze-dried within a week, and kept at -30°C until analysis.



1: R=COCH₃ shermilamine B

2: R=COCH₃ kuanoniamine D

3: R=H deacetylshermilamine B

4: R=H deacetylkuanoniamine D

Fig. 1 Structures of the four main alkaloids isolated from the purple morph of *Cystodytes*. (**1**) Shermilamine B; (**2**) kuanoniamine D; (**3**) deacetylshermilamine B; (**4**) deacetylkuanoniamine D

Chemical Extraction

Approximately 250 mg of each freeze-dried colony were weighed and extracted $\times 3$ in a 1:1 (v:v) mixture of dichloromethane and methanol and passed through a 20- μm polytetrafluoroethylene (PTFE) filter. The resulting solution was dried by vacuum rotary evaporation. Each crude extract was redissolved in methanol, and the final volume was adjusted to 10 ml. An aliquot of 1 ml was passed through a 13-mm, 0.20- μm PTFE syringe filter before high-performance liquid chromatography (HPLC) injection. The injection volume was fixed at 20 μl .

HPLC Analysis and Quantification

The HPLC elution conditions consisted of eluants A (water/methanol/acetic acid, 9:1:0.1 v:v:v) and B (methanol), an elution profile based on a linear gradient from 0% B to 100% B in 10 min, and a flow rate of 0.8 ml min⁻¹, with a fixed temperature of 30°C. We used an Agilent Eclipse XDB-C8 (4.6 mm ID \times 15 cm) analytical column. Analyses were performed with a Waters Alliance 2695 Separations Module with a Waters 996 photodiode array detector. The sulfur-pyridoacridine purifications and identifications were performed as described by (López-Legentil et al., 2005a). Shermilamine B, kuanoniamine D, and their deacetylated forms were detected at 308 nm. The peak area obtained for each compound was integrated and compared to a calibration curve obtained by using each of the isolated pyridoacridines as an external standard. The final amount of each compound was calculated by averaging three replicate injections. All analyses were processed with Empower software.

Quantification of Total Organic Matter

After chemical extraction, the remaining colony pieces were oven-dried to a constant weight (60°C for at least 24 hr), placed in aluminum cups, weighed, and burned in a furnace oven at 500°C for 12 hr. Total organic matter content for each colony was obtained by subtracting the ash from the total dry mass weight. We standardized the metabolite concentration in relation to organic mass, as variations in inorganic material (e.g., spicules) could confound the results.

Data Analysis

For the analysis of the production of shermilamine B, kuanoniamine D, and their deacetylated forms over time, we used one-way analysis of variance (ANOVA) with time (month) as factor after checking for normality and homoscedasticity of the data. Nonparametric equivalents (Kruskal–Wallis test) were applied when necessary (detailed in the next section).

Results and Discussion

All samples showed a low chemical diversity, with four peaks, identified as deacetylkuanoniamine D (retention time, 4.5 min), deacetylshermilamine B (retention time, 5.4 min), kuanoniamine D (retention time, 7.2 min), and shermilamine B (retention time, 8.1 min). Changes in the concentrations of kuanoniamine D, shermilamine B, and their deacetylated forms (mean \pm SE) are shown in Fig. 2 as the percentage of each compound relative to

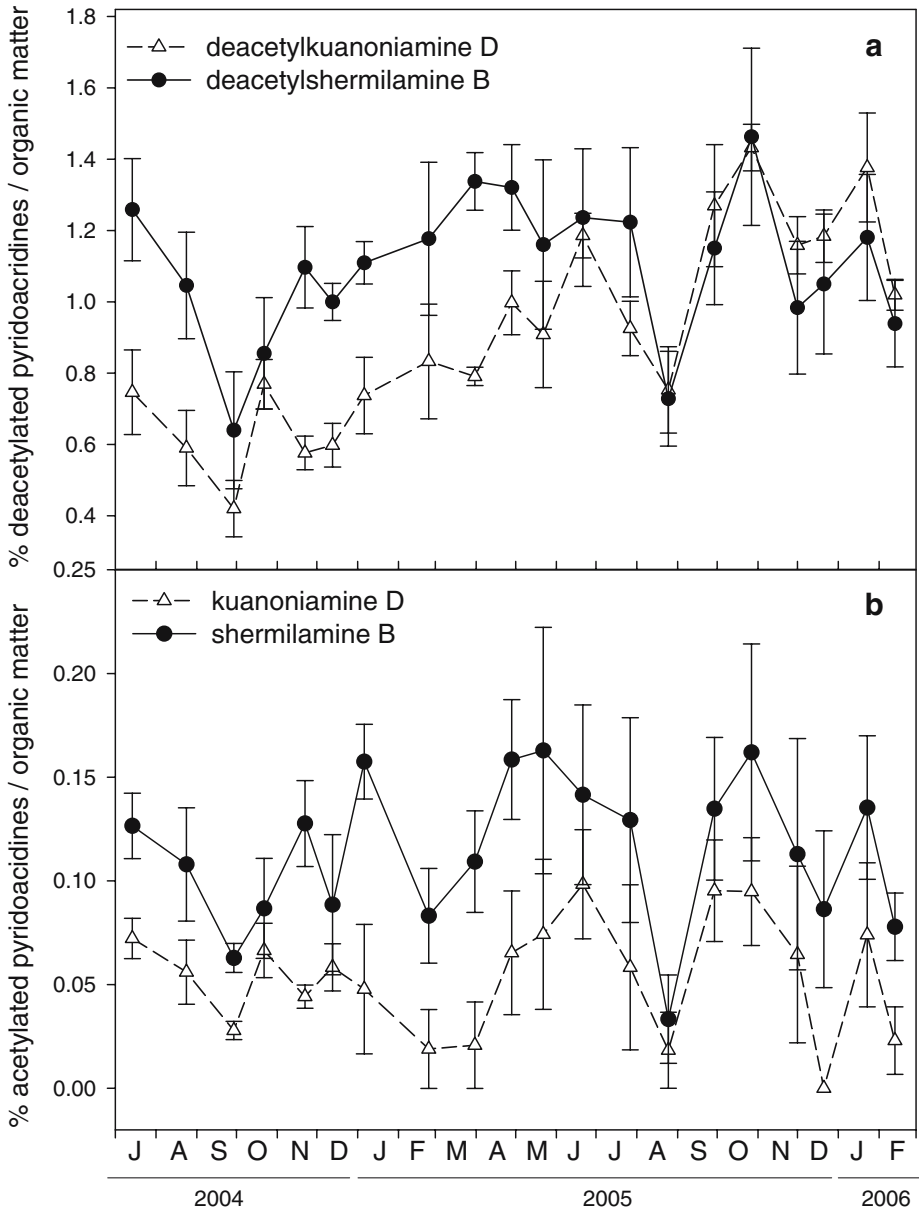


Fig. 2 Time course of the percentage of the four sulfur-containing pyridoacridines studied relative to total organic matter of each colony from July 2004 to February 2006. (a) Deacetylkuanoniamine D (dotted line) and deacetylshermilamine B (solid line); (b) kuanoniamine D (dotted line) and shermilamine B (solid line). Bars indicate standard errors (SE)

total organic matter. Deacetylshermilamine B was the most abundant compound, whereas kuanoniamine D was the least abundant. The percentages of deacetylkuanoniamine D and deacetylshermilamine B showed similar trends, with minima in September 2004 and August 2005 (Fig. 2a). However, only the production of deacetylkuanoniamine D significantly varied over time (Kruskal–Wallis, $P < 0.001$). Pairwise multiple comparison

procedures (Dunn's method) revealed that values in September 2004 were significantly lower, and values in October 2005 significantly higher. No significant difference was detected for the production of deacetylshermilamine B over time (ANOVA, $P = 0.073$). The concentration of shermilamine B showed no significant variation with time (Kruskal–Wallis, $P = 0.197$), although a minimum was observed in August 2005 and a less marked one in September 2004 (Fig. 2b). There was also no clear temporal pattern in the percentage of kuanoniamine D (Kruskal–Wallis, $P = 0.158$), but minima were found in September 2004, February and August 2005, and December 2006 (Fig. 2b). The large standard errors found in the production of these compounds showed that there was noticeable interindividual variability, which is commonplace among sessile invertebrates. All in all, there was no clear seasonal pattern in the production of any of the four compounds studied, except for the presence of minimum values in late summer 2004 and 2005. There was no evidence either of a biotransformation between acetylated and deacetylated compounds (or *vice versa*) that would have been reflected by an inverse time course of their abundances.

López-Legentil et al. (2005b) observed that the purple morph of *Cystodytes* had marked seasonal cycles for reproduction and growth, with larval release in summer (July–August) followed by a period of asexual growth. Investment in reproduction in this species is high, as the weight of larvae produced by a single zooid is almost equal to the weight of the zooid itself (Tarjuelo and Turon, 2004). Therefore, larval release (assuming they take secondary metabolites with them) could contribute to the low values in the production of deacetylshermilamine B and deacetylshermilamine D observed after sexual reproduction. However, these deacetylated compounds are present in both tunic and zooids (López-Legentil et al., 2005a), and the total mass of larvae is only 4% of the colony weight (Tarjuelo and Turon, 2004). A combination of larval release and “sexual exhaustion” after reproduction (Berrill, 1935) may account for the decrease in secondary metabolite concentration recorded in late summer 2004 and 2005.

Interacting organisms may also influence the production of secondary metabolites in *Cystodytes*. Predation and competition for space and food, if seasonally variable, may modulate the production of defensive secondary metabolites. Finally, abiotic factors should also be taken into account. Summer is an unfavorable season for many sessile filter feeders in the Mediterranean due to the existence of a food shortage period (Coma et al., 2000). This unfavorable season may be reflected by a decrease in investment in secondary metabolite production. A mixture of biotic interactions, and physiological and physical factors may all have shaped the trends observed in the production of the main pyridoacridine alkaloids.

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ultimately be regarded as interesting subjects for scientific investigations, with little real value. (Ridgway et al., 1990).

In fairness, for a number of insect species, mating disruption and other methods of insect control based on pheromones are simply not practical or economical, as opposed to being not efficacious. For example, a number of pheromone structures are too complex to synthesize economically, or too unstable to survive long periods in the field without degradation. However, for other species, pheromones are or could be readily accessible for large-scale use, but repeated efforts to make mating disruption work have been at best only partially successful. One likely cause for some of these failures was voiced by Sanders (1996) in the introduction to his chapter on mechanisms of mating disruption in the book *Insect Pheromone Research: New Directions*. Sanders stated:

Unfortunately, the mechanisms of how mating disruption works are still largely unknown. Because the technique involves complex behavioral interactions between insects in a communication mode (olfaction) to which humans have little inherent affinity, understanding disruption is not easy. (Sanders, 1996).

Remarkably, these comments were made some 30 years after the first mating disruption trial, and now, some 10 years later, our understanding of how and why mating disruption does or does not work with a particular species remains rudimentary. Possible mechanisms by which mating disruption might work (as discussed in the next two articles) were proposed more than 30 years ago (Bartell, 1982). The subject continues to be contentious because it has proven difficult to design experiments which unequivocally show that one mechanism or another, or a combination of mechanisms, are operative. Furthermore, it is unlikely that all insects will be affected in the same way by pheromones. For example, some species are much more sensitive to high rates of pheromone release than others, suggesting that they might be most susceptible to sensory fatigue or habituation. In contrast, species that tolerate relatively high pheromone release rates may be more susceptible to false trail following.

In the two papers that follow, Miller and his coworkers have developed a theoretical framework and mathematical models that, to our knowledge, represent the first attempt to deconstruct the results of mating disruption trials with the goal of determining which possible mechanisms of mating disruption were operative. The editors recognize that these articles may be controversial, and the models and their underlying assumptions may ultimately require extensive revision or modification. Nevertheless, we feel that these manuscripts represent an important first step in reenergizing the debate about the mechanisms of mating disruption. Researchers have indulged in speculation and hand-waving arguments about these mechanisms for several decades, with little actual research being done to prove that one or more mechanisms are indeed operative. Thus, the editors hope that the following papers will revitalize this area of research, and stimulate the design of experiments to specifically test the predictions and postulates deriving from the mathematical models of Miller and his colleagues.

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attractiveness of traps, females, and dispensers is known. The absolute value of the slope of the Miller-de Lame plot is a measure of each dispenser's activity (D_a) for suppressing catch of male moths in traps. An application activity ($D_{\bar{A}a}$) for a given dispenser can be calculated by multiplying D_a by the number of such dispensers applied per hectare of crop.

Keywords Mating disruption mechanisms · Disruption kinetics · Mathematical modeling · Percent disruption · Dispenser activity

Introduction

Mating disruption as currently applied to insects is the practice of continuously dispensing synthetic sex attractants into a crop for extended periods so as to suppress pest reproduction by interfering with mate finding. This pest management tactic holds considerable promise for management of moths and other pests that rely on long-distance chemical communication. In the United States, the demands for pest control by behavioral modification are rising as availability of conventional insecticides dwindles in the face of increasingly stringent regulatory policies. However, protocols for pheromone disruption of many important moth pests have not been optimized. In some cases, this tactic works robustly (e.g., redbanded leafroller, Novak and Roelofs, 1985; also in oriental fruit moth, Rice and Kirsch, 1990; Trimble et al., 2004, and other examples in Cardé and Minks, 1995), whereas in other cases results are mixed (e.g., obliquebanded leafroller, Agnello et al., 1996; Lawson et al., 1996; spruce budworm, Seabrook and Kipp, 1986).

General agreement has not been reached as to whether it is better to broadcast pheromone uniformly throughout a crop (e.g., microencapsulated formulations, Balkan, 1980; Vickers and Rothschild, 1991; Waldstein and Gut, 2003), or whether pheromone dispensers should be discrete sources spaced regularly throughout. In orchards, a spacing of 1–2 dispensers per tree is recommended for long-lasting, hand-applied pheromone dispensers like polyethylene tubes (ropes) (Rothschild, 1975; Nagata, 1989; Knight et al., 1998). The disruptive products used in some other crops favor an intermediate density of small and highly distributed point sources (e.g., hollow fibers, Doane and Brooks, 1981; Swenson and Weatherston, 1989; or laminated flakes, Tcheslavskaja et al., 2005). Likewise, there is divergence of opinion regarding the quantity of pheromone necessary for high-performance disruption and whether it is better to use the complete natural blend or just one or two of the major pheromone components (Knight et al., 1998; Minks and Cardé, 1988; Evenden et al., 1999). Different release devices, spacings, and chemistries may be called for (Gut et al., 2004) depending on the mechanism(s) of disruption to which a particular pest species is thought to be vulnerable.

It has often been pointed out that optimizing mating disruption will be aided by understanding the actual behaviors evoked or suppressed when insects are exposed to particular formulations of synthetic pheromones (e.g., Valeur and Löfstedt, 1996; Cardé et al., 1998; Evenden et al., 2000; Gut et al., 2004). The major mechanisms postulated to explain communicational disruption (Rothschild, 1981; Bartell, 1982; Cardé, 1990) include (formal definitions below): (1) false-plume-following; (2) camouflage; (3) desensitization—including adaptation and habituation; (4) sensory imbalance; and (5) combinations thereof. However, the evidence for which mechanism is mainly responsible for communicational disruption for particular pests under particular regimes of disruption remains largely circumstantial. We are aware of no example where a particular mechanism has been definitively proven to be the leading cause of mating disruption for any insect under actual field conditions.

Some might argue that biological phenomena like mating disruption are too complex to be usefully represented by simplistic explanatory models. Complications in interpretation are easy to envision if several disruption mechanisms operate simultaneously. However, we see value in attempting to break this seemingly complicated phenomenon into defined component causes, and assessing how each could shape overall outcomes. Recognizing and understanding patterns in disruption outcomes associated with individual explanatory models should facilitate recognizing and understanding outcomes from mixed models.

This analysis emphasizes competitive attraction as a primary contributor to mating disruption of moths by pheromone point sources. Competitive attraction has been variously referred to as: confusion (Knipling, 1979 *et ante*; Bartell, 1982), false-trail-following (Maini and Schwaller, 1992), and false-plume-following (Cardé, 1990; Stelinski et al., 2004a). Our attention was drawn to this mechanism when direct behavioral observations (Stelinski et al., 2004a, 2005a,b) revealed that oriental fruit moth (*Grapholita molesta*), obliquebanded leafroller (*Choristoneura rosaceana*), redbanded leafroller (*Argyrotaenia velutinana*), and codling moth (*Cydia pomonella*) all approached their respective polyethylene tube dispensers of pheromones at frequencies rivaling catches of moths occurring simultaneously in monitoring traps. Combined with earlier reports of attractiveness of dispensers used in mating disruption (e.g., Bartell, 1982; Barrett, 1995; Cardé et al., 1998; Witzgall et al., 1999; Maini and Accinelli, 2000), there is mounting evidence that competitive attraction is involved in communicational disruption of various moth species. In this paper, we: (1) explicitly define and formalize the competitive attraction explanatory model; (2) detail its predictions; and (3) determine where these predictions do or do not overlap with non-competitive explanatory models. This work sets the stage for a companion paper (Miller et al., 2006) that analyzes case studies from the literature for fit to competitive vs. noncompetitive disruption.

Definition, Assumptions, and Predictions of the Competitive-Attraction Explanatory Model for Communicational Disruption

Definition

We take *competitive attraction* to mean that the frequency with which male moths find calling females or monitoring traps (proxy for calling females) in a crop under disruption is reduced because males are diverted from orienting to females or traps due to preoccupation with nearby attractive or arrestive plumes from dispensers of synthetic pheromone. Accordingly, communicational disruption by sex attractant pheromones utilizes the same behavioral mechanisms normally enabling the responder to *find* (*sensu* Miller and Strickler, 1984) a calling female. These mate-finding mechanisms are mainly positive, plume-following anemotaxis combined with arrestment. Female moths under pheromone disruption by competitive attraction are envisioned to compete with sources of synthetic pheromone for responses from males.

Mating System in Which Pheromone Disruption by Competitive Attraction Operates

The mating system of moths using sex attractants for long-distance mate finding has been labeled *scramble polygyny* (Thornhill and Alcock, 1983) to describe the situation in which males compete heavily during limited windows of time for a share of calling females whose individual availability is fleeting. The sex ratio in a moth population is usually approximately one, but this does not mean that every male succeeds in mating. Individual males are rewarded for proficiency in detecting pheromone plumes and arriving at their sources.

Provided he is not physically or behaviorally defective, the first-arriving male usually mates with the calling female. Moreover, some males may mate numerous times, whereas others may never mate. When females mate infrequently, intrasexual competition among male moths is high, whereas that among females is comparatively low. This is because mated females cease calling (at least for a time), whereas all able-bodied males continue competing sexually throughout their lives. Under a regime of pheromone disruption by competitive attraction, scramble competition would be intensified for males by the addition of “false females” that make it more difficult for males to find authentic females. In addition, intrasexual competition would be intensified for females by deployment of many false females into the system. The effect is analogous to diminishing male density, or lowering the ratio of males to females. In the natural situation and with appreciable moth populations, any calling female is expected to be quickly found and mated. However, the signals of females could go unheeded under mating disruption operating by competitive attraction because males would be preoccupied by visits to the more abundant false females.

In the several cases where it has been quantified, fecundity of both male and female moths diminishes substantially with age (Knight, 1997; Jones and Aihara-Sasaki, 2001; Torres-Vila et al., 2002). Thus, delaying mating can contribute to pest control. However, when each mated female produces 100 or more viable eggs and mortality of larvae is low, efficacy in mating disruption must exceed ca. 98% for the pest population to decline (Knipling, 1979).

Assumptions of a Probability Model for Competitive Attraction

Figure 1 provides the spatial and numerical context in which we envision moth mating disruption to occur in one small portion of an orchard crop. This figure depicts a 0.3-ha plot of fruit trees infested with a tortricid moth pest. Active spaces of pheromone plumes generated by hand-applied pheromone dispensers in such an orchard vary from ca. 5–30 m in length, as estimated by portable electroantennogram devices (EAGs) (Suckling et al., 2006). For ease in viewing and interpreting Fig. 1, the density of dispensers (D) (Table 1 provides a key to abbreviations) is reduced from the usual 1–2 dispensers per tree. Instead, 24 dispensers of pheromone are spaced regularly throughout the 72-tree plot, which also contains 20 male (M) and 20 female (F) moths. One monitoring trap (T) baited with an optimized pheromone lure is deployed centrally in this plot, as is usual for assessing the effectiveness of communicational disruption, measured as inhibition of catch of males in traps relative to catch in an equivalent control plot not treated with pheromone. Wind, currently from the top left of the orchard, generates a pheromone plume from each dispenser and the trap. Active spaces are approximated by the light-gray ellipsoids snaking across tree rows. Shifts in wind direction over time would shift the direction and shape of plumes such that pheromone from individual sources would waft over varying sets of trees and moths through time. As females begin calling, their plumes mingle and compete with those from pheromone dispensers and the trap. Over a moth's lifetime, the encounters of males with pheromone sources (T , F , and D) are envisioned to occur in proportions approximating the relative densities and strengths of pheromone sources of each type in this orchard. The densities of male and female moths in this orchard would shift across time in accordance with their respective curves for entrance into and exit from the local reproductive pool. Variation in nightly reproductive activity would be influenced by abiotic factors like weather. However, orientational outcomes averaged across a full moth generation are envisioned to stabilize sufficiently to permit meaningful contrasts among different disruptive treatments sharing common conditions.

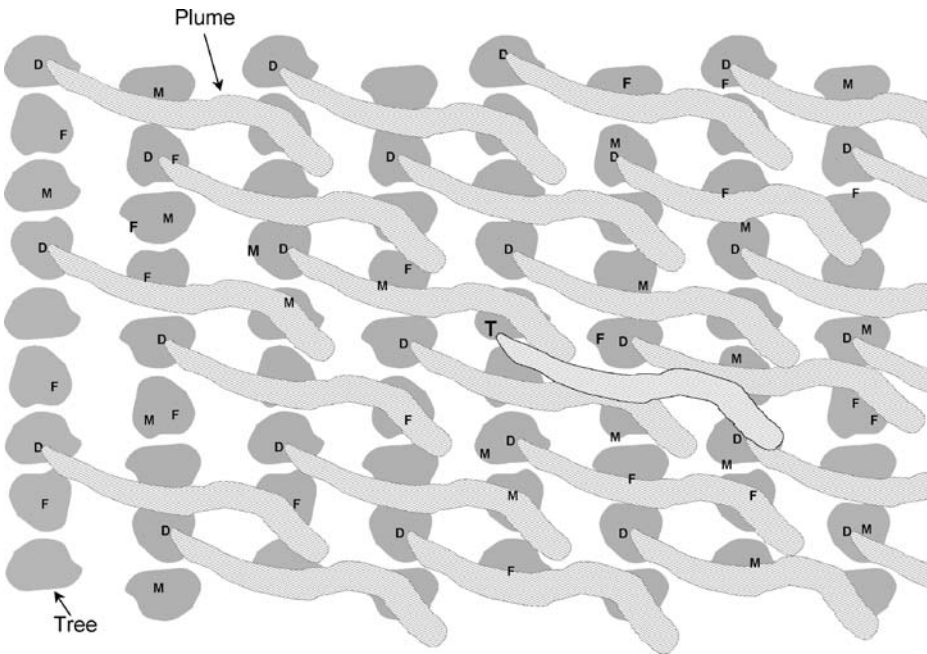


Fig. 1 Diagram of a 0.3-ha orchard plot of 72 trees containing 24 evenly distributed pheromone dispensers (D), 20 randomly distributed male moths (M), 20 randomly distributed female moths (F), and one centrally located monitoring trap (T). Wind originates from the top left.

Predictions of time-averaged outcomes from orientational events of male moths under disruption by competitive attraction are easiest to understand for the simplified, hypothetical situation where: (1) attractiveness is equal for *F*, *D*, and *T*; (2) each male engages in one attraction event per night; (3) the number of males removed by the one sticky trap has negligible impact on the male population; and (4) all females call and remain attractive through most of a several-hour reproductive period. Complexity will be systematically added once predicted outcomes of the simpler case are described.

Mathematically, this problem corresponds to that of loading a bag with 20 white spheres [= density of moth females (F_D) in Fig. 1], 24 green spheres [= density of dispensers (D_D)], and one red sphere [= density of traps (T_D)] and thoroughly mixing these spheres of equal size and mass. One male orientational event corresponds to reaching into the bag, randomly withdrawing one sphere, and then replacing it. The probability one draw will yield a white sphere is: $20/45 = 0.44$. Likewise, the probability one draw will yield a green or red sphere is: $24/45 = 0.53$, or $1/45 = 0.022$, respectively. With sufficient replication, the number of spheres of a given color drawn per set of draws equals the probability of drawing the given color at each draw multiplied by the number of draws per set, which equals the number of males in the plot when each male is allotted only one attraction event per night.

Translating the probability principles of the scenario described above to Fig. 1, the theoretically predicted male visitation rate per night to the trap is taken as synonymous with catch (*C*) to yield:

$$C = T_D M_D / (T_D + F_D + D_D) \tag{1}$$

Catch in this case = $1 * 20 / (1 + 20 + 24) = 0.44$ males/trap/night. Similarly, the predicted male visitation rate per night to the 20 females and 24 dispensers of Fig. 1 = 8.9 and 10.7, respectively.

There is ample precedent for applying standard probability theory to predict time-averaged male catches when females call in competition with attractive traps. Knippling (1979) and coworkers did so when exploring the possibilities of annihilating insect populations using a trap-out tactic. Other researchers (e.g., Mertins et al., 1967; Hardee et al., 1969; Roelofs et al., 1970; Nakasuji and Fujita, 1980) built on this approach to modeling competitive attraction and found its predictions realistic in field tests.

Predictions of Competitive Attraction Models

1. General predictions from the mass trapping literature.

Key predictions already validated in literature (above references) taking this probabilistic approach to competitive attraction are as follows: (1) efficacy is strongly and positively correlated with the density of traps baited with artificial pheromone; (2) efficacy of competitive attraction is strongly and negatively correlated with pest density; and (3) the density of traps required to control a substantial population of pest moths is prohibitively high due to material costs.

Table 1 Key to abbreviations

Term	Definition
A	Total area of crop (ha)
A_c	Area of crop covered by camouflaging pheromone plumes
A_{uc}	Area of crop not camouflaged by pheromone plumes
$C = V_{Tt}$	Catch (males/trap/time)
C_{max}	Male catch in traps placed into crop not receiving pheromone dispensers
D	Dispenser of synthetic pheromone deployed for disrupting sexual communication
D_a	Dispenser activity = suppressive effect of a dispenser on ability of males to find females or monitoring traps; the units are area (ha) over which one dispenser can theoretically reduce C_{max} by 50%
$D_{\bar{a}a}$	Dispenser application activity = $D_a * D_D$ = potency of a given pheromone formulation as applied to one ha
D_D	Dispenser density (dispensers/ha)
D_{sa}	Dispenser specific activity = $D_a/\mu\text{g}$ pheromone released by 1 dispenser/hr; the units are $\text{ha} * \text{hr}/\mu\text{g}$
F	Female moth
F_D	Female moth density (females/ha)
k_D	Attractiveness of a pheromone dispenser relative to that of a trap or female moth
k_F	Attractiveness of a female moth relative to that of a trap or dispenser
k_T	Attractiveness of a trap relative to that of a female or dispenser
M	Male moth
M_D	Male moth density (males/ha)
T	Trap baited with a pheromone lure; attracts and captures male moths
T_D	Trap density (traps/ha)
$V_{/D}$	Visitation rate of male moths to a dispenser (male visits/dispenser/time)
$V_{/F}$	Visitation rate of male moths to a female moth (male visits/female/time)
$V_{/T} = C$	Visitation rate of male moths to a trap (males/trap/time)

2. Predictions of mating disruption outcomes from our competitive-attraction model as applied to Fig. 1.

According to Equation (1), the nightly probability that a male will visit a given trap or a given female under the competitive-attraction assumptions of Fig. 1 is 0.44. If each visit to a female resulted in mating, 8.9 out of the 20 total females would mate on night 1. The comparable number of male visits to a given female in a control plot equivalent to that of Fig. 1, but without pheromone dispensers can also be calculated using Equation (1) as: $1T * 20M / (1T + 20F) = 20/21 = 0.95$. Mating disruption outcomes are typically scored by % inhibition of male catch in traps, calculated as: $[1 - (\text{mean catch per trap in the pheromone-treated plot} / \text{mean catch per trap in the check plot})] \times 100\%$ (Roelofs and Novak, 1981). That value for the current example is: $[1 - (0.44/0.95)] 100\% = 54\%$.

From the perspective of pest control, a 54% mating disruption efficacy per night for the 24 pheromone dispensers per 72 fruit trees is inadequate. Moreover, having nine of the 20 females mate on the first night lowers the total density of attractant sources for the second night: 11F, 24D, and 1T = 36 total. The calculated male catch on the second night for one trap or male visitation rate to a given female is: $1 * 20/36 = 0.56$, a value slightly higher than 0.44 male visits per female for night 1. It follows that 6.1 females of the 11 remaining virgins would mate on night 2, leaving only 4.9 of the original 20 virgin females after two nights. After night 3, the preponderance of females would have mated. The number of pheromone dispensers in Fig. 1 is clearly too low to control the pest under competitive attraction operating within the above assumptions.

A key prediction of competitive attraction is that moth catch per trap will be negatively correlated with density of competing attractant sources. This relationship is specified in Fig. 2a for the conditions of Fig. 1. However, densities of dispensers, moths, and traps are now scaled per hectare. An identical profile would result if male visitation rate per 3.3 virgin females per night were plotted on the *y*-axis of Fig. 2a. The effect of increasing dispenser density is nonlinear for competitive attraction. Initially, the impact of small increases in dispenser density is great; however, the return for a given increment in dispenser density progressively diminishes. Unfortunately for pest management, complete cessation of male visits per female or trap under a regime of competitive attraction is approached only asymptotically and never reached. The shape of this response profile is an inherent property of inverse functions, where a swelling D_D in the denominator of Equation (1) initially overwhelms but never completely negates T_D and M_D in the numerator.

Inverse functions lend themselves well to graphical analyses after an inverse transformation. Indeed, the simulated competitive attraction data from Fig. 2a when plotted as the inverse of male catch/trap/night ($1/C$) on the *y*-axis vs. D_D on the *x*-axis (designated hereafter as a Miller-Gut plot) yield a straight line. Equation (1) rearranged into the $y = mx + b$ format yields:

$$1/C = 1/T_D M_D * D_D + (T_D + F_D) / T_D M_D \quad (2)$$

For a given moth and trap density, the greater the slope of a Miller-Gut plot, the greater is the disruption per dispenser. Moreover, when attractiveness of $T = F = D$, the inverse of this slope equals $T_D M_D$ and hence reveals M_D , when T_D is known. Furthermore, the absolute value of the *x*-intercept of a Miller-Gut plot of competitive-attraction data equals $T_D + F_D$, when attractiveness of $T = F = D$. Even more importantly, this value also equals the number of dispensers D per hectare (D/ha) required to reduce male catch by $1/2C_{\text{max}}$, under these specified conditions. Larger absolute values for *x*-intercepts equate to lower disruptive

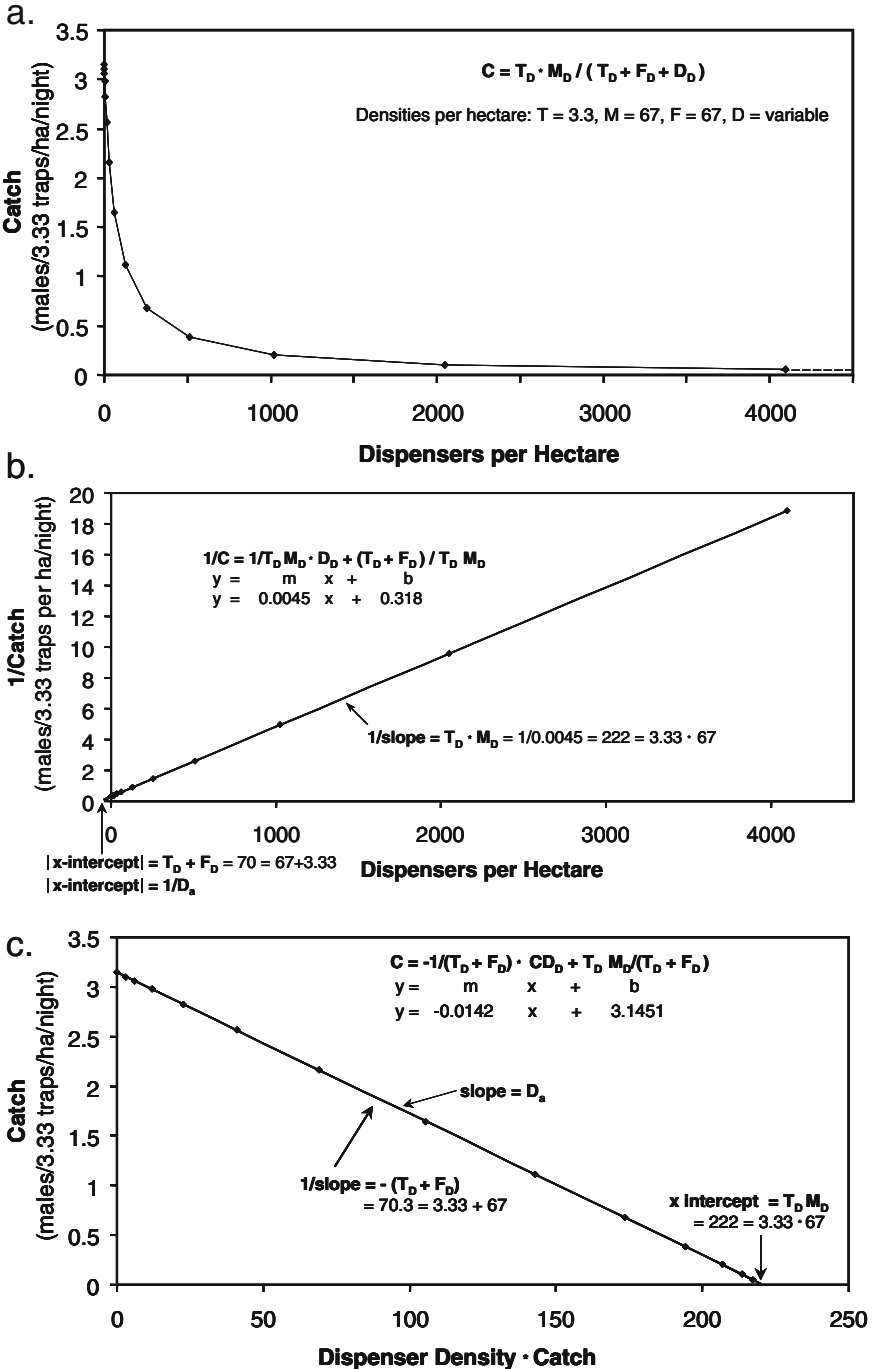


Fig. 2 Plots of a simulated communicational disruption profile for competitive attraction operating under the conditions of Fig. 1 and the assumptions given in text. (a) Untransformed plot; (b) Miller–Gut plot; (c) Miller–de Lame plot. See Table 1 for definition of abbreviations. Mathematical simulations were performed in Microsoft Excel.

potency per dispenser. The reciprocal of the absolute value of the x -intercept offers the most useful measure of disruptive activity per dispenser (D_a) expressed as the fraction of one ha over which a given dispenser would be capable of reducing catch by $1/2C_{\max}$. In Fig. 2b, $D_a = 1/70 = 0.0142$. Theoretically, each dispenser would be capable of suppressing catch in 0.042 ha (420 m²) by 50%. An increase in D_a for a given T_D and F_D reflects an increase in a dispenser's competitive attractiveness relative to the traps and females against which it was competing. D_a can be calculated variously:

$$\text{absolute value of the Miller – Gut } x\text{-intercept} = D_D \text{ yielding } 1/2 C_{\max} = 1/D_a \quad (3)$$

The x -intercept is calculated from the $y = mx + b$ equation by setting $y = 0$ and solving for x , which yields $x = y\text{-intercept}/\text{slope} = 1/D_a$. Rearrangement yields

$$D_a = \text{Miller – Gut slope} / \text{Miller – Gut } y\text{-intercept} \quad (4)$$

Substituting terms from Equation (2) yields, for the special case when attractiveness of $T = F = D$:

$$D_a = (1/T_D M_D) * T_D M_D / (T_D + F_D) = 1 / (T_D + F_D) \quad (5)$$

Under competitive attraction, we suggest that $D_{\bar{A}a}$ (dispenser application activity) become the designation for the disruptive activity of a given number of dispensers per designated area of treated crop and with each dispenser sharing a common D_a ; thus:

$$D_{\bar{A}a} = D_a * D_D \quad (6)$$

$D_{\bar{A}a}$ is unitless, since it results from multiplying ha/dispenser by dispensers/ha. Use of standard units of measure (i.e., hectare) will facilitate comparisons of disruption potencies across experiments and pest species. Substitution of $1/T_D + F_D$ from Equation (5) for D_a in Equation (6) yields:

$$D_{\bar{A}a} = D_D / (T_D + F_D) \quad (7)$$

In what we designate as a Miller-de Lame plot, male catch (C) is plotted on the y -axis against dispenser density * male catch ($D_D C$) on the x -axis. The resultant graph (Fig. 2c) yields a straight line; C_{\max} is the y -intercept, $T_D * M_D$ is the x -intercept, and the absolute value of the slope directly reveals D_a . Equation (1) can be rearranged into the $y = mx + b$ format as:

$$C = -1 / (T_D + F_D) * C D_D + T_D M_D / (T_D + F_D) \quad (8)$$

We suggest that the Miller-de Lame plot will be the more stringent test of whether actual experimental results from the field fit the theoretical predictions of competitive attraction. However, before that fit can be judged, assurance will be needed that deviation from the linear profile indicative of competitive attraction is not primarily attributable to sampling error inherent in low male catches. In field experiments, sampling error will rise as catch of males drops to tiny values because many dispensers are present. Moreover, this error will be compounded as increasingly imprecise values of C are multiplied by a swelling D_D . Thus, adherence of actual field data to a Miller-de Lame function like that of Fig. 2c is expected to deteriorate when moving from left to right on such a graph.

The principle that competitive attraction outcomes vary strongly with density of the target pest is demonstrated in Fig. 3a, as calculated under the conditions of Fig. 1 and its stated assumptions. Application of attractive pheromone sources at 50 dispensers per 72 trees of Fig. 1 predicts ca. 95% trap catch suppression for a moth population of 5 males and

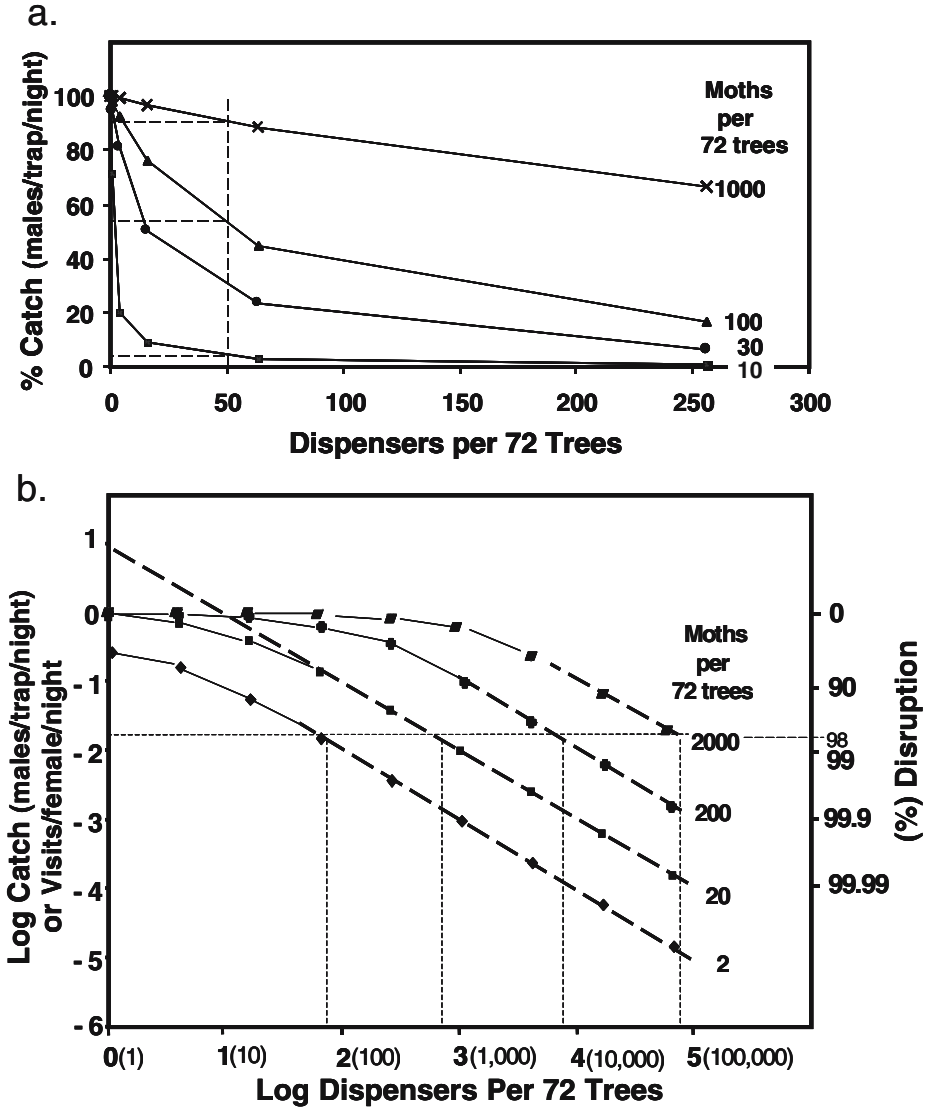


Fig. 3 Efficacy of simulated communicational disruption by competitive attraction as influenced by moth density under the conditions of Fig. 1 and its assumptions and a 1:1 sex ratio. Percent catch is expressed relative to catch in an equivalent plot not receiving pheromone dispensers. (a) Plot on untransformed axes; (b) plot on $\log_{10} \times \log_{10}$ axes; numbers within parentheses along the x -axis are the untransformed densities of pheromone dispensers.

5 females per 72 trees (Fig. 3a). However, the predicted disruption efficacy for 100 and 1000 moths per 72 trees is only ca. 45% and 10%, respectively.

A $\log_{10} \times \log_{10}$ plot of communicational disruption as a function of pheromone dispenser density displays this relationship across a wide range of pest and dispenser densities (Fig. 3b). Under competitive attraction, $\log \times \log$ plots for all pest densities become linear with a slope of negative 1 when the numbers of female moths become

inconsequential relative to the total number of attractant sources. Furthermore, the elevation (projected y -intercept) of each linear portion (bold) of the various profiles graphed in Fig. 3b reveals the density of males in the system. Conveniently, Fig. 3b also prescribes the number of dispensers required for a desired percent reduction in male visits (mating) across the various pest densities. This is accomplished by projecting a horizontal line across the graph at the desired value of percent disruption and reading out values of x at the intersections. For example, the horizontal dashed line in Fig. 3b indicates a 98% suppression of nightly male catch of males or visits per female. Values of x at the intersections along the dashed horizontal line prescribe that ca. 90, 900, and 9000 dispensers per 72 trees would be required to achieve 98% communicational disruption for moth densities of 2, 20, and 200 per 72 trees. This equates to 1.3, 12.5, and 125 dispensers per tree. These numbers are strikingly high but they are similar to the densities of traps Knipling (1979) and Roelofs et al. (1970) concluded from experimental data would be required for competitive trapping to control moth populations actually encountered in the field. Effective densities of traps proved cost-prohibitive. Fortunately, technologies are available (e.g., Miller et al., 2006) for deploying attractive pheromone dispensers at costs of materials and labor much less than required for a sticky trap.

A subset of data from Fig. 3b is displayed as a Miller-Gut plot in Fig. 4a. This plot is not as immediately useful as the $\log \times \log$ plot (Fig. 3b) in prescribing dispenser densities needed for desired levels of disruption for particular pest densities. However, a distinct advantage of Miller-Gut and Miller-de Lame plots is that all data contribute to shaping a straight line, including the zero-pheromone treatment, where numbers of males caught are maximal (C_{\max}). Considering all data simultaneously maximizes precision when deriving secondary measures from each data set, such as D_a .

Inspection of Equation (5) and Fig. 4a reveals that D_a will rise as the density of calling females declines during a given moth generation. However, D_a is independent of male density. We suggest that sufficiently stable measures of D_a to permit efficacy comparisons of different disruption formulations can be obtained from tests averaging results across a full moth generation. Initial low D_a values obtained when the density of calling females is maximal will be averaged with more abundant higher D_a values from later in that generation. However, the best direct comparisons of disruption efficacy under competitive attraction will be those made with comparable moth populations, confirmed by reliable direct measurements of both male and female densities.

Simulations varying T_D , F_D , and D_D and theoretical calculations established that the x -coordinate of the point of convergence for Miller-Gut plots of varying moth densities (Fig. 4a) equals T_D (Fig. 4b), whereas the y -coordinate of the point of convergence equals $(F_D/T_D M_D)$ when $M_D = F_D$. Moreover, in simulations including no traps and where females and dispensers competed only with one another, Miller-Gut plots for male visitation rate to females under differing moth densities converged on the y -axis.

A final and the most obvious prediction of the competitive attraction model of pheromone disruption is that males approach pheromone dispensers in the field. It should be possible to document such attraction by direct visual observations, or indirectly, by trapping. The relative attractiveness of dispensers, lures, and females can be ascertained by deploying and observing them simultaneously under equivalent male populations and for equivalent times. For most experiments reported in the literature, traps collecting males operate continuously whereas females are thought to call for only part of the time that males are responsive. Such tests reveal time-averaged rather than instantaneous attraction of traps relative to females. Full understanding of attractiveness of traps vs. females will be necessary for a full interpretation of competitive-attraction data.

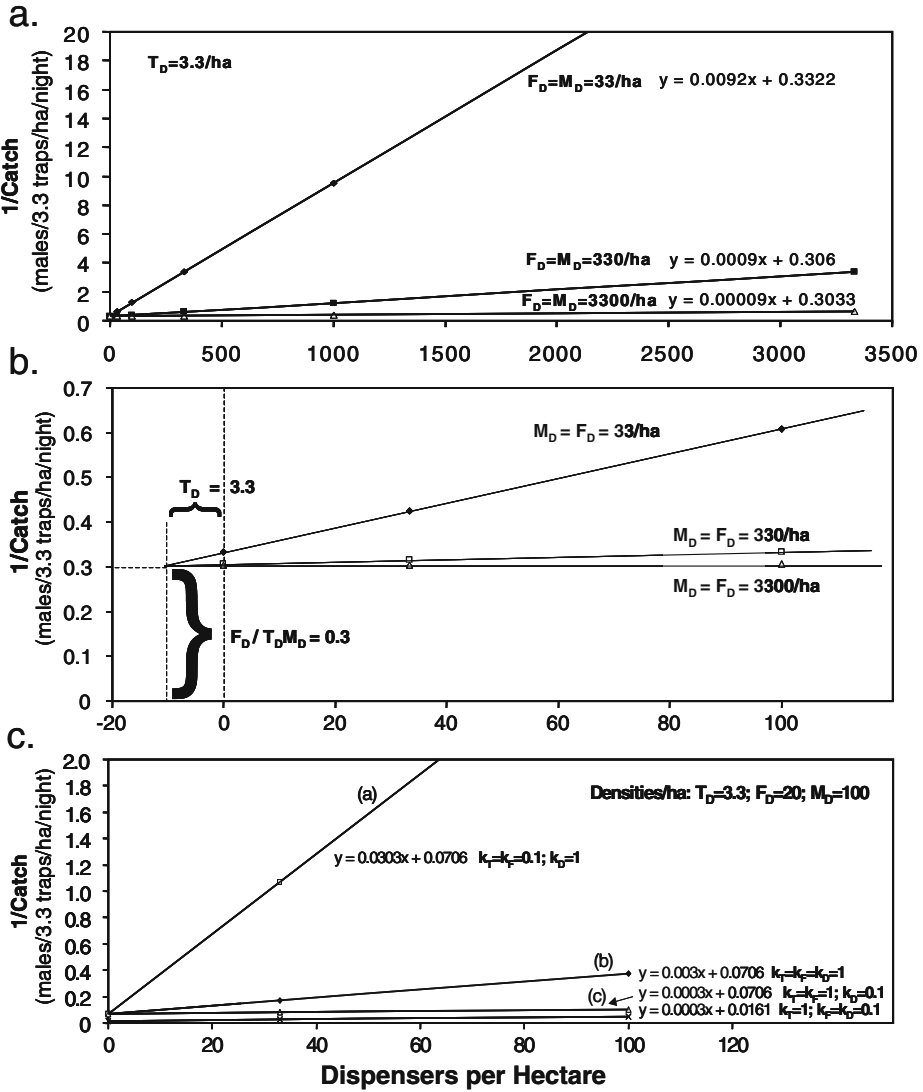


Fig. 4 Miller-Gut plots of simulated outcomes of communicational disruption by competitive attraction. (a) Influence of moth density; (b) expansion of the x - y intersection of (a); (c) influence of varying values of k_T , k_F , and k_D . See Table 1 for definition of abbreviations.

Variations from Initial Assumptions

Inequality of T , F , and D Attractiveness

Accommodating inequalities in attractiveness of pheromone point sources requires insertion of factors adjusting attraction strength into Equation (1). The expanded equation is:

$$C = k_T T_D M_D / (k_T T_D + k_F F_D + k_D D_D) \tag{9}$$

where k_T , k_F , and k_D are positive numbers representing the attractiveness of T , F , and D , respectively, relative to the most attractive of the trio, whose value is taken here as 1.0. When attractiveness of $T = F = D$, all k values equal one and drop out of the equation.

Changes in outcomes of competitive attraction due to shifts in relative attractiveness of D , F , and T can be calculated as follows. If attractiveness of D is 10-fold less than that for F and T , then $k_F = k_T = 1$, but $k_D = 0.1$. When all k values are expressed in terms of k_T , the equation becomes $C = k_T T_D M_D / (k_T T_D + k_T F_D + k_T D_D / 10) = k_T T_D M_D / k_T (T_D + F_D + 0.1 D_D) = T_D M_D / (T_D + F_D + 0.1 D_D)$. On the other hand, if attractiveness of D is 10 times greater than that of T or F , the k_T -substituted equation reduces to $C = T_D M_D / (T_D + F_D + 10 D_D)$. Effects of other changes in relative attractiveness of T , F , and D on male catch or visitation rate per female can likewise be calculated. In all cases, the graphical shape of the output remains unaltered, i.e., Miller-Gut (Fig. 4b) and Miller-de Lame plots yield straight lines (Fig. 4c). However, slopes and intercepts are shifted by alterations in k values (Fig. 4c). Values of k need to be considered when deriving values of $T_D M_D$ or $T_D + F_D$ from Miller-Gut or Miller-de Lame plots as per Fig. 2b and 2c. The following rules apply when $k_T = 1$:

$$1/\text{Miller-Gut slope} = \text{Miller-de Lame } x\text{-intercept} = T_D M_D / k_D \tag{10}$$

$$\text{Miller-Gut } x\text{-intercept} = 1/\text{Miller-de Lame slope} = -(T_D + k_F F_D) / k_D, \tag{11}$$

or when $k_T < 1$:

$$1/\text{Miller-Gut slope} = \text{Miller-de Lame } x\text{-intercept} = k_T T_D M_D k_D \tag{12}$$

$$\text{Miller-Gut } x\text{-intercept} = 1/\text{Miller-de Lame slope} = -(k_T T_D + k_F F_D) k_D \tag{13}$$

For example, the equation for graph (c) in Fig. 4c is: $y = 0.0003x + 0.0706$. It was generated from a relationship where $k_T = k_F = 1$, and $k_D = 0.1$; thus, attraction of $T = F = 10D$. Applying Equation (10), $1/\text{Miller-Gut slope} = 1/0.0003 = 3333 = T_D M_D / k_D$. This value matches (within rounding error) the expected value of 3300, where $T_D = 3.3$, $M_D = 100$, and $k_D = 0.1$, as per Fig. 4c. Applying Equation (11) to this same equation of Fig. 4c (c) yields: $\text{Miller-Gut } x\text{-intercept} = -0.0706/0.0003 = -235 = (T_D + k_F F_D) / k_D$. This value matches (within rounding error) the expected value of -233, where $T_D = 3.3$, $M_D = 100$, and $k_D = 0.1$, as per Fig. 4c. Thus, in addition to sensitivity to densities of moths and traps, slopes of transformed plots for competitive attraction data and x -intercepts are affected by the relative attractiveness of T , F , and D . When this experimentally accessible relationship is measured, densities of male and female moths can be computed with confidence from Miller-Gut or Miller-de Lame plots of experimental data.

The density of traps (T_D) in disruption protocols is usually low relative to F_D and D_D . Under this condition, altering attractiveness of T relative to F and D has negligible effect on mating disruption of female moths. However, varying the potency of the lure in a monitoring trap relative to that of F and D can markedly shift male catch. If nightly attractiveness of traps monitoring disruption efficacy exactly equaled nightly attractiveness of the average female, inhibition of male catch would be an exact proxy measure of mating disruption. When traps are more attractive than females, actual mating disruption will be under-estimated, but sampling will be more sensitive. Conversely, using monitoring traps that are less attractive than females could over-estimate the efficacy of mating disruption. Thus, knowledge of the relative attractiveness of T , F , and D is essential in order to accurately predict disruption efficacy via catch of males in traps under competitive attraction.

Raising the density of monitoring traps in a crop under disruption by competitive attraction will increase sample size and thus accuracy of the measured value for disruption.

However, mating disruption as measured by suppression of catches of males in traps deployed in disruption plots relative to control plots is not independent of trap density under competitive attraction. This is demonstrated in Fig. 5. Variation in relative inhibition of catch with varying trap density is greatest (albeit never extreme) for low moth densities and low densities of pheromone point sources. Percent disruption values shift with trap density because of inequalities in the total number of attractant sources in the control vs. treated crop. As was acknowledged above for D_a , the percent disruption measure will vary with T_D and F_D under competitive attraction. Thus, under disruption by competitive attraction, it is not surprising that discrepancies in disruption efficacy are reported for the same pest by different investigations by using a common disruption formulation but operating under different pest densities.

Multiple Attraction Events by Males per Diel Cycle

Assuming that most male moths engage in only one bout of orientation to a source of pheromone per day is an over-simplification leading to a liberal assessment of disruption efficacy in the above simulations. The effect of multiple orientations of males to pheromone sources per night can be accounted for by multiplying the above-calculated outcomes by the mean number of orientations/male/d. For example, if the average male successfully found four different pheromone sources per day, that disruption outcome is approximated by calculations as described above, but using four times the original density of males. In this example, the resultant diminution in disruption efficacy due to repeated male orientations could be offset by quadrupling the density of pheromone dispensers assuming that disruption occurs primarily by competitive attraction.

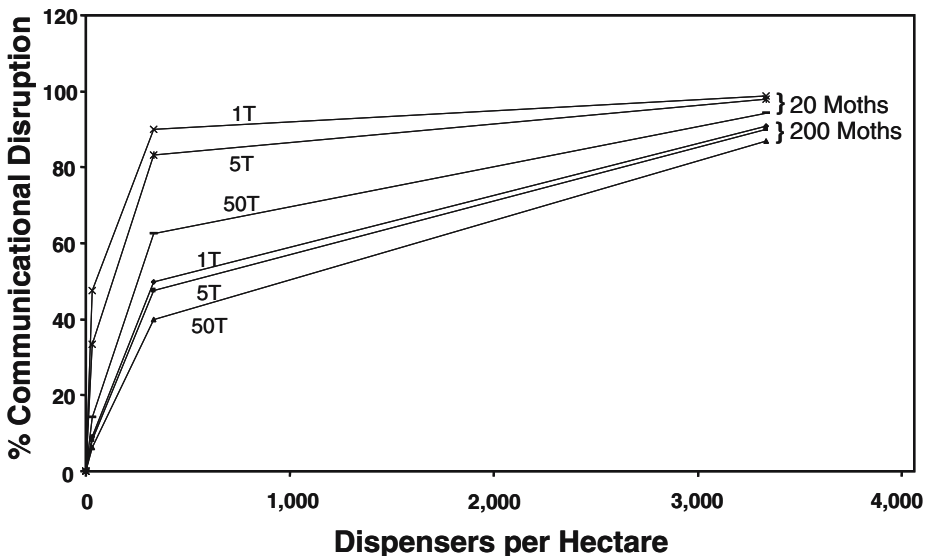


Fig. 5 Influence of trap and moth densities per hectare on simulated outcomes of communicational disruption by competitive attraction. Percent disruption in treated plots is expressed as male catch relative to that in equivalent control plots not receiving pheromone dispensers.

Variation in the Density of Females Calling

Increasing the density of calling females decreases simulated male visitation rates to a given female (Fig. 6). A similar pattern would emerge for catch of males per monitoring trap. This effect is pronounced only at low dispenser densities. During early emergence of adult moths of a given generation, the proportion of females in the population that call would be maximal and then drop as females exit the reproductive pool. A large number of females competing with traps could suppress male catch markedly in the beginning of the adult generation. The simulations in Fig. 3b suggest that, early in a moth generation, male catch could be slightly less than 1 male/trap/night for moth densities varying across 3 orders of magnitude at low pheromone dispenser densities. On a night when many females call, a catch of 1 male/trap/night indicates that every calling female would mate that night. However, as the majority of females became mated and few or none were left to call, male catch/trap/night could increase by orders of magnitude. This would result in the disruption profiles of Fig. 3b becoming increasingly linear and their actual y-intercepts would more nearly match their projected y-intercepts, i.e., the female depressive effect on male catch would be removed (Fig. 3b).

The idea that calling females can depress catch of male moths in pheromone-baited traps has been documented in the field. For example, Kehat et al. (1985) recorded a strong depressive effect of abundant virgin females on catch of *Spodoptera littoralis* males in pheromone-baited traps deployed in cotton. Catch of males was initially very low when males and virgin females began eclosing simultaneously in the crop and abundant mating pairs were directly observed. Catch in pheromone-baited traps began increasing and peaked

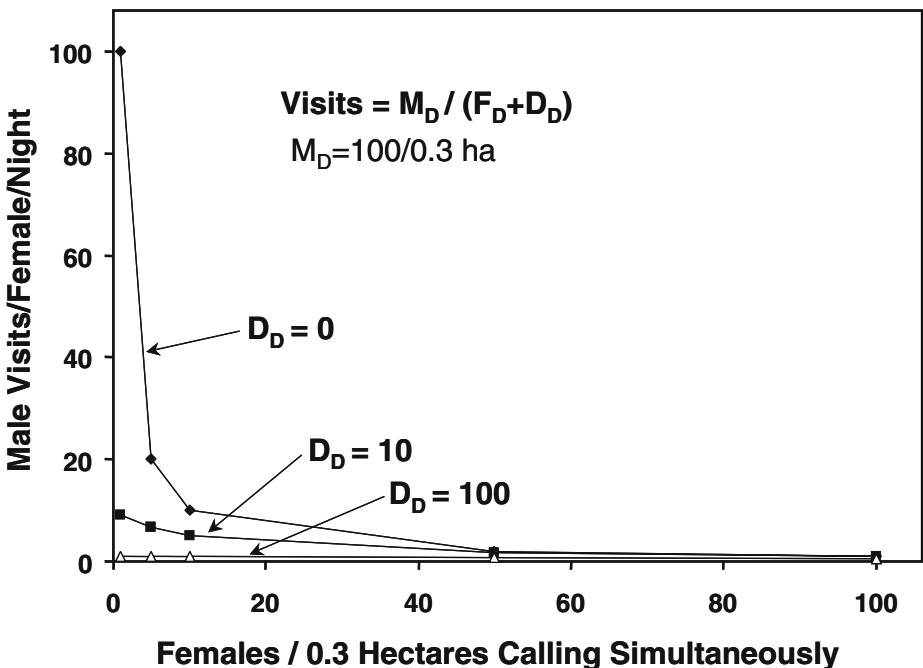


Fig. 6 Influence of dispenser and female moth densities on male visitation rates per female in simulated outcomes of disruption by competitive attraction.

5–6 after peak abundance of visible mating pairs. These authors cite similar findings for other moth species, where temporal patterns in appearance and abundance of virgin and mating moths were compared with highly resolved trapping data.

Shifting female competition for males over time may explain why catch of male moths in monitoring traps has proven to be poorly or indirectly correlated with crop damage (Knight, 1995; Gut et al., 2004). A catch of 1 male/trap/night can be obtained from the same moth population that later yields e.g., 30 males/trap/night, depending on how many females are unmated. However, the number of females mating on nights yielding catches of one or fewer males per night could have been many times higher than on nights yielding catches of 30 males/trap/night. Inconsistency between catches in pheromone monitoring traps and actual moth mating frequencies is an explicit prediction of competitive-attraction phenomena. A poor or offset relationship between male catch and crop damage can follow.

Variation in Moth Spatial Distribution

Barclay (1992) and Barclay and Judd (1995) pointed out that outcomes of competitive attraction vary depending on whether senders and receivers are regularly dispersed or clumped. Translated to the context of Fig. 1, it follows that mating would be more likely if multiple males and females were found on a given tree than when randomly distributed as in Fig. 1. Barclay (1992) explored the magnitude of this effect through simulation modeling and “found that control [by competitive attraction] would be about four times as difficult for a population that is highly clumped (k of the negative binomial distribution = 0.25) as for a regularly dispersed population.” Thus, where pests clump, the density of pheromone dispensers operating by competitive attraction will need to be increased at least several fold over the value suggested by our simulations that assumed a regular or random distribution.

Predictions of Alternative, Non-Competitive Models

The tradition of *strong inference* (Platt, 1964; Giere, 1984) recommends that researchers detail the predictions of all the alternative models potentially explaining a phenomenon and determine where the sets of predictions for explanatory models do or do not overlap. Strong tests aim at non-overlapping predictions. Although it is not feasible here to detail the predictions from all explanatory models and their combinations that might possibly contribute to pheromone disruption, we address those for which overlap in predictions is critical to valid interpretation of field data to be analyzed in the following paper (Miller et al., 2006).

Camouflage (Signal Jamming) and Its Predictions

Camouflage is proposed as the explanation for mating disruption where a calling female cannot be recognized and located by males encountering her pheromone plume because its guiding boundaries are obscured by ubiquitously present synthetic pheromone. Such a mechanism of communicational disruption seems better explained as *jamming* of the female’s signal—making it unintelligible by broadcasting signal of the same type—rather than blending it into a natural, heterogeneous background, as the term *camouflage* usually implies. Nevertheless, *camouflage* is entrenched in the literature and useful if defined. Under a pure camouflage model of pheromone disruption, males would not be attracted to pheromone dispensers. However, they would always retain the ability to orient to females

and traps that happen to emit pheromone into air not already containing a camouflaging background dosage.

It follows that each camouflaging plume released from a dispenser precludes males from finding females in that plume. If plumes from camouflaging dispensers were of average area (x) and not so dense as to overlap, then the total area of crop camouflaged (A_C) would be approximated by the linear function:

$$A_C = D_D * x. \quad (14)$$

If moths did not exit camouflaging plumes, efficacy of disruption by camouflage could be calculated as:

$$\% \text{ Disruption} = (1 - P_{\text{Auc}})100 = P_{\text{Ac}} * 100 \quad (15)$$

where P_{Auc} is defined as the proportion of total crop area that is uncamouflaged and P_{Ac} is defined as proportion of total crop area that is camouflaged. Assuming an even distribution of male and female moths, an average of 16 females and 16 males would not mate if 80% of the crop shown in Fig. 1 containing 20 male and 20 female moths were camouflaged. Assuming that the four uncamouflaged females mated with the four available males, this would yield a disruption of 80%. Female visitation rate would decrease linearly with dispenser density and disruption would be independent of moth density. Both of these predictions differ strikingly from those of competitive attraction.

Movement of female and male moths without regard to the distribution of pheromone from camouflaging dispensers is likely to diminish rather than enhance mating disruption over the situation where moths do not exit camouflaging plumes. If even brief periods in clean air resulted in successful calling, any receptive female spending a portion of her diel reproductive period outside a camouflaging plume could potentially mate. If so, the probability of remaining unmated would mirror the probability of never exiting a camouflaging plume upon successive flights. Under this scenario, efficacy of mating disruption would initially increase slowly as a function of dispenser density and then increase dramatically as plume coverage approached 100%. Such a disruption profile differs dramatically from that predicted for competitive attraction (Fig. 2a).

Temporarily invoking mixed explanatory models, disruption efficacy could be dramatically inferior to that predicted by Equation (15) if virgin females were repelled or deterred from calling by plumes from camouflaging dispensers. On the other hand, disruption efficacy would be enhanced if males were attracted into and remained within camouflaging plumes, while females aggregated outside camouflaging plumes. Here, competitive attraction would again be a major component of that overall phenomenon.

Quantitative Contrast of Predictions of Competitive Attraction vs. Camouflage

The above discussion suggests that mating disruption by camouflage generates a set of predictions different from that of competitive attraction. Explanations involving moth movement (but not attraction) diverge even further from the predictions of competitive attraction than does Equation (15). Thus, Equation (15) was selected for further contrast with competitive-attraction predictions of Equation (9), so as to differentiate among them quantitatively.

Adding traps into crops disrupted by camouflage so as to measure mating disruption injects a small element of competitive attraction. This causes a male-catch measure of disruption to vary somewhat with moth density. Males not camouflaged can respond either to females or to traps that are not camouflaged. Thus, for trapping data, the camouflage

equation needs to be modified to reduce male visitation rate to females by a proportion attributable to that absorbed on average by the trap used to measure the experimental outcome. This adjustment can be made as follows where $V_{/F}$ represents male visits/female/night and A the total crop area:

$$V_{/F} = M_D / (F_D + T_D) (A - A_c) \quad (16)$$

We simulated camouflage outcomes by two methods applied to Fig. 1. First, Equation (16) was employed after the proportion of Fig. 1 that was camouflaged was calculated by multiplying the measured area of one dispenser plume by the number of plumes to be overlaid and then subtracting the product from total crop area. Second, the proportion of area not covered in Fig. 1 by the various densities of plumes was measured experimentally, before invoking Equation (16). Using Microsoft PowerPoint™, varying densities of dispenser plumes were overlaid in a regular pattern onto the 72-tree orchard plot of Fig. 1, while minimizing plume overlaps. Areas covered by plumes from dispensers were blackened, whereas uncovered areas remained white. The percentage of total area that was white vs. black in each treatment picture so generated was quantified by using Scion Image™ software (National Institute of Standards and Technology, Gaithersburg, MD, USA; <http://www.nist.gov/lispix/imlab/>).

Outcomes of our first approach to camouflage simulations are compared in Fig. 7 with those for competitive attraction when both moth and dispenser density were variables. Camouflage profiles fell linearly with increasing dispenser density (Fig. 7a); the reduced slopes with reduced moth densities reflect the competitive effect of the monitoring trap. A similar outcome resulted from the overlay simulations (hence data not shown). However, beyond the highest dispenser densities of Fig. 7a, appreciable overlap of plumes was unavoidable. The effect was that additional crop coverage per added dispenser progressively declined. It was difficult to blot out the last vestiges of white from Fig. 1 even when 200 plumes were overlaid with the restriction that they must originate from a tree and not the aisles between rows of trees. Thus, both camouflage and competitive attraction simulations approached 100% disruption asymptotically.

We caution that experimental proof of an asymptotic profile for camouflage is required before much weight can be placed upon pictorial over-lay simulations. We have little confidence that pheromone dispersal can be approximated by such elementary modeling. It seems likely that turbulence and diffusion would homogenize pheromone distribution within the crop over time, particularly if fields were large and dispenser densities were high. Moreover, the simulations were conducted in two- rather than three-dimensional space. To disappear from the crop, pheromone would need to rise above the treated crop, exit from field edges, or be irreversibly absorbed or degraded. Alternatively, pheromone molecules could reversibly become adsorbed on solid surfaces (Karg et al., 1994; Gut et al., 2004), allowing buildup of pheromone to some above-threshold steady-state. Thus, camouflage should result in total communicational disruption. So, where moth densities were high and sample sizes robust, a linear or near-linear descent of a disruption profile to zero male visits per trap or female can be taken as strong evidence for one of the noncompetitive disruption mechanisms, like camouflage. Such a profile is inconsistent with competitive attraction. However, until the ambiguity is removed as to whether or not plumes from point-source dispensers of pheromone do or do not become spatially homogenized over time, an asymptotic approach of disruption profiles toward an efficacy of 100% cannot be used as a criterion to unequivocally differentiate between competitive vs. noncompetitive disruption mechanisms.

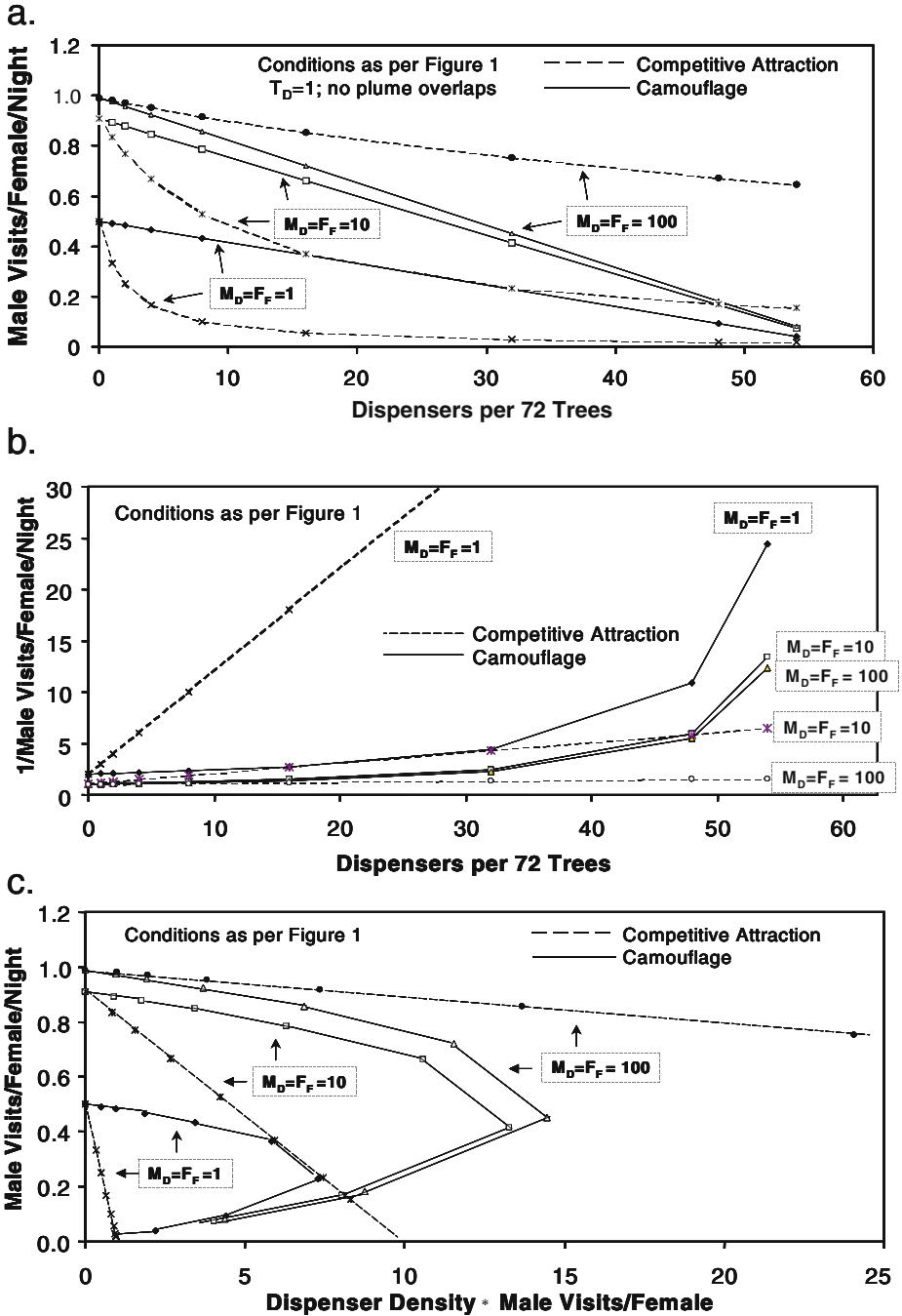


Fig. 7 Comparison of dosage–response profiles for simulated communicational disruption outcomes mediated by competitive attraction vs. camouflage. Plot types are (a) untransformed; (b) Miller–Gut; and (c) Miller–de Lame. In (b), outcomes for the lower two camouflage plots fall mostly on top of one another.

Nevertheless, Fig. 7 offers other distinctive predictions for such differentiation. The high sensitivity of competitive attraction outcomes to pest density has already been emphasized. With successive increases in moth density, disruption profiles become increasingly less concave on graphical plots (Figs. 3a and 7a). In contrast, the shape of camouflage profiles of catches of males in traps changes only slightly when moth densities are appreciable (Fig. 7a). The greatest influence of moth density on male visits/female/d in the Fig. 7a simulations occurred at the extreme of only one female competing with one trap. As the density of female moths increased, presence of a trap had progressively less influence on measured outcome. It is important to recognize that relative efficacy of communicational disruption operating by camouflage would not be influenced by pest density if pheromone dispensers were completely unattractive and no monitoring traps were inserted into the system. This is yet another case where applying an instrument (attractive trap) for measuring the performance of a system slightly alters the system.

Under extremely high pest densities, the predictions of competitive attraction and camouflage by large plumes become increasingly similar (Fig. 7a). Furthermore, simulations suggest camouflage would out-perform competitive attraction at high dispenser and pest densities. At very high pest densities, it is also possible that male and female moths may begin finding one another by non-chemical means (e.g., visual search; Levinson and Hoppe, 1983), further blurring interpretation of disruption mechanisms. If data analysis were limited only to untransformed plots like Fig. 7a, differentiation of camouflage vs. competitive attraction mechanisms would be best achieved at low pest and low dispenser densities.

Simulated camouflage and competitive-attraction outcomes were compared by using both Miller-Gut (Fig. 7b) and Miller-de Lame (Fig. 7c) plots in preparation for pattern recognition during comparisons of disruption data from the field (Miller et al., 2006) with data from simulations. A key distinction between mechanism classes is that competitive attraction yields straight lines on both types of secondary plots, whereas camouflage yields concave profiles on Miller-Gut plots (Fig. 7b) and distinctively recurved profiles on Miller-de Lame plots (Fig. 7c). Mathematical simulations confirmed a general rule that, when D_D causes a linear decrease in C , the maximum $D_D C$ for plots of C vs. $D_D C$ always equals $\frac{1}{2}C_{\max}$. This is because $D_D C$ values are being generated by the quadratic equation— $D_D C = -mD_D^2 + C_{\max}D_D$ —resulting from substituting $-mD_D + C_{\max}$ for C in the equation $y = CD_D$. Quadratic equations always yield symmetrical plots with their maximum midway between their intercepts, in this case 0 and C_{\max} .

Finally, the most direct way to differentiate whether camouflage or competitive-attraction mechanisms of disruption are operating under field conditions is to observe whether males orient to and closely approach pheromone sources. Such occurrences are inconsistent with pure camouflage, but are required by competitive attraction. However, experimenters would need to guard against false negatives. Under a high density of pheromone dispensers and a moderate pest density, the probability that males would be attracted to a dispenser under observation is low for competitive attraction. However, overall visitation rate to this type of source could be high when considering all such dispensers. Observational time required to acquire strong evidence either for or against competitive attraction or camouflage by directly observing occurrence or absence of orientations by male moths would be equally great.

Desensitization and Its Predictions

We take *desensitization* to mean that synthetic sex pheromone released from point sources in a crop causes male moths exposed to the pheromone to become less sensitive and/or less

responsive than they normally would be to female-produced pheromonal signals or their equivalents. Adaptation of olfactory receptor neurons of male moths can occur following continuous exposure of antennae to pheromone (Kuenen and Baker, 1981; Baker et al., 1988, 1989). This phenomenon is dosage-dependent, but reversible in pheromone-free air. A “long-lasting” adaptation has been described for several moth species (Kaisling, 1986; Stelinski et al., 2003a,b; Judd et al., 2005); it lasts for 10 to >60 min after the removal of moths from air containing high concentrations of pheromone. Pre-exposure to continuous or pulsed pheromone can decrease behavioral responsiveness to normal pheromone signals in many moth species (Bartell and Roelofs, 1973; Bartell and Lawrence, 1976; Sanders, 1985; Rumbo and Vickers, 1997; Daly and Figueredo, 2000). These behavioral modifications after exposure to volatilized pheromone last much longer than antennal adaptation, suggesting habituation (Bartell and Roelofs, 1973; Figueredo and Baker, 1992; Rumbo and Vickers, 1997; Stelinski et al., 2003a,b; Judd et al., 2005).

Despite the considerable attention desensitization has received as a candidate for explaining mating disruption, there is substantial evidence in the literature against it. The major inconsistency is that effective mating disruption occurs in crops at pheromone concentrations much lower than those required for desensitizing moths by adaptation or habituation (Schmitz et al., 1997; Rumbo and Vickers, 1997; Judd et al., 2005). Such data suggest that desensitization is not a leading contributor to mating disruption. For example, male grapevine moths (*Lobesia botrana*) were able to find pheromone-baited traps in the field following 8 hr of confinement in vineyards treated with mating disruption dispensers (1 dispenser/5 m²; each dispenser contained 500 mg of (E7,Z9)-dodecadienyl acetate; Schmitz et al., 1997). Reduction in the numbers of males captured in optimized pheromone traps in the field was obtained only when males were pre-exposed in the laboratory to pheromone at 4 µg/l air. This concentration vastly exceeds the average airborne concentration of pheromone (1–2 ng/m³) achieved in a crop treated with hand-applied mating disruption dispensers (Koch et al., 1997, 2002). In another example, reduction of male oriental fruit moth (*Grapholita molesta*) captures in optimized monitoring traps in the field occurred only after 1 hr of laboratory exposure to pheromone at 65 µg/m³ (3200 female equivalents/m³) (Rumbo and Vickers, 1997). Again, this high atmospheric concentration of pheromone required to desensitize *G. molesta* males far exceeded that achieved under typical mating disruption conditions in the field plots.

Long-lasting peripheral desensitization in obliquebanded leafroller (*C. rosaceana*) occurred only after minutes-long confinement in the laboratory in containers with pheromone concentrations of at least 1 ng/ml, or in the field after 1 d of confinement a few centimeters from polyethylene tube dispensers (Stelinski et al., 2003b). Given that feral *C. rosaceana*, *A. velutinana*, *G. molesta*, and *C. pomonella* approach and remain near such pheromone dispensers for only ca. 30 sec in apple trees (Stelinski et al., 2004a, 2005a), it is unlikely that this level of pheromone exposure is sufficient to induce long-lasting adaptation.

Nevertheless, key predictions of the desensitization model of mating disruption are: (1) efficacy should increase with dosage of pheromone released and thoroughness of coverage; (2) compared to that for competitive attraction, efficacy for desensitization would be relatively independent of pest density at low to moderate pest populations for reasons identical to those presented above for camouflage; and (3) the overall frequency with which male moths are observed orienting toward pheromone point sources in disruption plots would be greatly reduced relative to responses to similar sources in plots not treated with pheromone. Under increasing dispenser densities, male moths would have commensurately less chance to find pheromone-free spaces where they could recover normal sensory and behavioral capability. Thus, at high dispenser densities, disruption profiles for desensiti-

Table 2 Distinguishing traits of the two major categories of mating-disruption mechanisms

Trait	Competitive ^a	Non-competitive ^b
Shape of disruption profiles	Concave profile on untransformed plot; straight line with positive slope on M–G ^c plot; straight line with negative slope on M–de L ^d plot	Straight line on untransformed plot; concave profile on M–G plot; recurved profile on M–de L plot ^e
Convergence of efficacy profiles	Converge at coordinates $-T_D$, $+(F_D/T_D)/M_D$ on M–G plot when attractiveness of $T = F = D$	No regular pattern of convergence on M–G plots
Influence of pest population on disruption efficacy	Strong	Little to none except when pest density rises to where mate-finding is possible without long-range pheromone
Sensitivity to plume size	Efficacy not highly sensitive to plume size so long as plume size for $T = F = D$	Efficacy directly proportional to plume size

^a Competitive attraction.

^b Includes camouflage, desensitization (adaptation; habituation), and sensory imbalance.

^c Miller–Gut plot = 1/catch on y-axis vs. dispenser density on x-axis.

^d Miller–de Lame plot = male catch on y-axis vs. dispenser density * catch on x-axis.

^e Certain types of non-competitive disruption are possible that deviate from these given predictions in ways that make them more dissimilar to the predictions of competitive attraction.

zation could drop even faster than those for camouflage. Such profiles should reach 100% efficacy at high densities of pheromone point sources.

Sensory Imbalance and Its Predictions

Sensory imbalance (Bartell, 1982; Flint and Merkle, 1983) is defined here as *disrupting mate-finding not via adaptation or habituation but by interfering with the male's ability to perceive (as opposed to receive) the normal sensory inputs associated with their species' sex pheromone*. For example, dispensing only one of the major components of a pheromone blend from dispensers could adulterate the normal blend of a female's plume that commingles with the dispenser plume, rendering her plume unrecognizable to males. Sensory imbalance shares the above predictions for desensitization.

Combinations of Explanatory Models

Until now, this paper has emphasized non-overlapping explanatory models. However, we recognize that, for moths under field conditions, these mechanisms may come into play sequentially or in combinations. For example, competitive attraction may bring males close to dispensers where desensitization might occur for certain pests. Thus, in evaluating actual field data, care needs to be taken to recognize patterns in outcomes indicative of single mechanisms acting alone, as well as multiple mechanisms perhaps acting sequentially or concurrently.

Conclusions

This analysis revealed that mating disruption by competitive attraction operates predominantly by division; the attention of males is literally divided between a given

female and nearby dispensers. On the other hand, non-competitive disruption operates predominantly by subtraction; moths in effectively treated areas of crop are literally subtracted from the total pool. Key traits useful in differentiating between these mechanisms are summarized in Table 2. They will be used to judge which category of mechanism is supported by actual experimental disruption profiles from the pheromone literature (Miller et al., 2006).

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disruption by pheromone point sources are listed. We believe that the congruence of diverse sets of mating disruption field data with explicit *a priori* predictions validates competitive-attraction theory. The analytical tools and principles governing competitive attraction that were uncovered during this study of mating disruption of moths should be generally applicable to competitive-attraction phenomena.

Keywords Mating disruption mechanisms · Profile analysis · Percent disruption · Dispenser activity · Dispenser application activity

Introduction

Miller et al. (2006, this issue) reported analytical procedures and criteria whereby mechanisms of mating disruption by point sources of sex pheromone can be differentiated into two main categories: competitive (false-plume following) vs. non-competitive (camouflage, desensitization, and sensory imbalance). In this report, we apply those procedures to case studies mainly from the published literature on mating disruption of moths. The objectives were to: (1) determine to what extent experimental disruption profiles were congruent with the various theoretical predictions detailed by Miller et al. (2006); (2) determine whether one category of disruption is more common than the other; and (3) begin to interpret the practical consequences of the results of these analyses.

Methods

We searched the literature for experimental data sets allowing graphical analysis of suppression of catches of male moths in monitoring traps vs. density of point sources of synthetic sex pheromone. A target of at least three densities of pheromone dispensers in addition to zero pheromone narrowed the prospective cases. Ideally (Miller et al., 2006), such experiments would have recorded dispenser density (D_D), trap density (T_D), relative attractiveness of traps (T), females (F), and dispensers (D), catches of males (C) over most of a moth generation, and densities of female (F_D) and male moths (M_D) (see Table 1 of Miller et al., 2006 for a key to abbreviations). However, we found no study where all these parameters were recorded. In all cases analyzed in this work, data were sufficient for valid determinations of competitive vs. non-competitive disruption. However, moth densities computed from the competitive-attraction equations of Miller et al. (2006) could be compared with measured densities in only a few cases because relative attractiveness of T , F , and D usually was not measured.

Disruption profiles were examined with three types of graphical plots: untransformed (C vs. D_D), Miller-Gut ($1/C$ vs. D_D), and Miller-de Lame (C vs. $C^* D_D$) (Miller et al., 2006). Patterns in profiles consistent with competitive vs. non-competitive disruption are summarized in Table 2 of Miller et al. (2006). Where applicable, straight lines were fit to data by weighted regression. In most cases, statistical analyses were not possible because the raw data were not accessible. For our own field experiments, statistical procedures are explained in the respective figure captions.

When estimating or interpreting male and female densities from given dosage–response profiles, we elected to first show the calculations most simply by operating on a starting premise that attractiveness of $T = F = D$. Judgments about fit of actual field data to theory should be withheld until the justification for departure from attractiveness of $T = F = D$ is

revealed and the appropriate mathematical adjustments are made. Most cases ultimately supported a competitive-attraction equation more complex than Equation (1) of Miller et al. (2006) where attractiveness of $T = F = D$.

Results and Discussion

Cases Consistent with Competitive Attraction and Inconsistent with Camouflage or Other Non-competitive Disruption Mechanisms

Oriental Fruit Moth (Grapholita molesta)

Rothschild (1975) conducted one of the most complete disruption experiments useful to the current analyses when he documented communicational disruption of oriental fruit moth (OFM) in Australian peaches. The pheromone formulation was 98% (*Z*)-8-dodecenyl acetate (2% *E*-isomer) dispensed from 1 ml closed polyethylene tubes (precursor to pheromone ropes) each releasing ca. 15 µg/hr of pheromone. This pheromone blend did not fully match that of the female, now known to also include ca. 1% (*Z*)-8-dodecenyl alcohol (Cardé et al., 1979). In a test originally aimed at determining the number of dispensers required to prevent males from finding pheromone-baited monitoring traps, dispensers were deployed in duplicate 100 tree plots (0.4 ha) at 0, 0.5 (every other tree), 1, and 2 dispensers per tree. Twelve monitoring traps were deployed in a 3 × 4 grid in each plot. Trap catches were reported as the total for all 12 traps/plot over three sampling periods of 20–30 d spanning each of three moth generations (Rothschild, personal communication). Before this test began, the OFM population was quantified at 0.6–1 pupae per tree based on tree banding, and subsequently at 0.5–1 moths per tree by counting OFM adults recovered from cloths laid under trees fogged with pyrethrum. Assuming an equal sex ratio, this equates to an F_D and M_D of 0.4/tree, or about 150 moths/ha of each sex.

Rothschild's disruption outcome is scaled to 1 ha and plotted on untransformed axes in Fig. 1a, as a Miller-Gut plot in Fig. 1b, and as a Miller-de Lame plot in Fig. 1c. Although few, the data support an interpretation of competitive attraction. The untransformed plot (Fig. 1a) is more curvilinear than linear. It resembles the competitive-attraction profile in Fig. 3a of Miller et al. (2006) for a simulated moth population of about 30 moths per 72 trees (0.4/tree). The initially steep drop in catches of males with dispenser density leveled out by ca. 1 dispenser per tree. This data set is reasonably linear on a Miller-Gut plot (Fig. 1b), which then justifies derivation of secondary measures. The density of males in this experiment can be estimated (procedures of Miller et al., 2006) from Fig. 1b as: $1/\text{slope} = M_D T_D = 1/0.0004 = 2,500$. Since $T_D = 30/\text{ha}$, calculated $M_D = 83$ males/ha compared to the measured value of 150 males/ha. Allowing for some mortality and a trap efficiency of less than 100%, it is not surprising that this derived value of M_D was somewhat lower than the initial M_D directly measured by Rothschild at the start of the adult generation. Female density in this test can be derived from the absolute value of the *x*-intercept = $0.0213/0.0004 = 53 = T_D + F_D$. Because $T_D = 30/\text{ha}$, $F_D = 23$ females/ha. The derived F_D/M_D ratio is $23:83 = 1:3.6$. A time-averaged F/M ratio favoring males is expected in the kinetics of competitive attraction for OFM, whose females are not known to mate frequently, whereas males continue to seek females throughout their lives. Therefore M_D would be the more stable measure of OFM density during a given generation. However, the accuracy of such F/M ratio calculations,

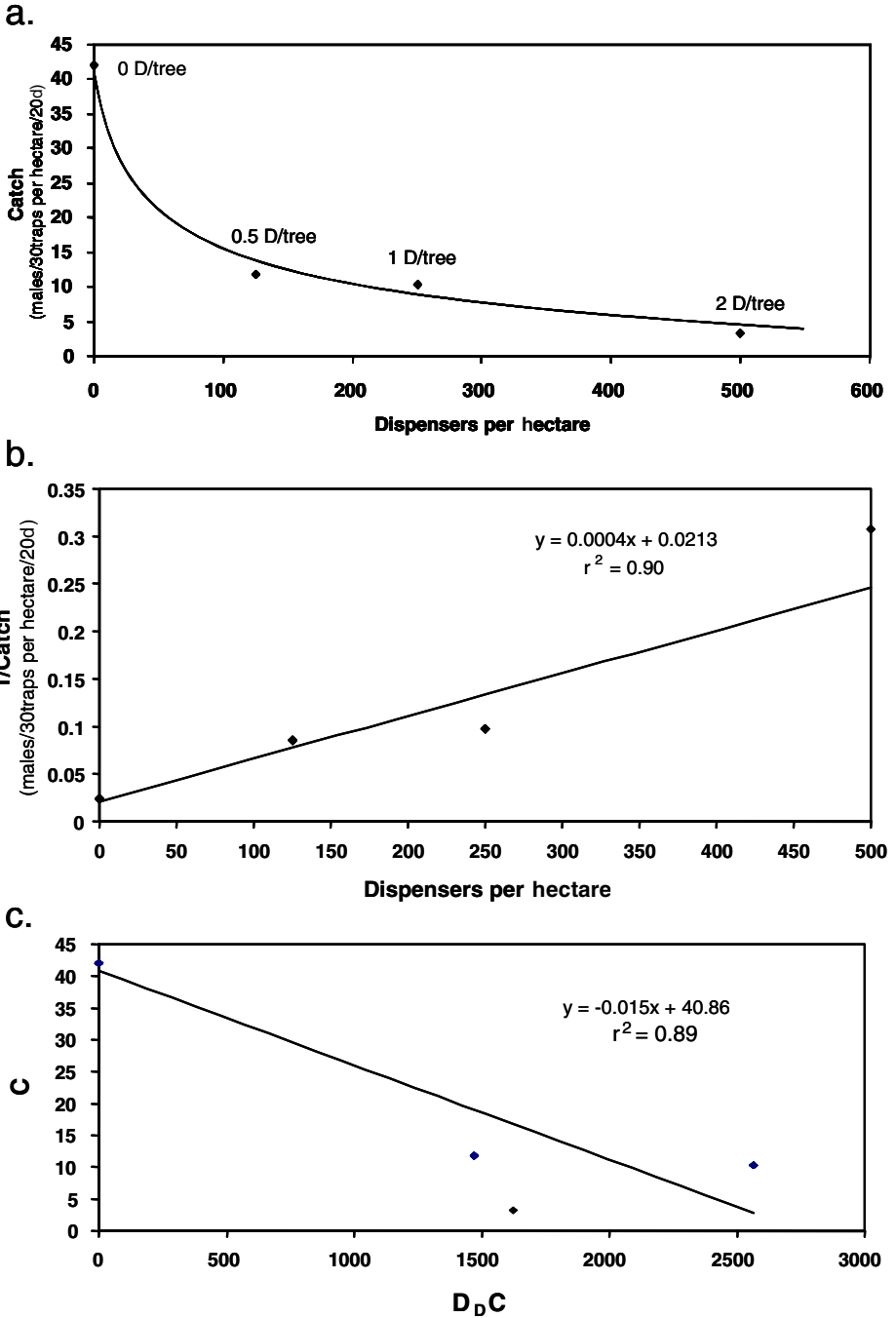


Fig. 1 Untransformed (a), Miller-Gut (b), and Miller-de Lamé (c) plots of the disruption profile from Rothschild’s (1975) test deploying polyethylene tube dispensers of pheromone targeting oriental fruit moth, *Grapholita molesta*, in peaches. The curve for panel (a) was hand-drawn and fit by eye. The straight lines in (b) and (c) (as well as those in subsequent figures) were fit by weighted regression

as well as moth density calculations, are based on the untested assumption that attractiveness of $T = F = D$ in Rothschild's test. The adequate agreement between calculated and measured M_D as well as a reasonable F/M ratio in this test supports approximate equivalency in attractiveness of T , F , and D in this particular case.

The Miller-de Lame plot of Rothschild's (1975) data (Fig. 1c) is also consistent with competitive attraction. The linear fit is reasonable given the few data and the susceptibility of the lowest catches of males to sampling error. There was no convincing evidence of a consistent recurve having its inflection near $1/2y_{\max}$ like that expected for non-competitive mechanisms such as, e.g., camouflage (Fig. 7c of Miller et al., 2006). However, the current analysis would have greatly benefited by including $D_D C$ values falling between 0 and 1400. The estimate of M_D as derived from the x -intercept of Fig. 1c = $T_D M_D$ is 91 males/ha compared to 83 for the Miller-Gut plot. This translates into an estimate of 0.23 males/tree, and falls near the measured range (0.25–0.5) for M_D . F_D can be derived from the inverse of the slope of this Miller-de Lame plot: absolute value of $1/\text{slope} = (T + F) = 67$. Since $T_D = 30$, $F_D = 37$. Thus, the F/M_D ratio derived from the Miller-de Lame plot is 37:91 or 1:2.5, which is similar to the Miller-Gut estimate as required by competitive attraction theory based on Equation (1) of Miller et al. (2006).

Collectively, Rothschild's OFM data are a reasonable fit to the full set of predictions generated from our competitive-attraction model of pheromone disruption. The probability of having the data fit all these predictions by chance alone appears small. No evidence was uncovered that refuted competitive attraction for this study. Conversely, this data set is inconsistent with camouflage or other non-competitive mechanisms of pheromone disruption.

Only after fit is found consistent with competitive attraction and inconsistent with other explanatory models is it appropriate to calculate a dispenser activity (D_a) value by using procedures unique to competitive attraction. Using Equation (4) of Miller et al. (2006), D_a for Rothschild's dispenser = slope (from Fig. 1b)/ y -intercept = $0.0004/0.0213 = 0.019$. D_a , as directly revealed by the slope of Fig. 1c, was 0.015. When using the latter value and invoking Equation (6) of Miller et al. (2006), D_{Aa} for Rothschild's dispenser densities of 125, 250, and 500 dispensers/ha = 1.9, 3.8, and 7.5, respectively.

As pointed out in Miller et al. (2006), D_a values are theoretically independent of the units used to record true catches. Nevertheless, all values accumulated in Table 1 were derived by using units of male catch/trap/d, for the sake of consistency. However, valid calculations of moth densities and sex ratios must be done with catch values for actual trap densities accumulated over the total elapsed time of the test, as per the Fig. 1 example.

We (Stelinski et al., 2005c) recently quantified OFM communicational disruption in response to an emulsified wax formulation (Atterholt, 1996, 1998; De Lame, 2003) of pheromone releasing a 93:6:1 blend of (*Z*)-8-dodecenyl-acetate: (*E*)-8-dodecenyl-acetate: (*Z*)-8-dodecenol, present in the wax at a total starting concentration of 5%. This experiment was conducted in 12-tree (0.05 ha) rectangular plots of apples in Western Michigan (USA). Drops of wax (0.1 ml) were applied by syringe to branches and twigs of the trees with the aim of achieving a uniform distribution from the bottom of basal limbs to treetop. There were five replicate plots with densities of wax drops per tree of: 0, 3, 10, 30, and 100. Two sticky traps deployed in the center two trees of each plot and baited with optimized OFM monitoring lures measured male catches. A new set of wax drops was applied after each OFM adult generation, when rising trap catches revealed waning efficacy due to pheromone depletion, confirmed also by gas chromatographic analysis. During the 40 d of effective disruption per application, release rate per 0.1 ml wax drop fell from ca. 0.4 to 0.1 $\mu\text{g/hr}$ /drop.

Table 1 Comparison of Moth Communicational Disruption Outcomes Mediated by Competitive Attraction

Insect	Reference	Pheromone composition	Type of dispenser	Release rate per dispenser ($\mu\text{g/hr}$)	Experimental plot size (ha)	Maximum dispensers/ha	Maximum % disruption ^a	C_{max} (males/trap/night)	D_a (M–G slope/ \pm -intercept) ^b	D_{sa} ($D_d/\mu\text{g}$ pheromone/hr)	Maximum D_{sa} (D_a^* D_b)
Oriental fruit moth	Rothchild (1975)	Z8-12:OAc 97% E8-12: OAc 3%	Closed polyethylene tube	15	0.4 (100 trees)	500 (2/tree)	92	1.7	0.021	0.0014	11
Oriental fruit moth	Stelinski et al. (2005b)	Z8-12:OAc 93% E8-12: OAc 6% Z8-12:OH 1%	Wax drops (0.1 ml)	0.1–0.4 (0.25)	0.05 (12 trees)	27,300 (100/tree)	99.4	5.1	0.0071	0.028	194
Pink bollworm	Flint and Mertle (1983)	Z7:Z11-16:OAc 50% Z7: E11-16:OAc 50%	Rubber septa	Unknown (1 mg load)	0.03	1,821	88	78	0.0038	–	6.9
Obliquebanded leafroller	Lawson et al. (1996)	Z11-14:OAc 83% E11-14:OAc 8% Z11-14:OH 9%	Polyethylene tube	25	0.04 (12 trees)	960 (3/tree)	70	0.9	0.0022	0.00009	2.1
Obliquebanded leafroller	Deland et al. (1994)	Z11-14OAc 93% E11-14: OAc 7%	Polyethylene tube	20	0.09	2,000	90	0.3	0.0052	0.00026	10
European leafroller	Deland et al. (1994)	Z11-14OAc 93% E11-14: OAc 7%	Polyethylene tube	20	0.09	2,000	96	0.8	0.0111	0.00056	22
Fruitree leafroller	Deland et al. (1994)	Z11-14OAc 93% E11-14: OAc 7%	Polyethylene tube	20	0.09	2,000	99.4	9.0	0.0982	0.00491	196
Gypsy moth	Tchelslavskaita et al. (2005)	\pm disparture	Laminated flakes	0.093	25	134,409	99.1	0.8	0.00052	0.00056	70
Light brown apple moth	Suckling and Angerelli (1996)	E11-14:OAc 71% E9, E11-14:OAc 3% Z9-14: OAc 26%	Polyethylene tube	10.5	0.5 (561 trees)	200	97.4	0.3	0.19	0.018	38
Beet armyworm	Shorey et al. (1994)	Z9:E12-14:OAc	2-cm-long Teflon™ capillaries	1 for 1.32 diam capillary	0.0056–0.053	6,429	98.9	18.3	0.02	0.02	129

^a % Disruption = (1 – (male catch under pheromone treatment/male catch for control)) 100.

^b Number of dispensers/ha required to suppress catch by 50% is given by $1/D_a$.

Periodically during this experiment, observers positioned themselves to be able to observe and record behaviors of male moths in the vicinity of wax drops. Moth behaviors were directly observed within tree canopies on 26 total nights throughout three generations of OFM flight. In each control plot, a single wax drop was randomly deployed in one tree for observation; these plots were otherwise untreated. Two or three observers worked simultaneously, rotating among plots and conducting 20-min observational bouts per treatment, such that multiple treatments were under observation concurrently. Behavioral events were dictated into a hand-held audio recorder by the observer standing ca. 1 m from a pheromone dispenser. Observations after dusk were assisted by night-vision goggles as described by Stelinski et al. (2004a).

Male OFM were commonly observed visiting wax drops containing pheromone (Stelinski et al., 2005c). More than 75% of these males approached within 20–60 cm of the pheromone source, and over 70% moved away from wax drops within 20 sec by flying upwind. None remained near pheromone dispensers for more than 2 min (Stelinski et al., 2005c). Similar results were documented for three other tortricid pests of Michigan fruit, all of which closely (ca. 0–100 cm) and briefly (ca. 2–30 s) approached Isomate-type, polyethylene tube dispensers (Stelinski et al., 2004a, 2005a,b). We conclude that males of all these tortricids are attracted to and briefly arrested by commercially available (Pacific Biocontrol Corp., Portland, OR, USA) synthetic pheromone dispensers releasing high amounts of their respective pheromones relative to females, but in a suboptimal blend.

The OFM disruption profile for wax drops, averaged across the three moth generations per season, well fits the predictions of competitive attraction. On an untransformed plot, the disruption profile was concave rather than linear (Fig. 2a). Moreover, disruption never reached 100% despite deployment of 100 wax drops per tree. However, catches of males at 30 and 100 wax drops per tree averaged less than 1 male/trap/20 d per OFM generation. Thus, to obtain a more reliable measure of central tendency, the data for the two highest dispenser densities were combined into a mean of 65 dispensers/ha. This disruption profile was perfectly linearized by a Miller-Gut plot (Fig. 2b). Analysis of these OFM data by the Miller-de Lame (Fig. 2c) plot was compromised by not having any $D_D C$ values fall near $1/2C_{\max}$.

Female vs. male densities in these plots were not directly measured experimentally. However, M_D can be calculated from Fig. 2c via the x -intercept of 820,218. Because attractiveness of D was measured at 0.1 that of T (Stelinski et al., 2005c), the intercept of $820,218 = T_D M_D / k_D$ [Equation (10) of Miller et al., 2006]. Because $T_D = 40$ traps/ha, and $k_D = 0.1$, M_D was 2,051 males/ha, or about 9 males/tree. If $k_T = k_F$, female density can be calculated from the absolute value of the slope of Fig. 2c; $1/\text{slope} = 1/0.0087 = 115 = T_D + F_D$. Because $T_D = 40/\text{ha}$, $F_D = 75$ females/ha, yielding an F/M activity-density ratio favoring males by 1:27. We consider it likely that attractiveness of females was somewhat less than that of the optimized trap that is constantly releasing pheromone. If k_F is taken at 0.1 k_T , the calculated F/M ratio becomes 1:3 and more nearly matches the Rothschild (1975) outcome. Only after this k_F value is established experimentally can this type of calculation be made with confidence.

The D_a for wax drops, as derived from the slope of Fig. 2c, was 0.009. It was 0.007 when male catch was graphed as males/trap/d (slight difference due to rounding error judged smaller for males/trap/d). The D_{sa} for wax drops (0.028) exceeded that for Rothschild's (1975) larger dispensers (0.0014) (Table 1). Perhaps this is because our formulation was a more complete blend that included the (*Z*)-8-dodecenol. Even at a considerably greater OFM density as judged by C_{\max} (Table 1) and calculations of M_D from the above respective Miller-de Lame plots, 10 wax drops per tree disrupted as well as did

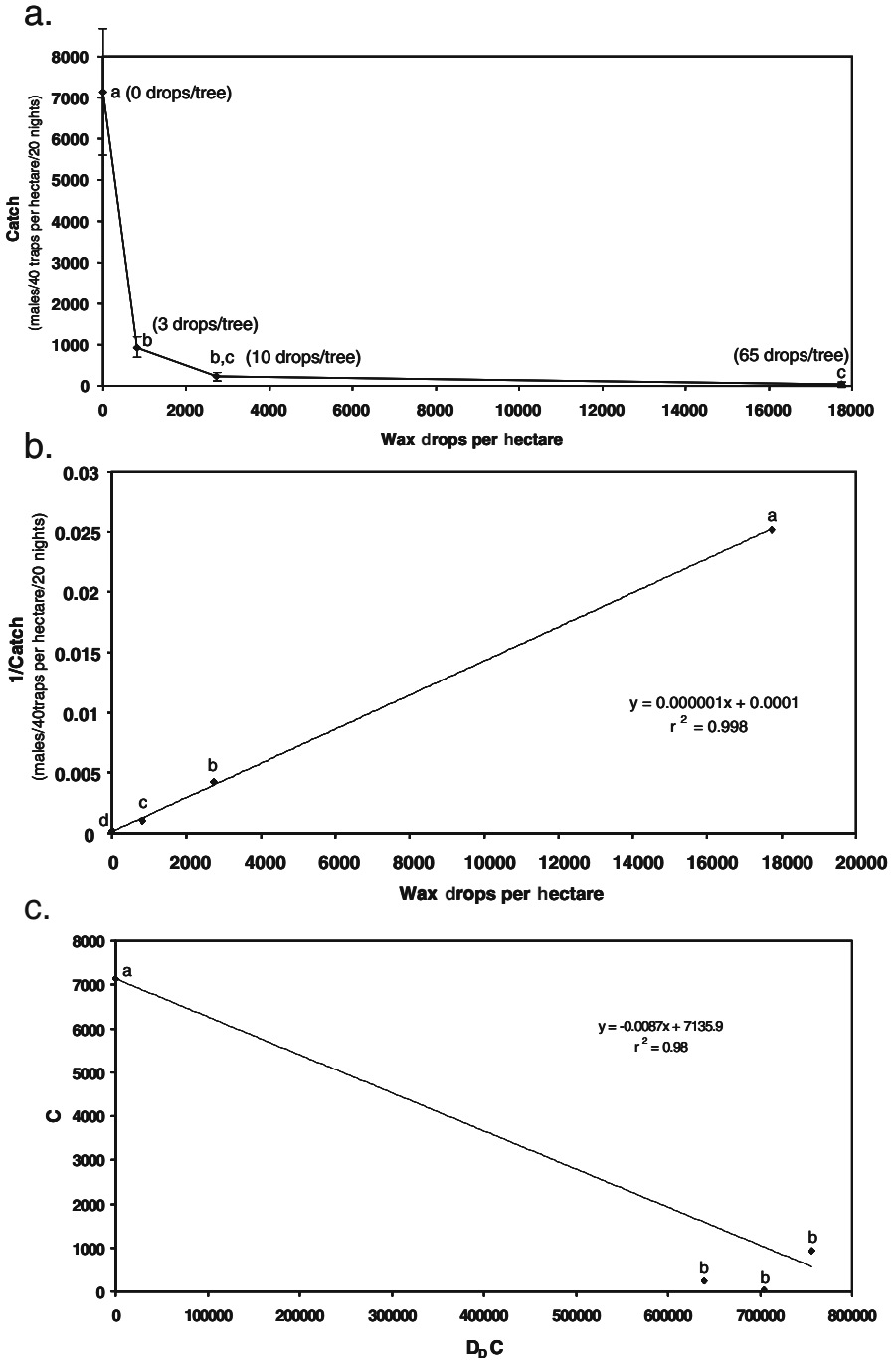


Fig. 2 Untransformed (a), Miller-Gut (b), and Miller-de Lame (c) plots of the disruption profile from Stelinski et al.'s (2005c) test deploying 0.1 ml wax drops targeting oriental fruit moth, *Grapholita molesta*, in apples. Within a panel, means not sharing a common letter are statistically different at $P < 0.05$ by an LSD test after ANOVA on data transformed to $(x+0.5)^{1/2}$ for (a) and (c), and $\ln(x+0.5)$ for (b)

Rothschild's two polyethylene tubes per tree while using 2.5 rather than 30 μg of total pheromone/hr/tree. However, each of Rothschild's dispensers was capable of containing up to 800 mg of pheromone compared to 4 mg per wax drop. Thus the larger dispensers had the advantage of lasting longer than one adult generation.

Pink Bollworm (Pectinophora gossypiella)

Flint and Merkle (1983) quantified the relationship between inhibition of pink bollworm (PBW) traps vs. density of rubber septa deployed on stakes in 0.03 ha plots of cotton in Arizona, USA. The pheromone was gossyplure, a 50:50 mixture of 16-carbon acetates listed in Table 1. Two monitoring traps were deployed per plot to yield a T_D of 6.7/ha. The study design was complex; the various pheromone dosages were tested sequentially through time, not simultaneously. Moreover, the population of PBW increased through time. Thus, to generate a continuous disruption profile, all catches needed to be scaled to the highest catch (78 males/trap/night) via the known ratio of catch in disrupted plots relative to its paired control, also set to 78.

On untransformed axes (Fig. 3a), the disruption profile for PBW was consistent with an inverse function. Catches of males initially fell steeply, leveled off, and then appeared to approach zero asymptotically. This profile was more linear in a Miller-Gut plot (Fig. 3b) than concave as a non-competitive profile would be (Fig. 7b of Miller et al., 2006). The Miller-de Lame plot would have benefited from more data particularly at low values for $D_D C$ (Fig. 3c), but even so, the few and scattered data are not inconsistent with competitive attraction. Moth densities were not calculated for this case because the units for trapping data were unclear and the relative attractiveness of T , F , and D was not determined. D_a for these gossyplure-loaded septa was 0.0038 (slope of Fig. 3c). A D_{sa} could not be calculated because there was no report of the pheromone release rate per septum. The D_{Aa} for a D_D of 1821/ha was 6.9 (Table 1), which corresponded to 88% inhibition of catches of males relative to traps in the control plots. Disruption of PBW in response to septa loaded with gossyplure was expected to be mediated by competitive attraction, because similar dispensers had strongly attracted males. The current analysis is consistent with that expectation.

Obliquebanded Leafroller (Choristoneura rosaceana)

Lawson et al. (1996) measured obliquebanded leafroller (OBLR) disruption efficacy in 0.04-ha plots of apples in Western New York (USA) in response to four densities of polyethylene tube dispensers releasing 25 $\mu\text{g/hr}$ of total pheromone blend [83% (*Z*)-11-tetradecenyl acetate, 8% (*E*)-11-tetradecenyl acetate, 9% (*Z*)-11-tetradecenol]. This formulation was not highly disruptive; it yielded a slightly concave profile for dispenser density vs. inhibition of catch in monitoring traps (Fig. 4a) akin to that for competitive attraction under a high moth population (Fig. 3a of Miller et al., 2006). This profile yielded a straight line in Miller-Gut and Miller-de Lame plots (Fig. 4b and c), providing the strongest profile evidence for competitive attraction out of all the case studies.

Male density was not directly measured in this study. $T_D M_D$ as calculated via the x -intercept of Fig. 4c was extremely high (2,193,214) under an assumption that attractiveness of $T = F = D$. Given the T_D of 50/ha, M_D computes to 43,864 males/ha, a value incongruent with a C_{\max} of only 6,100. We suspect that attractiveness of these rope dispensers was considerably less than that for the optimized traps. If k_D were taken as 0.2 of

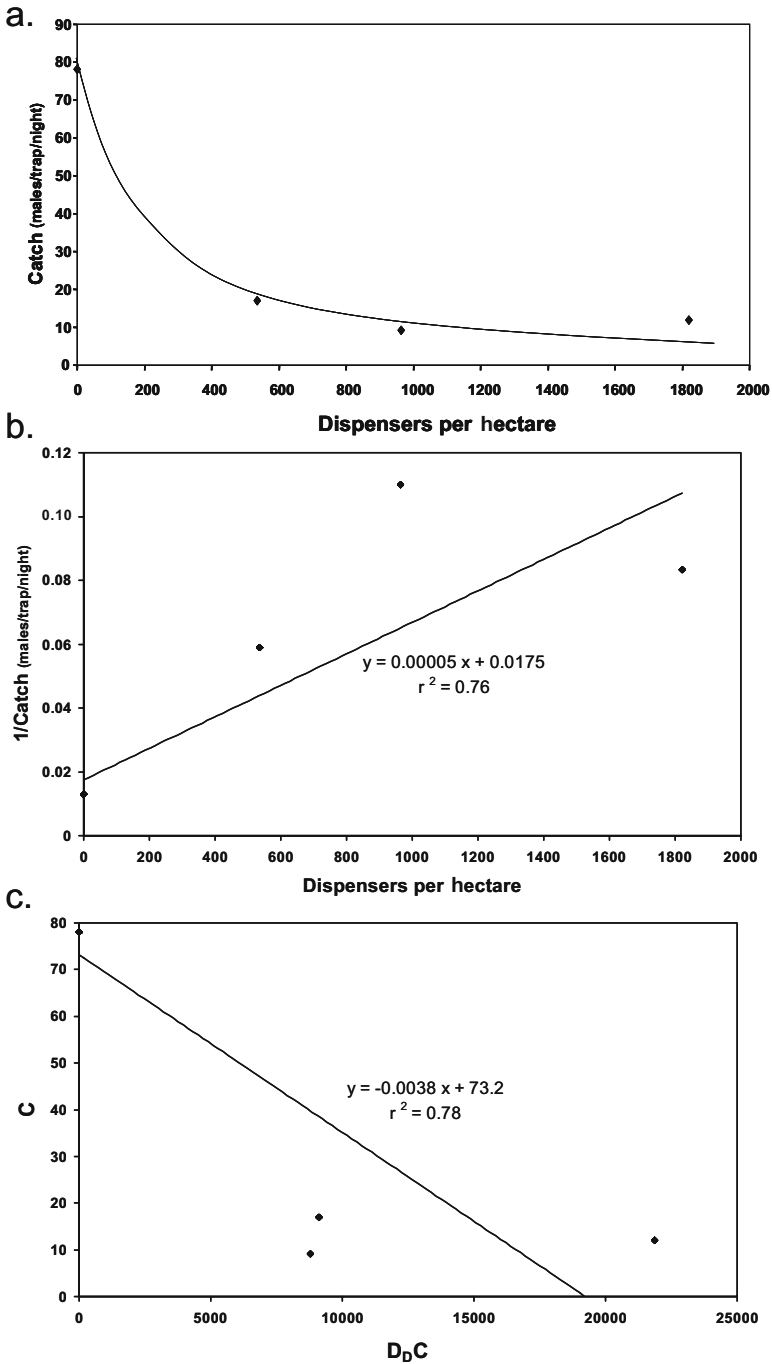


Fig. 3 Untransformed (a), Miller-Gut (b), and Miller-de Lame (c) plots of the disruption profile from Flint and Merkle's (1983) test deploying rubber septa dispensers targeting pink bollworm, *Pectinophora gossypiella*, in cotton

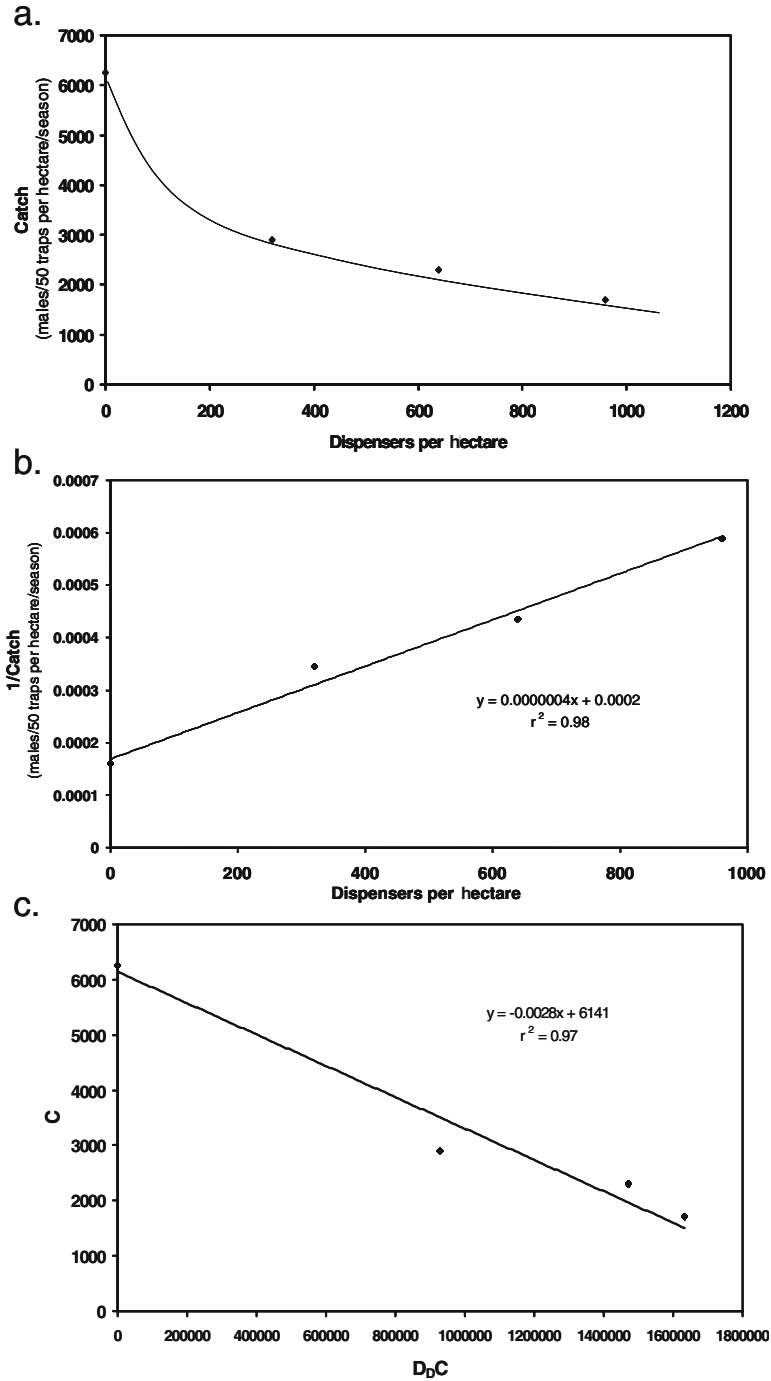


Fig. 4 Untransformed (a), Miller-Gut (b), and Miller-de Lame (c) plots of the disruption profile from Lawson et al.'s (1996) test deploying polyethylene tube dispensers targeting obliquebanded leafroller, *Choristoneura rosaceana*, in apples

k_T and k_F , the calculated M_D would be $43,864 * 0.2 = 8,800$ males/ha, which is more realistic. F_D as computed by the inverse of the Fig. 4c slope was 357 females/ha under an assumption that attractiveness of $T = F$.

D_a for OBLR responding to pheromone ropes was calculated as 0.0052 from a plot expressing catch as males/trap/d, and D_{sa} was a mere 0.0001. Thus, it is not surprising that D_{Aa}^- , even for 960 ropes/ha, was only 2.1 (Table 1) and efficacy as measured by % inhibition of male catch in these plots relative to that in untreated plots was only 70%.

Better disruption of OBLR has been reported in the Western United States and Canada. Deland et al. (1994) achieved ca. 90% disruption by using 1,000 and 2,000 Hamaki-con polyethylene tube dispensers per hectare. These data (plots not shown) were a better fit to competitive than non-competitive mechanisms, e.g., the Miller-de Lame plot did not recurve. D_a and D_{sa} were calculated at almost three times the corresponding values for Lawson et al.'s (1996) OBLR test (Table 1). However, whether this difference in disruption potency was attributable to the pheromone formulation or physiological/behavioral differences across OBLR races is unknown.

Deland et al. (1994) collected data for two other leafroller species along with OBLR. Disruption of both the European leafroller (*Archips rosana*) and fruittree leafroller (*A. argyrospila*) conformed to competitive attraction better than to non-competitive disruption mechanisms (plots not shown). Disruption outcomes were superior to that for OBLR (Table 1). The D_{Aa}^- for fruittree leafroller was a remarkable 196 at 2,000 dispensers/ha. Notably, the relative attractiveness of optimized pheromone lures in traps was found to be 3.3 times greater than male catch in traps baited with virgin females, i.e., $k_F = 0.3k_T$.

Gypsy Moth (Lymantria dispar)

Tchesslavskaja et al. (2005) recently reported dose–response data on gypsy moth (GM) disruption by using a 3 × 3 mm Hercon (Hercon Laboratories, Emigsville, PA, USA) Disrupt[®] II flake formulation of racemic disparlure, emitting at ca. 93 ng/flake/hr (Webb et al., 1988). This formulation was applied at five dosages by airplane to replicated 25 ha forest plots in Virginia. Communicational disruption was evaluated by suppression of catch in eight (+)-disparlure-baited traps per plot (0.32 traps/ha). When this test site was found to have few feral gypsy moths, ca. 150 laboratory-reared males were released in the center of each plot each week of the 6-wk test. Because gypsy moth males live ca. 2 wk (Capinera and Barbosa, 1975), the mean male population density interacting with attractant sources over this test should have stabilized around 300 males per plot (ca. 12 males/ha). Catches of ca. 270 males per plot averaged across this test suggest emigration was not substantial.

The untransformed dosage–response profile (Fig. 5a) fits competitive attraction. Male catch dropped precipitously in response to the lowest densities of flakes applied. Thereafter, efficacy increased modestly with order-of-magnitude increases in the density of flakes. Nevertheless, trap catch was suppressed to remarkably low levels (up to 99% trap inhibition). However, traps were never completely inhibited, despite the very high densities of applied flakes.

Although there was scatter in the data, the gypsy moth disruption profile was reasonably linear on Miller-Gut and Miller-de Lame plots (Fig. 5b and c). Evidence was lacking for a concavity in Fig. 5b or a recurve in Fig. 5c. D_a from a Miller-de Lame plot expressing catch as males/trap/night was 0.00052. D_{sa} was somewhat higher (0.006), because the release rate per flake was less than 1 µg/hr (Table 1). Even though the density of dispensers was 5-fold higher for GM than OFM disrupted with wax drops, D_{Aa}^- was lower for GM than that for the

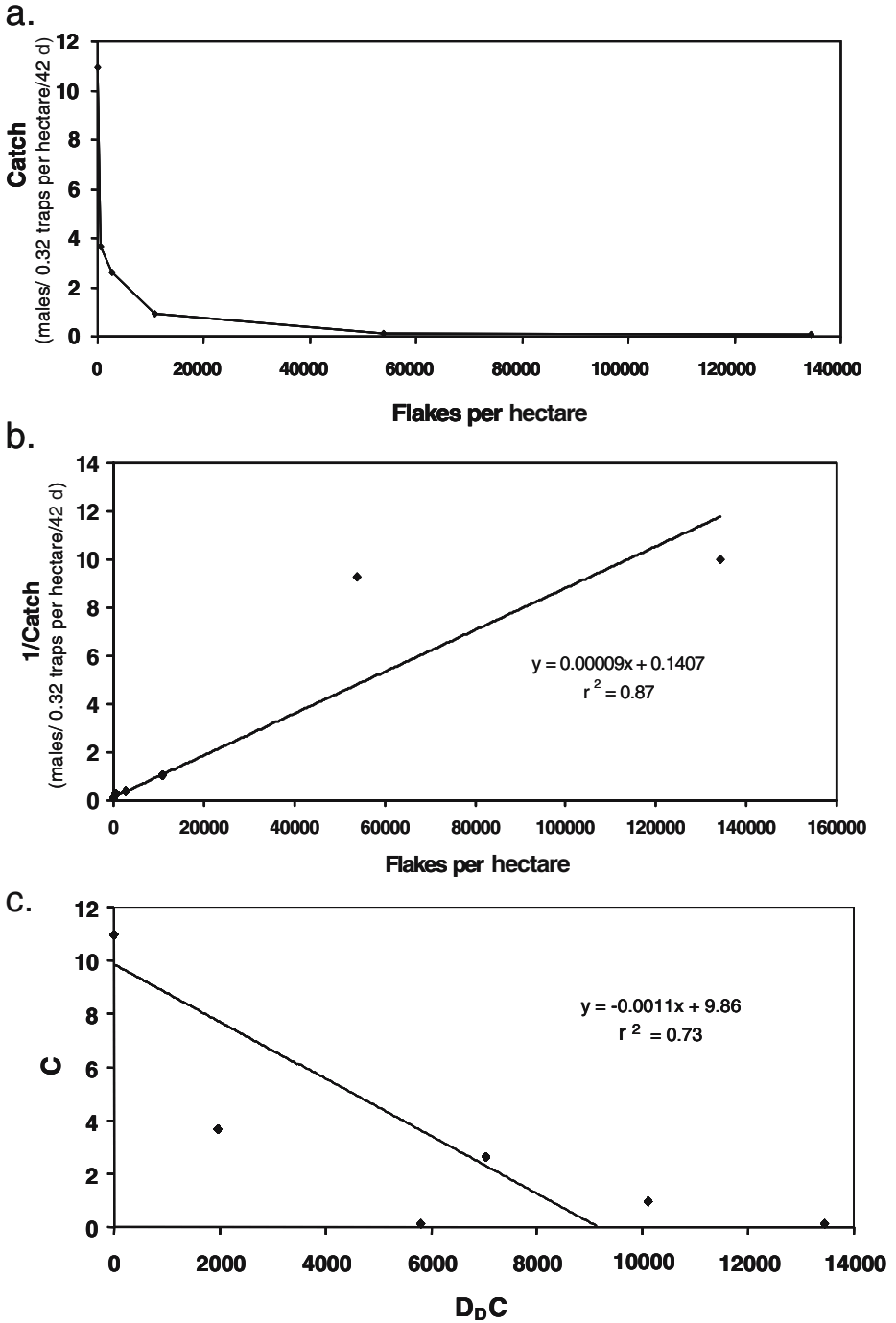


Fig. 5 Untransformed (a), Miller-Gut (b), and Miller-de Lame (c) plots of the disruption profile from Tcheslavskaja et al.'s (2005) test deploying laminated flake dispensers targeting gypsy moth, *Lymantria dispar*, in forest plots

Stelinski et al. (2005c) OFM test (Table 1) because the potency of each flake was 1/13 of that for each wax drop. It would be interesting to document how much greater D_{sa} for GM would be for flakes charged with the natural and more attractive (as well as costly) (+)-rather than (\pm)-disparlure (Miller et al., 1977).

The $T_D * M_D$ value calculated from the x -intercept of Fig. 5c was 8,964. Because $T_D = 0.32$ traps/ha in this case, M_D calculated on an assumption that attractiveness of $T = F = D$ is 28,000. This number is far higher than the 12 males/ha calculated to be in these plots based upon the male releases. The most likely explanation for this incongruence is that each flake was less attractive than a trap. We found no published data on the attractiveness of GM pheromone flakes relative to monitoring traps or females. However, by using Equation (10) of Miller et al. (2006), the k for flakes can be calculated at about 0.0004 relative to k for traps. With this adjustment, a calculated M_D becomes a reasonable 11 males/ha, or 280 males/plot. Putting this value for k_D into Equation (11) of Miller et al. (2006) yields $k_F F_D = 0.04$; so, if $k_F = 1$, $F_D =$ nearly zero, as was known to be true in this test. Thus, here is a case where attractiveness of D relative to T can be calculated with confidence from competitive attraction equations because both M_D and F_D were known, along with T_D and D_D . Although attractiveness of flakes seems trivial relative to traps, this test established that even weakly attractive (or perhaps arrestive) sources of pheromone can have important practical impact when dispensed at very high densities.

A note of caution needs to be sounded. This test was conducted in the absence of a normal density of female moths; this would inflate % inhibition of male catch and D_a . Both of these measures of disruption efficacy are sensitive to T_D and F_D , but not M_D (Miller et al., 2006). A somewhat less optimistic outcome than reported above would likely be obtained under a normal F/M ratio for GM.

Studies Varying Density of Release Sites along with Release Rate per Site

Suckling and Angerelli (1996) recorded a disruption profile for polyethylene tube dispensers targeting the lightbrown apple moth (LBAM), *Epiphyas postvittana*, in 0.5-ha plots of New Zealand apples. The pheromone formulation (Table 1) contained (*Z*)-11-tetradecenyl acetate as an “antagonist” in addition to the natural pheromone blend. This study held the overall density of sources to 200 dispensers/ha, while varying the number of release sites/ha: 0, 2, 18, and 200. This design now permits quantification of D_a and D_{Aa} when dispensers were deployed individually vs. clusters of 11 and 100 per release site.

Surprisingly, given inclusion of an “antagonist”, the disruption profile for LBAM (Fig. 6a) was consistent with competitive attraction rather than camouflage or some other non-competitive mechanism. Polyethylene tube dispensers thought to contain “antagonists” of OBLR (Stelinski et al., 2004a,b) and codling moth (CM) (Witzgall et al., 1999) have likewise been found to be attractive to males. In the LBAM study, one 100-dispenser cluster yielded 67% inhibition of male catch compared to 97% for 100 dispensers deployed individually. Thus, clustering the dispensers greatly reduced the overall treatment potency.

The Miller-Gut plot of the LBAM disruption profile was convex rather than straight (Fig. 6b). This pattern is consistent with competitive attraction where, as a group, each cluster of dispensers was more disruptive than an individual dispenser; however, the density of individual dispensers was higher than the density of clusters. We broke down the convex profile of Fig. 6b into three segments for calculations of D_a under the three different dispenser distributions. D_a for individual dispensers was 0.19 as calculated by dividing the slope of segment A–D (Fig. 6b) by its intercept [Equation (4) of Miller et al., 2006]; D_{Aa}

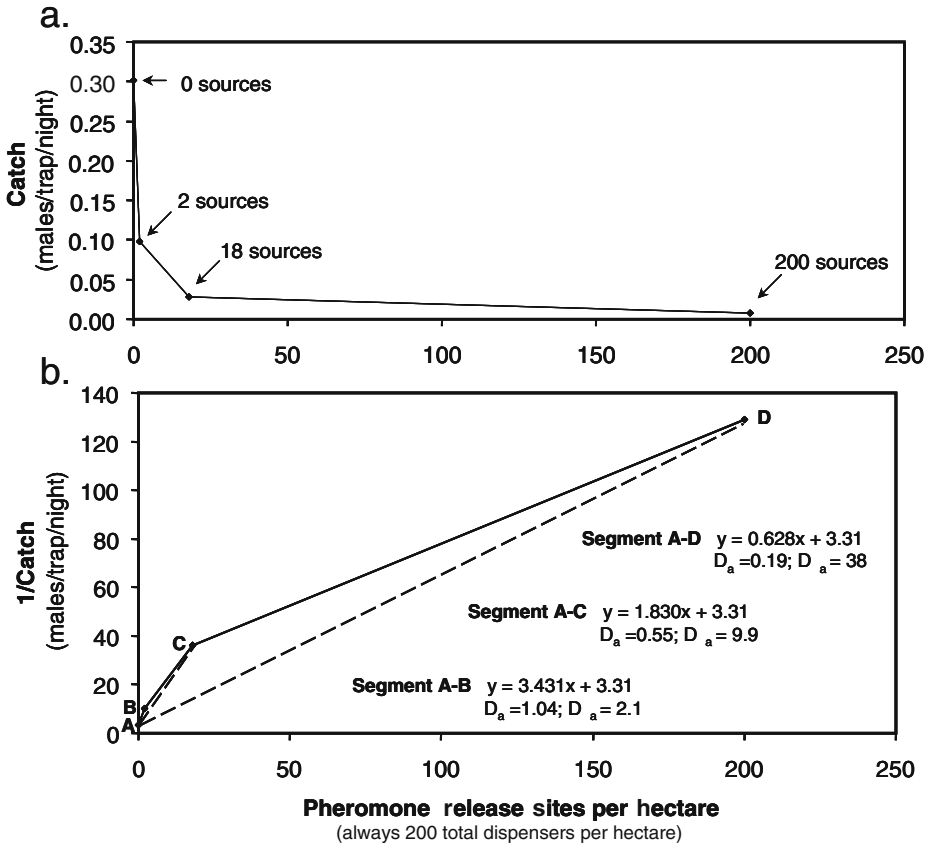


Fig. 6 Untransformed (a) and Miller-Gut (b) plots of the disruption profile from Suckling and Angerelli’s (1996) test deploying polyethylene tube dispensers targeting lightbrown apple moth, *Epiphyas postvittana*, in apples

was 38. As dispensers were clustered, D_a /source (Fig. 6b) increased by only 5.5-fold, but D_{Aa} diminished by 18-fold. This outcome confirms previous knowledge of a tradeoff between dispenser density vs. disruption strength per release site. However, the current analysis emphasizes that the loss in potency due to clumping dispensers may be greater than previously understood. Under competitive attraction, the first few dispensers deployed into a plot will dramatically decrease male catch (e.g., Fig. 2; Miller et al., 2006), except when moth density is extraordinarily high. This advantage of being the first (even though few) release sites into a plot may obscure the offsetting dramatic decline in D_a and D_{Aa} when dispensers are clumped and the density of release sites is kept low because the overall release rate of pheromone is already high.

The Miller-de Lame plot (not shown) for these LBAM was concave, as expected for competitive attraction under source clustering with a concomitant reduction in D_a per cluster. The potency of single LBAM dispensers deployed at 200 per ha was excellent ($D_a = 0.19$) and superior to that of any other dispenser in Table 1. D_{sa} of LBAM dispensers was surpassed only by that for wax drops deployed for OFM. This finding suggests that

mating disruption of LBAM would be highly effective under the approach of using thousands rather than hundreds of point sources of pheromone per hectare.

Other investigators have recorded disruption outcomes while varying pheromone release rates along with density of release sites. For example, Charlton and Cardé (1981) used 0, 668, and 1,336 release sites per ha (plot size was 0.03 ha) to measure OFM disruption in apple plots in response to Albany International Corp. (no longer available) hollow fibers releasing the three components of this moth's pheromone (see Table 1), but not in the precise natural blend. The densities of fibers per source were 0, 16, 96, and 880. These data (graphs not shown) were also consistent with competitive attraction. D_a per fiber (calculated as described above for LBAM) was 0.0019, in line with other OFM pheromone release devices (Table 1). D_a per fiber declined from 0.0019 for 16 fibers per source to 0.00034 for 880 fibers per source. This OFM research confirms that increasing pheromone release per site did not fully compensate for a reduction in the density of release sites. Reducing the density of release sites comes at a cost of reduced D_{sa} and $D_{\bar{a}}$. Both release rate per source and density of sources matter in communicational disruption mediated by competitive attraction. For LBAM and OFM within the parameter ranges tested, however, source density was more influential than overall release rate of pheromone per source.

Shorey et al. (1994) conducted an elegant study conducive to evaluation of the relationship between density of pheromone release sites and release rate per site. They arranged a test where (in our terminology) k_D and D_D were experimentally manipulated, whereas k_T and k_F , along with moth density, remained constant. This test was conducted on the beet armyworm (BAW), *Spodoptera exigua*, in plots of tomato and cotton. Only the major pheromone component, (Z9,E12)-tetradecadienyl acetate, was released from 2-cm-long Teflon™ capillary tubes varying in diameter from 0.30 to 1.32 mm. Release rate was purportedly directly proportional to capillary diameter. Single capillary tubes attached to wooden stakes were deployed into the crop, always in a 36×36 grid, but with variable separation between stakes: 4.6, 3.0, and 1.5 m. Therefore, plot size varied from 0.006 to 0.05 ha, and D_D varied from 0 to 6,429/ha. One monitoring trap was deployed at the center of each treatment and control plot (five replicates). Because T_D varied somewhat in these tests, we made no attempt to calculate or interpret moth densities.

With rises in k_D relative to k_T and k_F , the competitive-attraction simulations of Fig. 4c of Miller et al. (2006) predicted a series of diverging straight lines with increasing slopes on Miller-Gut plots. The Shorey et al. (1994) BAW disruption profiles fit this prediction well (Fig. 7), except for the profile for the 0.56-mm capillary, which was flatter than expected. D_a increased directly with pheromone release rate from the capillaries ($D_a = 0.018D_{sa}$; $r^2 = 0.99$), revealing that D_{sa} remained a constant 0.018, irrespective of capillary diameter. $D_{\bar{a}}$ was 129 for the largest diameter capillary (1.32 mm) releasing pheromone at $1 \mu\text{g/hr}$ and deployed at 6,429 capillaries per ha (Table 1).

Shorey et al. (1994) seem to have over-interpreted their BAW data when they concluded that release rate of pheromone point sources was much more important than density of release sites. By starting with a relatively high density of dispensers (679/ha) and progressing only upward, these investigators realized only a small gain in % disruption with additional increases in dispenser density. This probably was because their lowest treatment density occurred at a point on this disruption profile beyond the rapidly falling phase and in the asymptotic phase. On the other hand, the pheromone release rates per release site in this experiment began very low (83 ng/hr) and progressed only to $1 \mu\text{g/hr}$. D_a increased steadily across this narrow range of release rates. However, there is no guarantee that disruption potency would have increased indefinitely with increasing

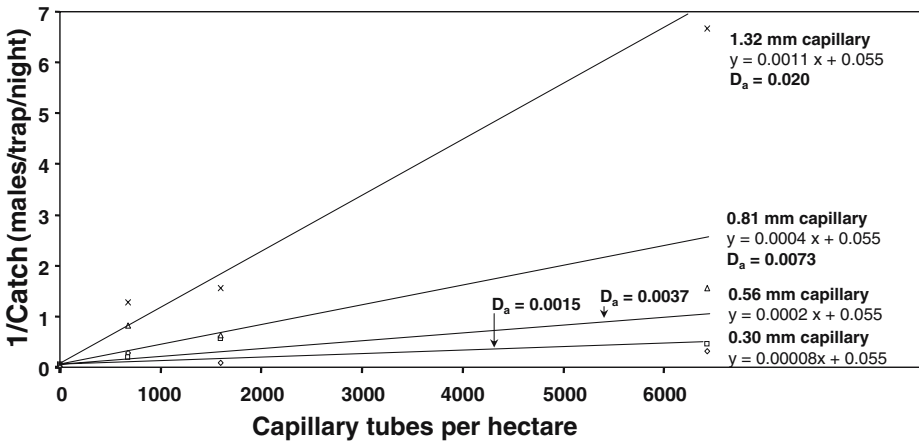


Fig. 7 Miller-Gut plot of the disruption profile from Shorey et al.’s (1994) test deploying four differently sized capillary tube dispensers targeting beet armyworm, *Spodoptera exigua*, in tomato and cotton

release rate per release site. Not realizing that this test favored showing a strong release-rate effect and a weak dispenser-density effect, these authors suggested that, for BAW, “if a certain amount of pheromone component is to be evaporated per hectare per day, it may be more effective to do so from relatively few evaporators, each releasing large amounts of pheromone and spaced far apart, than for small evaporators releasing lesser amounts of chemical and positioned correspondingly closer together” (Shorey et al., 1994). From here, it was a short step to aerosol and similar dispensers (Mafra-Neto and Baker, 1996; Shorey and Gerber, 1996; Shorey et al., 1996; Isaacs et al., 1999) releasing very high rates of pheromone from few point sources per hectare.

A more complete picture can now be constructed from the classic data of Shorey et al. (1994) for the roles of dispenser density (D_D) and rate of pheromone release per site (k_D) in influencing disruption efficacy. Under this example of competitive attraction appearing to conform to Equation (9) of Miller et al. (2006), both factors are mutual partners interacting multiplicatively to reduce the probability of a male visit to a female from ca. 1 to $1/k_D D_D$. As such, any increase in k_D is multiplied by D_D , and *vice versa*. The mathematics indicate it is maximization of $k_D D_D$ that matters, and that this could be done variously, e.g., by using a high density of point sources, each with low attractiveness. Here, an additional benefit is that spatial coverage of the crop would be thorough. In reality, however, the pheromone release rate per dispenser cannot be permitted to fall below some minimal level required to generate an attractive plume. Conversely, few point sources releasing pheromone at high rates might theoretically generate a very high $k_D D_D$. However, deploying just a few large plumes may leave gaps in coverage, especially when fields are small. Moreover, there are usually limits to pheromone concentrations that can attract male moths. Releasing pheromone at rates too high to be attractive could nullify a competitive-attraction mechanism in favor of an alternative disruption mechanism. Maximizing $k_D D_D$ as well as D_{sa} for expensive pheromone compounds will be a worthy economic goal of applied pheromone research for the foreseeable future. It will require experiments like that of Shorey et al. (1994), but with a wider range of release rates per dispenser to reveal shifts and limits in k_D . Also, dispensers will need to be observed to determine to what degree they are attractive to males.

Summary of Case Studies Supporting Competitive Attraction

More than 10 disruption profiles were uncovered that fit the set of predictions for competitive attraction detailed by Miller et al. (2006). This set of cases can be further tested for internal consistency by correlating values for $D_{\bar{A}a}$ and % disruption (Table 1). If all cases were properly classified as competitive attraction, a consistent and interpretable

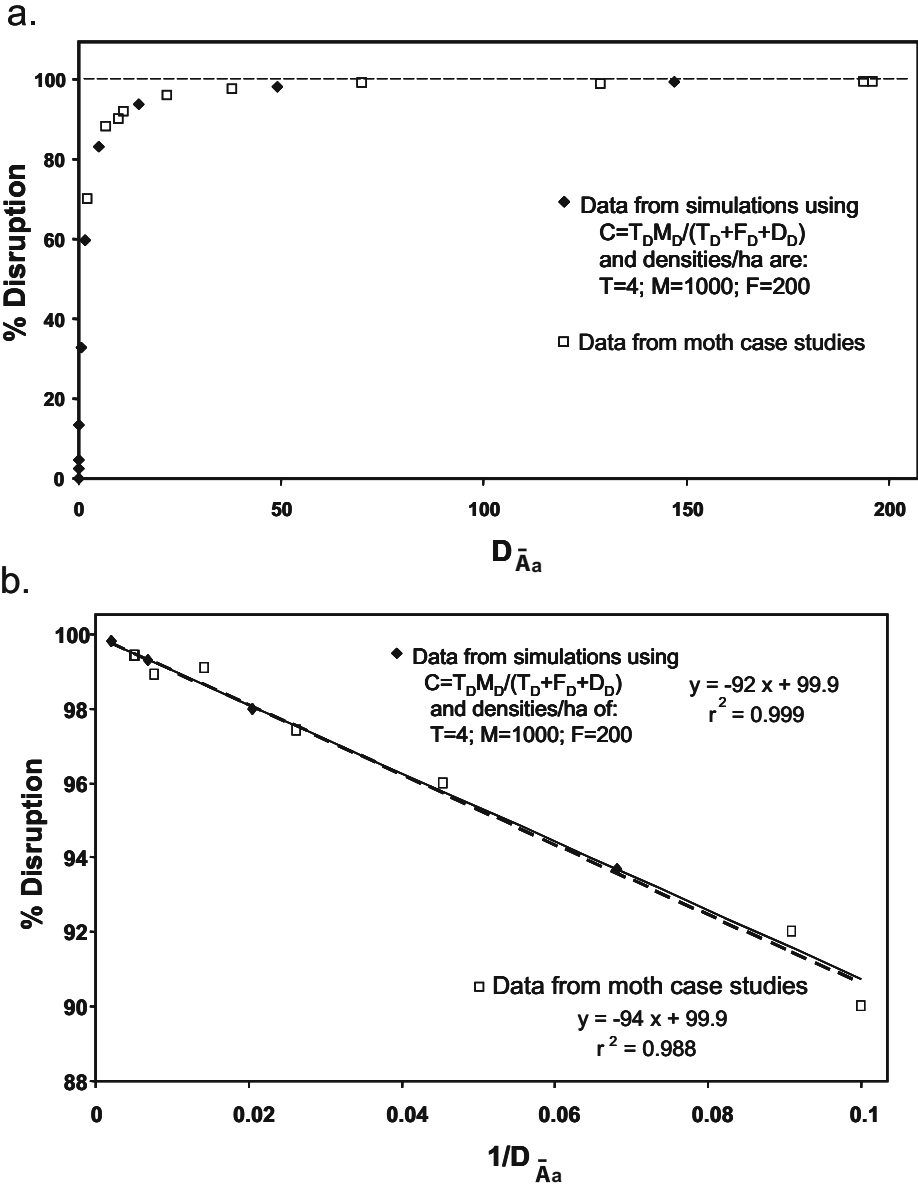


Fig. 8 Graphical comparison of measures for disruption potency arising from Table 1. (a) Relationship between % disruption vs. $1/D_{\bar{A}a}$ (b) Untransformed plot of % disruption vs. dispenser application activity ($D_{\bar{A}a}$)

relationship should be found when % disruption is plotted vs. $D_{\bar{A}a}$. This prediction is confirmed by Fig. 8a. Values of % disruption for both simulated and actual data rose dramatically with increases in $D_{\bar{A}a}$; then they rapidly tapered off and approached 100% disruption asymptotically. The fit between real and simulated data under conditions detailed in Fig. 8a was excellent.

Figure 8a demonstrates that % disruption and $D_{\bar{A}a}$ offer strikingly nonparallel measures of disruption potency. Tiny numerical differences in % disruption near 100% obscure very large differences in disruption potency as measured by $D_{\bar{A}a}$. Expansion at the bottom end and compression at the top end of the % disruption scale can distort judgments of the relative potency of disruptive formulations operating by competitive attraction. For example, an alteration in a formulation that increases disruption from 50% to 90% appears to be considerably more impressive than an increase from 98% to 99%. However, a rise from 50% to 90% inhibition of male catch in traps corresponds to an increase in $D_{\bar{A}a}$ of only 8, whereas the rise from 98% to 99% corresponds to a $D_{\bar{A}a}$ increase of 50. From the perspective of practical pest control, this suggests that % disruption values below 90% may all reflect trivial disruptive potency under competitive attraction, unless pest density is very high.

Percent disruption of male catch in traps has been a useful measure of practical outcomes of disruption experiments, but its proper interpretation requires reevaluation. We recommend that consideration be given to additional measures of disruption potency and efficacy beyond % disruption. As research fields mature, it is usual that multiple measures are devised for a given phenomenon, e.g., degrees Fahrenheit, centigrade, and Kelvin for temperature. D_a and $D_{\bar{A}a}$ can serve as alternative disruption measures for cases of disruption operating by competitive attraction. These variables seem better suited than % disruption to differentiate among the high-performance treatments (> 98% disruption) that will increasingly become the practically relevant zone of interest for pest managers and the pheromone industry.

Given that D_D occurs only in the denominator of Equation (1) of Miller et al. (2006), evidence should be found that D_D reduces catch by some consistent inverse function. Indeed, when % disruption values in Table 1 were plotted vs. the inverse of the corresponding $D_{\bar{A}a}$ values, a precise relationship was uncovered when % disruption exceeded 90% (Fig. 8b). The regression equation was: % disruption = $94(1/D_{\bar{A}a}) + 100$; the r^2 value was 0.99. This outcome was also virtually identical with that for the simulated data of Fig. 8b. Rearrangement and rounding up the slope yields (equation numbering continued from Miller et al., 2006):

$$\% \text{ Disruptions} \approx 100 - 100/D_{\bar{A}a} \quad (17)$$

Similarly, if catch in control plots is normalized to 1,

$$\text{Catch in crop receiving pheromone} \approx 1 - 1/D_{\bar{A}a} \quad (18)$$

These equations should prove useful in interconverting the mass of % disruption data already in the literature. However, Equations (17) and (18) are applicable only when disruption operates by competitive attraction and D_D values are very high so as to dominate the generative equations. Finding a consistent and precise relationship between $D_{\bar{A}a}$ and % disruption across cases classified as competitive attraction offers powerful evidence for internal consistency and hence validity of the current approach to classifying disruption mechanisms.

Cases Consistent with Non-competitive Disruption Mechanisms and Inconsistent with Competitive Attraction

Our literature search uncovered only one case supporting a non-competitive disruption mechanism. This test was conducted by McLaughlin et al. (1972) in Southern California (USA) on pink bollworm in cotton. This work pre-dated identification of the PBW's pheromone (Table 1) and used a related compound (*Z*-7-hexadecenyl acetate; hexalure) attractive to males but less so than extracts of female pheromone glands. Hexalure was released at 6 $\mu\text{g}/\text{dispenser}/\text{hr}$ from stainless steel planchets attached to wooden stakes deployed at crop height in a 10×10 array. Inter-stake distances varied (3, 10, and 30 m); thus, plot sizes varied from 0.09 to 9 ha. Disruption in each plot was evaluated from catch of males in one central sticky trap baited with several virgin females. The results were presented only as % reduction in catch relative to control plots; actual catches were not reported.

It was not apparent from the untransformed plot of McLaughlin et al.'s (1972) test (Fig. 9a) whether the data fit competitive or non-competitive disruption. Catch did not initially drop precipitously with initial increases in dispenser density, but this pattern also can be realized under competitive attraction when moth density is very high (Fig. 3a of Miller et al., 2006). However, the subsequent approach to the x -axis was quicker than expected for competitive attraction under high moth density (Fig. 9a vs. Fig. 3a of Miller et al., 2006).

Secondary plots of this disruption profile were consistent with a non-competitive disruption mechanism. The Miller–Gut plot was concave (Fig. 9b), as expected for non-competitive disruption (Fig. 7b; Miller et al., 2006). The Miller-de Lame plot was recurved and its inflection point occurred near $1/2y$ -maximum (Fig. 9c), as is diagnostic for non-competitive disruption (Fig. 7c of Miller et al., 2006). Because this PBW profile was a better fit to non-competitive than competitive disruption, the steps taken above to calculate D_a and D_{sa} could not be taken.

The release rate in this PBW study was 8.2 mg/ha/hr; it yielded 99% communicational disruption. For comparison, 6.8 mg of pheromone/ha/hr in Stelinski et al.'s (2005c) test with OFM yielded 99.4% disruption, whereas 12.5 mg of pheromone/ha/hr yielded 99% disruption in the GM test of Table 1. Thus, disruption efficiencies may not vary greatly between competitive and non-competitive disruption when rates of pheromone dispersed/ha are high as in Table 1. This finding is consistent with the simulations of Fig. 7a of Miller et al. (2006). However, these simulations suggested that efficiency of disruption by competitive attraction would be superior to that for non-competitive mechanisms, when moth and dispenser densities are moderate to low.

A previously unpublished data set of our own also fits non-competitive disruption. This test targeting OFM was conducted over the full 2003 growing season in 0.15-ha, plots (45 trees) distributed across a 3-ha, recently abandoned apple orchard in Southeastern Michigan. The pheromone blend and dispenser formulation were identical to those reported by Stelinski et al. (2005c; see Table 1). However, rather than 0.1 ml wax drops, 12-ml dollops of wax were applied to a tree branch ca. 2 m above ground level as described by De Lame (2003). Dispenser densities were 0, 50, 130, and 230/ha. The experimental design was randomized complete block with four replicates. Two monitoring traps (Stelinski et al., 2005c) spaced ca. 12 m apart and near the center of each plot assessed catch of male moths across the full 2003 growing season. Traps were checked weekly and lures were replaced at the beginning of each OFM flight (every 6–8 wk). These emulsified wax dollops were sufficiently large to remain disruptive throughout the season. Release rate per dollop slowly

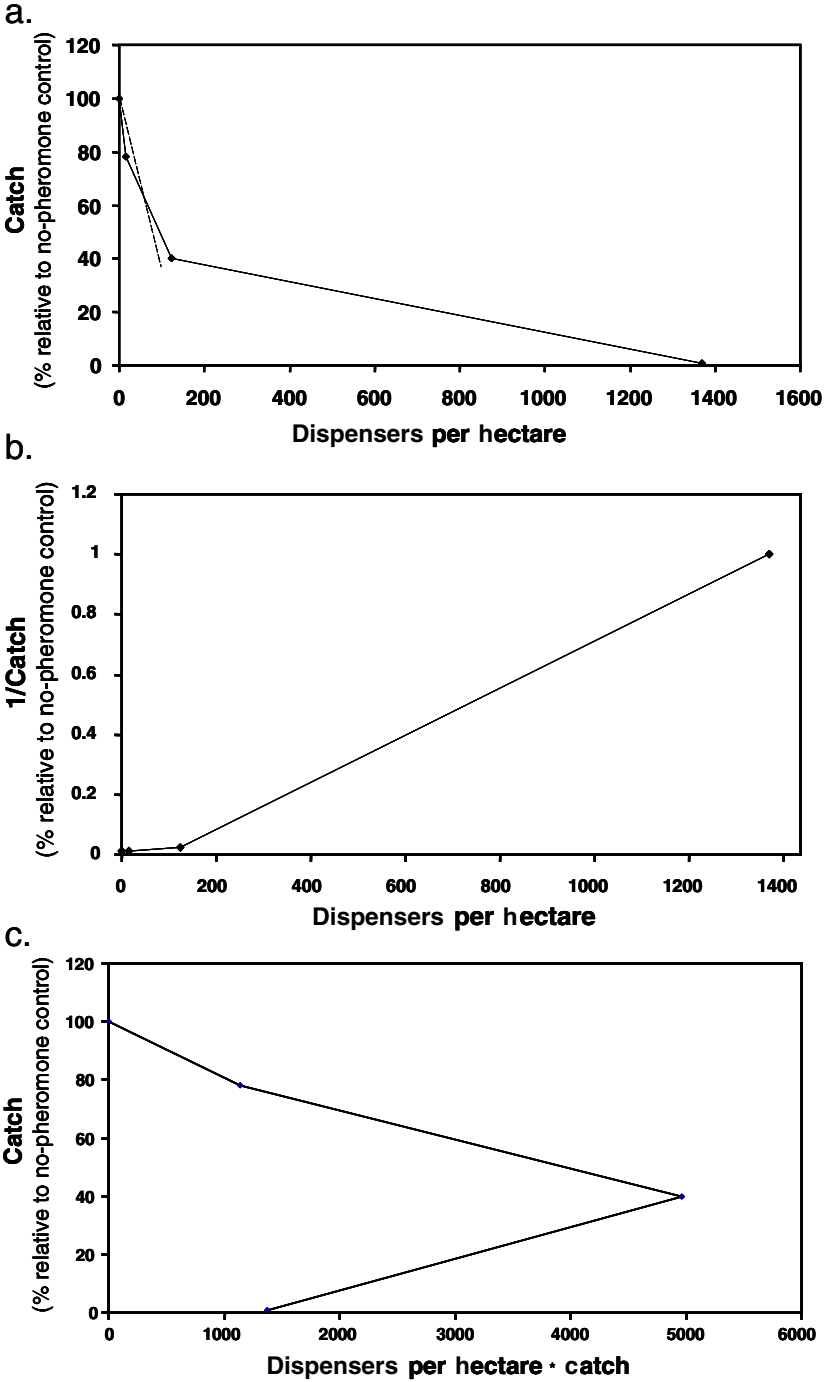


Fig. 9 Untransformed (a), Miller-Gut (b), and Miller-de Lamé (c) plots of the disruption profile from McLaughlin et al.'s (1972) test deploying planchette dispensers targeting pink bollworm, *Pectinophora gossypiella*, in cotton

declined from ca. 150 to 40 $\mu\text{g/hr}$ as determined by solvent extraction (De Lame, 2003) followed by gas chromatography (Stelinski et al., 2005c).

The season-long disruption profile for this test is plotted in Fig. 10a. With initial increases in dispenser density, catch dropped almost linearly until 130 dollops/ha. However, disruption did not increase significantly with the final increment in dispenser density. Having only one datum after the slope change was insufficient for assessing whether or not the approach to total inhibition of male catch was asymptotic (Fig. 10a).

Both secondary plots of the data supported a non-competitive disruption mechanism. The Miller-Gut plot (Fig. 10b) was concave, as expected for non-competitive disruption (Fig. 7b of Miller et al., 2006). Moreover, the Miller-de Lame plot (Fig. 10c) recurved significantly ($P < 0.05$) and according to the signature of non-competitive disruption (Fig. 7c of Miller et al., 2006). Finding some cases consistent with non-competitive disruption and inconsistent with competitive attraction, as well as *vice versa*, helps to authenticate the quantitative tools proposed by Miller et al. (2006) for differentiating between the two major classes of disruption mechanisms.

Release rate is the most obvious reason for the switch from competitive to non-competitive disruption mechanisms when using 0.1-ml wax drops vs. 10-ml wax dollops to disrupt OFM. As measured by male catch in traps, attraction of OFM is strongest when sources release between 0.07 and 0.72 $\mu\text{g/hr}$ (Baker et al., 1980). Release rate from our wax dollops ($> 40 \mu\text{g/hr}$) far exceeded OFM's upper limit for attraction of males very close to a source. Graphical analysis of the resulting disruption profile suggests that attraction was nullified, and plumes were generated that disrupted moth communication by some unspecified non-competitive mechanism, such as camouflage or desensitization. During this test, we unfortunately did not yet realize the importance of observing dispensers for evidence of male attraction. Thus, critical evidence that male OFM did not orient to these wax dollops is lacking.

This experiment documented a small efficiency improvement in OFM disruption when mediated by competitive attraction vs. a non-competitive mechanism. Dollops (230) releasing pheromone at 6.4 mg/ha/hr gave 96% disruption, a questionable level for practical control. Pheromone released at 6.8 mg/ha/hr from 27,300 wax drops effected an encouraging 99.4% disruption, also under high pest pressure. However, the large wax dispensers lasted all season, whereas a new set of drops had to be applied for each moth generation.

The slope derived from the first three points of Fig. 10a was 0.055; it reflects the decline in male catch per trap per wax dollop added per hectare, when starting catch was 8.2 males/trap/night for the control plot receiving no synthetic pheromone. Thus, each dispenser diminished catch by 0.67% ($0.055/8.2$) during the linear portion of Fig. 10a. Under a pure camouflage model of pheromone disruption, Equations (14) and (15) of Miller et al. (2006) proposed that each equivalent dispenser would camouflage an equal area (x) of crop, and that the total area camouflaged would be $D_D * x$. Combining this proposition with the measured Fig. 10a slope of 0.67% suggests that each wax dollop in this OFM test operated on 0.67% of 1 ha, which equates to only 67 m^2 /dollop. Under the spacing of 225 trees/ha, 67 m^2 equates to camouflaging plume coverage of only 1.5 trees/wax dollop. Accordingly, it would take a minimum of 150 dollops to completely cover the 1 ha under a scenario of perfect packing and no plume overlaps. However, even the highest dispenser density of 230 dollops/ha allowed a catch of 4% relative to the control catch. Thus, there must be a difference between plume sizes required to impact catch vs. inhibit it completely. The current OFM experimental data suggest there were small spaces within the treated crop that were difficult to disrupt by non-competitive mechanisms. Perhaps it is the zones immediately downwind of traps and females, where their pheromone plume concentrations are highest, that are not being covered by sufficient dosages of background pheromone to effect complete non-competitive disruption.

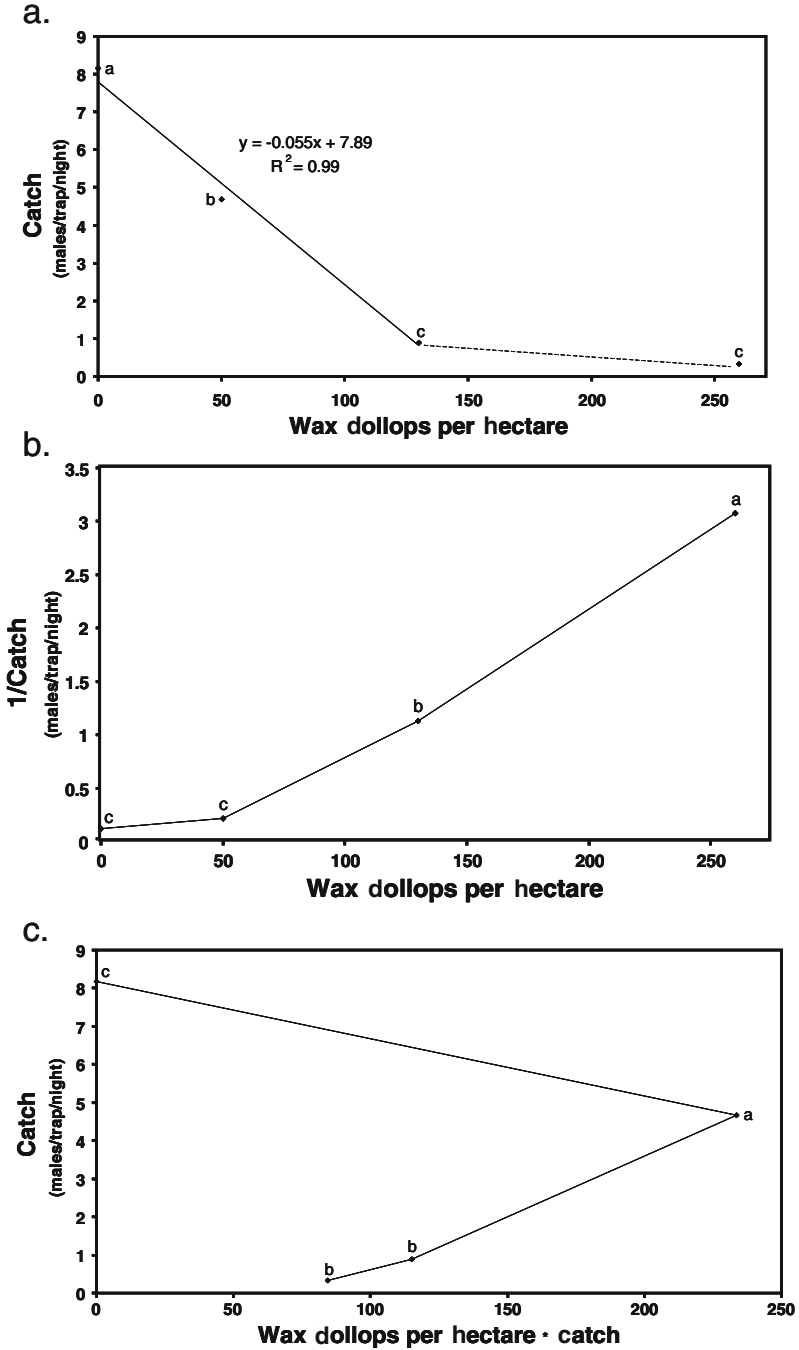


Fig. 10 Untransformed (a), Miller-Gut (b), and Miller-de Lame (c) plots of the disruption profile from our own test deploying 12 ml emulsified wax-dollop dispensers targeting oriental fruit moth, *Grapholita molesta*, in apples. Within a panel, means not sharing a common letter are statistically different at $P < 0.05$ by an LSD test after ANOVA on data transformed to $(x+0.5)^{1/2}$ for panels (a) and (c), and $\ln(x+0.5)$ for (b)

Alternatively, even our highest density of dispensers may have been insufficient to eliminate small gaps in coverage far from dispensers.

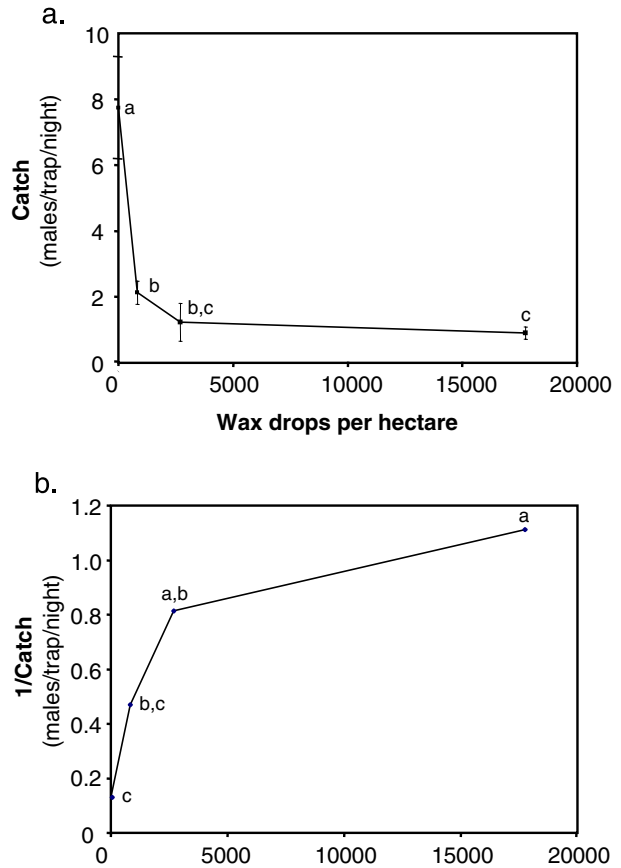
The capacity to shift from a competitive to a non-competitive disruption mechanism by using a common dispenser medium, but varying the deposit size, bodes well for emulsified wax as a flexible disruption formulation useful across a range of pest densities. By varying dispenser size and/or release rate, it might be possible to tailor the disruption mechanism for a given pest based upon its particular density, as suggested by Miller et al.'s (2006) Fig. 7a. At very high pest densities, it could be advantageous to disrupt pests by a non-competitive mechanism. On the other hand, competitive attraction appears to be the more efficient disruption mechanism for moderate to low pest populations (Fig. 7a of Miller et al., 2006). However, these decisions will also be influenced by the desired longevity of a given pheromone application. Small, but densely-distributed dispensers are likely to give the most efficient disruption for many pests, but reapplication will be required if small dispensers have limited longevity.

A Case Possibly Blending Competitive and Non-competitive Disruption Mechanisms

During the 2004 field season, we tested efficacies of varying densities of 0.1 ml emulsified wax drops in disrupting CM (*Cydia pomonella*) sexual communication. The methods were identical to those reported by Stelinski et al. (2005c) for OFM, with a few exceptions. Plots consisted of 16 rather than 12 trees, and only the major pheromone component for CM [(E8,E10)-dodecadienol] was incorporated into the wax at a starting concentration of 5%. Catch of male moths in each plot was monitored with two centrally located sticky traps baited with red rubber septa loaded with 0.1 mg of pure codlemone. This test ran for only 14 d during the first generation of moths, after which efficacy of wax drops noticeably diminished and the test was terminated. Release rate of codlemone over this period averaged 3 $\mu\text{g/hr/wax}$ drop as quantified by gas chromatography with the methods of De Lame (2003).

The resulting disruption profile for CM was a better fit to competitive attraction than to a non-competitive mechanism. The untransformed plot was sharply concave and not linear (Fig. 11a) and the Miller-de Lame plot did not recurve (data not shown). However, the Miller-Gut plot (Fig. 11b) was convex, much like that for LBAM (Fig. 6b). This is curious, because there was no clumping of wax drops as was true for ropes in Suckling and Angerelli's (1996) test. In contrast to the other competitive-attraction cases compiled in Table 1, disruption potency per dispenser declined for CM as density of wax drops rose. Emission rate of pheromone from wax drops was probably not influenced by their density, and so it is more likely that the response of CM males to monitoring traps was somehow shifted under the increasing density of pheromone plumes. One possibility is that CM males became partially desensitized to the emissions from high densities of wax drops, but not to the higher emissions from the monitoring traps, i.e., the threshold for response to pheromone point sources shifted upward (less sensitive) as the density of point sources increased. If so, visitation rate to the traps may have risen relative to weakly competing wax drops. Not having assessed the relative attractiveness of traps vs. females vs. wax drops, or deployed virgin females to assess mating, we cannot say whether disruption of females exactly reflected the disruption of traps. Disruption of females needs to be measured, as it was perhaps better or worse than disruption measured for traps. Although we are unable to fully interpret the disruption profile for this CM study, this case demonstrates the utility of these new analytical procedures in extracting potentially useful information hidden within a raw disruption profile (Fig. 11a). We expect other cases will be found

Fig. 11 Untransformed (a) and Miller-Gut (b) plots of the disruption profile from our own test deploying 0.1 ml emulsified wax-drop dispensers targeting codling moth, *Cydia pomonella*, in apples. Within a panel, means not sharing a common letter are statistically different at $P < 0.05$ by an LSD test after ANOVA on data transformed to $(x+0.5)^{1/2}$



that do not fit pure competitive or non-competitive disruption, and that more powerful analyses of these profiles will aid recognition and interpretation of mixed as well as pure disruption mechanisms.

Listing of Some Practical Ramifications When Competitive Attraction is the Leading Mechanism of Moth Mating Disruption

Pheromone Formulations for Disruption

If disruption primarily involves attraction, attractiveness of pheromone formulations should be optimized, e.g., match the natural blend of the female and optimize the release rate so as to generate plumes matching or exceeding those of females. In some cases, it may be cheaper to use an off-blend, i.e., if attractiveness of dispensers releasing abnormally high rates of a cheaper pheromone component can match attractiveness of females releasing a blend containing expensive minor components.

The density of point sources is strongly and positively correlated with the efficacy of a pheromone treatment. The higher the density of dispensers, the more effective is the treatment, provided that each dispenser remains highly attractive. However, it may be possible to apply dispensers at densities so high that they no longer attract. This could shift disruption from a competitive to non-competitive mechanism. To date, disruption efficacy per unit of pheromone used appears to be slightly higher for competitive vs. non-competitive disruption. Furthermore, it is advantageous for pheromone point sources to be thoroughly dispersed in the crop so as to avoid zones with no coverage. This is particularly important when field size is small. Very large fields may lend themselves to some reduction in dispenser density, provided pest density is low.

The release rate of individual pheromone point sources also matters; it interacts multiplicatively with dispenser density. Dispenser density and release rate should be manipulated so as to maximize $k_D * D_D$.

Formulations of pheromone to be used in disruption by competitive attraction should be flexible in the sense of offering opportunity to vary the density of dispensers and the timing of application. When pest densities are high, the number of dispensers will likely need to exceed the numbers affordable for manual application, i.e., future formulations will need to be amenable to application by machine. Desirable attributes for machine application are as follows: capability to be pumped or otherwise mechanically moved from storage reservoir to applicator; ability of the parent stock to be subdivided into particles or drops of desired sizes; transferability from applicator onto the crop; effective self-adhesion of the dispenser particles onto the crop; long-lasting release of the pheromone; avoidance of phytotoxicity or other undesired impacts of the formulation; ability to be biodegraded. An example of the type of formulation we foresee as viable for disruption that maximally exploits competitive attraction is the emulsified wax used in our OFM and CM studies. Exclusive of the active ingredients, the formulation is relatively inexpensive; it is formulable in large batches by using ordinary equipment; the product is storable in common containers; it is flowable and can be pumped to an applicator; techniques like extrusion through holes in a spinning hub can break the parent material into deposits ranging from droplets of microliter size through drops measured in hundreds of microliters, to dollops ranging up to several milliliters; this wax is self-adhering to diverse plant parts and adhesion can be increased by the addition of small amounts of benign glues; cleanup of equipment and clothing is easily accomplished using soap and water; the release rate of wax drops is surprisingly long and decreases slowly when the starting concentration of pheromone is kept below 5%; the wax carrier is not phytotoxic, as certain pheromones such as codlemone can be phytotoxic to leaves and fruits (Giroux and Miller, 2001); and these wax deposits eventually fall to the ground and biodegrade without known consequence. Other technologies with a similar set of desirable traits are the pheromone flakes (referred to above in the gypsy moth study) and pheromone fibers extensively explored several decades ago (Charlton and Cardé, 1981) and making a reappearance (Scentry Biologicals Inc., Billings, MT, USA; Knight, 2003).

Factors Influencing Performance of a Given Pheromonal Formulation

Disruption outcomes under competitive attraction are extremely sensitive to pest densities (Fig. 3; Miller et al., 2006). Generally, the density of pheromone dispensers should exceed the density of females by ca. 100-fold. Dispenser density can be somewhat lower, if attractiveness of each dispenser is commensurately greater than that of females or if a pest is

not highly fecund. We are aware of no case where an increase in the rate of pheromone released per dispenser fully compensated for reduction in dispenser density.

Measuring pest density in a crop is a required antecedent to optimizing a disruption protocol under competitive attraction.

Pheromone-baited monitoring traps might not always offer sufficient sensitivity and consistency for safely guiding real-time management decisions for mating disruption. Where attractiveness of traps equals that of females, obtaining a mean catch of one male per trap per several nights could indicate that disruption has failed, irrespective of an impressive value for % disruption. On the other hand, obtaining catches of zero in multiple traps known to be highly attractive to males constitutes the strongest evidence for success in mating disruption obtainable from traps.

Complexity, Reliability, and Sustainability of Mating Disruption

Under competitive attraction, it will probably not be possible to combine the pheromones of multiple species into one cocktail, as was explored for aerosol dispensers of pheromone (Isaacs et al., 1999). In crops like Michigan tree fruit where there are more than four key moth pests, this could translate into a substantial total number of pheromone applications over one season. Doing so will require convenient machine application of each pheromone and the capability to make applications in accordance with the appearance and abundance of each generation of each pest. This requirement adds complexity to pest management compared to application of long-lasting, broad-spectrum insecticides. Hopefully, this disadvantage for pheromones will be offset by absence of environmental hazards and sustainability of this pest management tactic.

One of the challenges to adoption of mating disruption has been reliability. In some situations, mating disruption has worked well for a given pest, whereas in other situations it has not. We suspect that widely varying pest densities explain much of this variability. With changes in formulation and application technologies, and attention paid to the principles of competitive attraction, substantial improvements in the reliability of mating disruption should be possible for many pests.

An advantage of competitive attraction is that it is already directed at forestalling the key behavior leading males to females—attraction. In contrast, when exploiting camouflage or desensitization, it will be the few males in the population capable of orienting to females despite the presence of the dispensed pheromone that will be resistant to the control measure and whose ethotype will dominate under this strong selection pressure. It may be better to sidestep this potential weakness of non-competitive mating disruption by engineering disruption to explicitly and immediately target attraction. Resistance is less of a threat under competitive attraction, if attractiveness of disruption formulations is adjusted to track the blend from authentic females over time. Nevertheless, rotating mating disruption with other management tactics is always advised.

Possible Outcomes

It is our hope that insights gained from this analysis will lead to developments that make mating disruption more practical and reliable at a time when the number of pest management tools is shrinking. Furthermore, the analytical procedures and principles arising from the study on moths may prove useful to competitive-attraction phenomena generally, be they,

e.g., use of attracticides (Eviden and McLaughlin, 2004), other kinds of insects (Polavarapu et al., 2002), competitive insect–plant interactions (Miller and Cowles, 1990), vertebrates responding to attractants (Li et al., 2002), sperm competition (Berkhead, 1998), or humans responding to competing products in the marketplace. This knowledge might also inform outcomes of the competitive interactions of inanimate homing devices involving attraction to or diversion from competing long-distance cues, e.g., <http://www.fas.org/spp/starwars/program/nmd/>).

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Introduction

A recurring need in practical entomology is to simulate attractive natural volatile sources with effective synthetic versions for use in insect management programs. Natural sources, often host-related, typically involve multiple chemical components, which greatly complicates simulation and behavioral analysis. A situation of this sort arose for an Australian fruit pest, *Carpophilus davidsoni* Dobson (Coleoptera: Nitidulidae). This article describes a research approach for developing a synthetic attractant based on a relevant natural model.

Nitidulid beetles are the most serious pests of ripening stone fruit in southern Australia, and *C. davidsoni* is the most significant of these (James et al., 1997; Hossain et al., 2006; and references therein). *Carpophilus* beetles cause damage by direct feeding (Hely et al., 1982) and by transmission of brown rot (*Monilinia* spp., Kable, 1969) and other harmful microorganisms. In recent years, growers have reported losses of up to 30% of the crop (Hossain et al., 2000).

Currently, no conventional insecticide is registered and practical for controlling *Carpophilus* spp. in stone fruit, but attractants have good potential in nitidulid management as alternatives to insecticides. It has been known for many years that *Carpophilus* beetles are attracted to odors from ripening fruit or fermenting foods (e.g., Wildman, 1933). In California, a mass-trapping program that used fermenting figs as bait led to a moderate damage reduction in fig orchards (Warner, 1961). In Australia, the native species are readily attracted to fermenting peach and apple juice, which have been used in traps for population monitoring (James et al., 1998; Hossain et al., 1999; Mansfield and Hossain, 2004). In general, attraction of *Carpophilus* spp. to food or fermentation odors is due to the emission of blends of small alcohols, aldehydes, and esters (Smilanick et al., 1978; Lin and Phelan, 1991; Phelan and Lin, 1991; Bartelt and Wicklow, 1999), although no specific information had been published regarding *C. davidsoni*.

In addition to host-related attractants, male-produced aggregation pheromones have been identified for the key Australian *Carpophilus* species, including *C. davidsoni* (Bartelt and James, 1994). The pheromones attract both males and females, and they function synergistically with food volatiles (reviewed by Bartelt, 1999). Baits containing both the pheromones and food-related attractants are more potent and have greater pest management potential than those containing food attractants alone.

Using attract-and-kill stations baited with *Carpophilus* pheromones, ripe and rotting peaches, and fermenting peach juice, Hossain et al. (2001, 2006) demonstrated protection of ripening stone fruit. The peaches contained within the stations were treated with insecticide, and the attracted beetles were killed when they landed and fed. The stations (three per hectare) were deployed well before fruit maturation in the orchard, and the concept was to remove as much of the local beetle population as possible before the fruit became susceptible to attack. From the standpoint of efficacy, the method has potential, but the large volumes of fruit and juice in the stations make them unwieldy and time-consuming to service. For the technique to become acceptable to growers, an easy-to-use alternative for the fruit and juice is required.

The specific objective of this research was to develop a synthetic food-related attractant that might replace the fruit and fruit juice in the attract-and-kill stations. Using fermented peach juice and intact fruit as natural models, volatile emissions were analyzed both qualitatively and quantitatively and characterized for attractiveness to *C. davidsoni* in a

laboratory wind tunnel. Synthetic blends were prepared that matched the natural sources, both in emission rates of key chemicals and in attractiveness. The activity of the final synthetic formulation was verified under field conditions.

Methods and Materials

Natural Volatile Samples

The laboratory experimentation was carried out in Peoria, Illinois, USA. Emissions from fermenting peach juice and whole peaches and nectarines were analyzed chemically and evaluated for attractiveness as the initial step of synthetic attractant development. The canned peach juice was the same type (Golden Circle™, Brisbane, Queensland) that had been used previously in field tests (Mansfield and Hossain, 2004). Fermentation was started by adding dried baker's yeast (0.25 g/50 ml of juice). Juice samples (10 ml) were placed in 50-ml Erlenmeyer flasks, which were equipped with glass inlet/outlet adapters (both the flasks and adapters had 24/40 ground glass fittings, so that connections would be air tight and chemically inert). All connections to flasks were made with 4-mm ID Teflon tubing. There was a constant flow of air (10 ml/min) through the headspace of each sample flask. Flasks were kept at room temperature. New juice samples were inoculated on consecutive days for 7 d and comparisons of all ages were performed on the eighth day, both with respect to chemical emissions and attractiveness to beetles. An unfermented juice sample was also included.

Peaches and nectarines of white-flesh and yellow-flesh varieties, from a local market in Peoria, were also studied. Fruit was unripe when purchased, and chemical and behavioral analyses were performed on consecutive days to document changes in chemistry and attractiveness over time. Individual pieces of fruit were placed in wide-mouthed canning jars of ca. 0.5- or 1-l volume, using the smaller size possible, and kept at room temperature. Fittings installed in the air-tight lids served as air inlets and outlets, and all external connections were with Teflon tubing of 4 mm ID. In addition, a piece of Teflon tubing extended inside the jar from the inlet fitting to the bottom, so that incoming air would circulate before exiting. There was a constant (24 hr/day) airflow of 10 ml/min through each jar so that emitted volatiles could not accumulate in the jar. With this configuration, the chemical emission rate at the outlet port fairly represented the emission rate from the piece of fruit, both qualitatively and quantitatively.

Synthetic Volatile Samples

Solutions of synthetic compounds were prepared to simulate natural samples in the emission rates of one or more components. Blends were formulated in 10 ml of water or oil and placed in 50-ml Erlenmeyer flasks with 10 ml/min airflow, as with fruit juice samples. A magnetic stirrer aided volatilization of compounds into the headspace. Compounds formulated in water were acetaldehyde, ethanol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol; compounds formulated in oil were methyl acetate, ethyl acetate, 2-methylpropyl acetate, 3-methylbutyl acetate, hexyl acetate, Z-3-hexenyl acetate, and ethyl octanoate. Synthetic chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI). Vacuum pump oil (Duoseal, Welch, Skokie, IL) served well because it had an extremely low volatile background. Based on solid-phase microextraction–gas chromatography (SPME-GC) analyses of volatiles from standard solutions, emission rate was nearly proportional to concentration in solution. Thus, the

concentration of a compound required to give a particular emission rate (i.e., to simulate a natural sample) could be readily calculated once baseline information was obtained. Dilute stock solutions of individual synthetic compounds (between 0.1 and 100 mg/ml, made up in advance) facilitated the preparation of target blends. Synthetic blends were generally prepared fresh each day.

Four individual samples of fermenting juice (1–3 d after inoculation) were chosen for simulation on 4 separate days. For each of these, an SPME-GC analysis was conducted on the natural sample early in the day, target composition of the synthetic blend was calculated, the synthetic blend was prepared and analyzed (and if necessary, adjusted), and the natural and synthetic materials were compared in the wind tunnel bioassay. A need was to complete sample preparation quickly, before the natural model had time to change significantly and to allow enough time for bioassays. The amount of time between the analysis of the natural sample and the beginning of wind tunnel bioassays was typically 2 hr.

In addition, a bioassay standard was prepared, adapted from earlier work with *C. humeralis* (Bartelt and Zilkowski, 1998). The standard consisted of an aqueous mixture of acetaldehyde (0.3 mg/ml), ethanol (90 mg/ml), and ethyl acetate (0.18 mg/ml). This mixture (10 ml in a 50-ml flask, magnetically stirred at 25°C, airflow through the headspace at 10 ml/min) delivered the three compounds at rates of 7000, 98,000, and 9500 ng/min, respectively.

Chemical Analysis

Gas chromatography and coupled GC/mass spectrometry were performed as previously described (Bartelt and Wicklow, 1999), using DB-5MS columns (30 m × 0.25 mm ID, with a 1 μm film thickness, J&W Scientific, Folsom, CA). The oven temperature program was 50°C for 1 min, increasing at 10°C/min to 250°C, with a final hold at 250°C for 3 min. Chemical identifications were made by reference to the Wiley mass spectral library and were confirmed by comparing GC retention times and mass spectra to authentic standards.

Quantitative sampling of emissions from the fruit, juice, and synthetic sources was performed by SPME. SPME is a simple, sensitive, and solventless analytical technique (Pawliszyn, 1997) where chemicals are absorbed (in this case, from the air) by a thin, polymer-coated, silica fiber, which is manipulated with a syringe-like holder (Supelco, Bellefonte, PA, USA). Absolute quantitation by SPME was based on previously established absorption-kinetics properties for fibers of the 100-μm poly(dimethylsiloxane) type (Bartelt and Zilkowski, 2000). Sampling was always performed at the air outlets of the containers with volatile sources. The coated portion of an exposed SPME fiber was situated 2 to 3 cm inside the open end of the Teflon outlet tube, along the central axis. Airflow rate in the outlet tube was always 10 ml/min, and room temperature during sampling was recorded. Sampling time was 30 min, and GC injection of the SPME sample followed immediately.

The absolute emission rates from the sources of volatiles (in ng/min) were calculated directly from the GC peak areas by using relationships developed previously (Bartelt, 1997; Bartelt and Zilkowski, 1999, 2000). The mathematical equations were programmed into the data system with the Hewlett Packard ChemStation macro language. Thus, chemical emission rates became part of the printed GC report as specific compounds were recognized by retention time.

Bioassay Equipment

A volatile delivery system (VDS), described previously (Bartelt and Zilkowski, 1998, “new system”), was used to deliver scents. Briefly, inputs for the VDS were the airstreams

emerging (at 10 ml/min) from containers with fruit, fruit juice, or synthetic compounds. The VDS allowed up to four inputs to be controlled at once, and any of these, or any combination of these, could be quickly directed to the wind tunnel by switching a valve. Furthermore, the levels of volatiles from the four inputs could be individually adjusted, so that any level between 5% and 100% of each source could be used in the bioassay. Thus, with a small number of physical volatile sources, a wide range of combinations and doses could be tested easily.

The wind tunnel was $0.7 \times 0.7 \times 1.3$ m in size. Airflow was laminar (ca. 0.3 m/sec); incoming air was warmed with an electric space heater to maintain a temperature of 28°C to 30°C in the wind tunnel, and exiting air was exhausted from the room. Lighting was from above with four 40-W fluorescent tubes. The outlet tube from the VDS, which carried the bioassay volatiles, opened into the wind tunnel at the center of a tan, circular cardboard disk, which served as landing target for responding beetles. The disk was 8 cm diam, mounted 40 cm above the wind tunnel floor and 20 cm from the upwind end, with its plane perpendicular to the direction of air movement.

Bioassay Insects and Procedures

A culture of *C. davidsoni* was established at National Center for Agricultural Utilization Research, Peoria, with adults sent from Australia. The beetles were reared on an artificial diet (Dowd, 1987; Bartelt and James, 1994). To prepare for wind tunnel bioassays, ca. 500 to 600 adults (of mixed sex and up to 4-wk old) were transferred from food cups into a 1-l glass jar in the evening before the tests were to be run. A crumpled paper towel was placed in the jar as a walking substrate, but no food or water was provided. The following morning, beetles were transferred into the wind tunnel, and bioassays began when 50 to 100 *C. davidsoni* were consistently in flight (see Results and Discussion).

Volatile samples were evaluated in the wind tunnel within 1 or 2 hr of chemical analysis, to establish a relationship between rates of compound emission and attractiveness. A test started when the output from the VDS was directed into the wind tunnel. Beetles landing on the cardboard disk during the next 2 min were counted, and then the flow from the VDS was switched away from the wind tunnel. Beetles remaining on the disk after a test were dislodged by gentle tapping. Time between tests was typically 2 to 5 min. Bioassay sessions lasted as long as 4 to 6 hr and included as many as 60 individual tests.

The volatile treatments of an experiment (up to 10) were tested consecutively in the wind tunnel in random order. This group was considered as a block for the purpose of statistical analysis. Data were acquired for additional blocks (also randomized) during the same day or on subsequent days, as needed. The counts of landings were transformed to $\log(x + 1)$ to stabilize variance and subjected to two-way analysis of variance (ANOVA). If the overall *F* test was significant, then the means were compared by the least significant difference (LSD) method at the 0.05 level.

A control (clean air delivered by the VDS) was included in every experiment, and the bioassay standard (see above) was sometimes included as well, as a basis for measuring day-to-day changes in treatments. An index of activity (*IA*) was used for expressing relative activity of treatments: $IA = 100 \times (T - C) / (S - C)$, where *T*, *C*, and *S* are the mean counts of landing beetles during the experiment for a treatment, the control, and the standard, respectively (Bartelt and Wicklow, 1999). *IA* expresses the attractiveness of a treatment as a percentage of the attractiveness of the standard, after correcting for the baseline (control) response. By definition, *IA* for the standard is 100, and *IA* for the control is 0.

Beetles were removed from the wind tunnel by allowing them to enter cups of diet set on the floor (typically complete within 1 hr). After at least 2 d of feeding, beetles could be reused in the bioassay. By alternating among several sets of insects, bioassays could be run each day. Newly emerged adults were added to the groups to replace dead ones as needed. The wind tunnel floor was swept with a vacuum cleaner, and the walls were wiped down with water between uses.

Field Experiments

Synthetic food attractant (SFA) was evaluated in Australia by one of us (M.S.H.) in two field experiments. Both were conducted in peach orchards (variety T204) in the Goulburn Valley, Victoria, Australia. The population of *Carpophilus* spp. was relatively low during the first experiment (December 2–13, 2002); fruit on the trees was green during this period. The second experiment (February 18 to March 21, 2003) was carried out after harvest at an orchard with a higher beetle population; some overripe and rotting fruit was still present on the ground.

The experiments included three treatments: The first was SFA (120 ml of an aqueous solution of ethanol, 35 mg/ml; ethyl acetate, 0.094 mg/ml; 3-methyl-1-butanol, 0.060 mg/ml; acetaldehyde, 0.051 mg/ml; 2-methyl-1-propanol, 0.027 mg/ml; and 2-methyl-1-butanol, 0.010 mg/ml). The second was a higher dose of the synthetic attractant (SFA10X), which was 120 ml of an aqueous solution prepared in the same way as SFA but the amount of each compound was 10 times greater. SFA and SFA10X were held in 200-ml glass jars. The third treatment was 120 ml of fermenting apple juice (FAJ), which was known to be attractive to the beetles (Mansfield and Hossain, 2004) and served as a standard for comparison. The traps were Magnet™ funnel traps (23 × 17 cm) and were hung at a height of 1.5 m. Each trap contained one bait jar, covered with fine mosquito net to exclude beetles, and a piece (1 cm²) of dichlorvos-impregnated insecticidal plastic strip.

Each test was set up as a 3 × 3 Latin square replicated twice ($N = 6$ for each treatment). Trap spacing was 20 to 24 m (traps in every fourth tree), and randomization was carried out at the beginning of each experiment. During the first experiment, all baits were replaced and trapped beetles were removed daily or on alternate days. During the second experiment, traps were serviced twice weekly. Trap catches were estimated volumetrically (200 beetles/ml), and species composition was determined (Dobson, 1954, 1964), based on a sample of 500 beetles per trap, or on the entire trap catch if it was <500. Trap catches were transformed to $\log(x + 1)$ to stabilize variance, and statistical analysis was carried out by using Genstat 5.42 (Genstat Committee, 2002). The Latin square design was subjected to ANOVA, using a repeated-measures model. Both treatment main effects and treatment-by-time interactions were fitted. Individual means were compared by *t*-tests when significant *F* values were obtained. The two field experiments were analyzed separately.

Results and Discussion

Initial Bioassay Observations

Responses of *C. davidsoni* in the wind tunnel were similar to results with *C. humeralis* (e.g., Bartelt and Zilkowski, 1998). Beetles initially crawled into corners and became motionless. However, with the lights on and the temperature at 28°C to 30°C, they began to fly spontaneously after 1 to 2 hr. Then, for as long as 4 to 6 hr, typically 50 to 100 beetles

would be airborne at any instant, even in the absence of attractants. Bioassay experiments were conducted only when this level of flight activity existed.

When an attractant was directed into the wind tunnel through the VDS, a characteristic response usually began within 20 to 30 sec. A “cloud” of hovering beetles formed at a location about 0 to 30 cm downwind of the paper disk and volatile outlet. Beetles slowly began to alight on the disk, and this landing activity intensified as the test continued. Beetles on the disk walked rapidly, and some attempted to enter the VDS outlet tube (prevented by a small-diameter opening). When the flow of volatiles from the VDS was turned off, the cloud of hovering beetles dissipated almost instantly and most of those on the paper disk took flight within a minute.

Beetles responded to the bioassay standard (containing acetaldehyde, ethanol, and ethyl acetate; see above). During 2 mo of bioassays, the standard mixture and control were compared 68 times, usually as part of larger experiments. Mean numbers of landings per 2-min test were 56.7 (\pm 28.5 SD) and 0.5 (\pm 0.6 SD), respectively (paired *t*-test on transformed counts, $t = 16.3$, $P < 0.001$). The absolute numbers of beetles responding to any treatment, including the standard mixture, differed somewhat between days and even from hour to hour because of variation in numbers of beetles in the wind tunnel, numbers actually in flight, and other factors. Blocking of experiments compensated for this variability.

Chemical Analysis and Attractiveness of Fruit Juice and Fruit

A key capability in this study was that airstreams from volatile sources could be both chemically analyzed and behaviorally evaluated within 1 or 2 hr, before an ephemeral volatile source had time to change. Thus, the chemical emission rate from a sample (in ng/min) became the chemical delivery rate into the wind tunnel, and a direct relationship could be established between chemical composition and behavior.

A typical GC profile for fermenting peach juice is shown in Fig. 1 (upper panel), and the chemical identifications, emission rates, and bioassay results are summarized in Table 1. This material was highly active in the wind tunnel, attracting nearly twice as many beetles as the standard. All six of the tested intact peaches and nectarines were demonstrably attractive in the wind tunnel, but attractiveness increased dramatically as the fruits became ripe or overripe (subjectively categorized by taste, smell, and softness). Correspondingly, chemical emissions became more abundant and more complex with ripening. GCs for two typical examples are shown in Fig. 1. Corresponding chemical identifications, emission rates, and bioassay results are summarized in Table 1. Based on bioassay landings, an unripe peach was about one fourth as attractive as the standard, whereas the overripe peach was over twice as attractive. All of the whole pieces of fruit showed the same general trend, but there were differences in the amounts and identities of compounds, especially the esters (data not given).

A property of this SPME method is that sensitivity increases dramatically with molecular weight (Bartelt, 1997; Pawliszyn, 1997), and this property must be kept in mind as chromatograms are examined; the largest GC peak does not necessarily represent the most abundant chemical. In the upper panel of Fig. 1, for example, the GC peak for ethyl octanoate (**15**) is far larger than that for acetaldehyde (**1**); yet, the actual emission rate for ethyl octanoate was less than one tenth of that for acetaldehyde (Table 1). Nevertheless, acetaldehyde (the most volatile analyte in this study) was still detectable by SPME whenever there was enough to elicit a behavioral response.

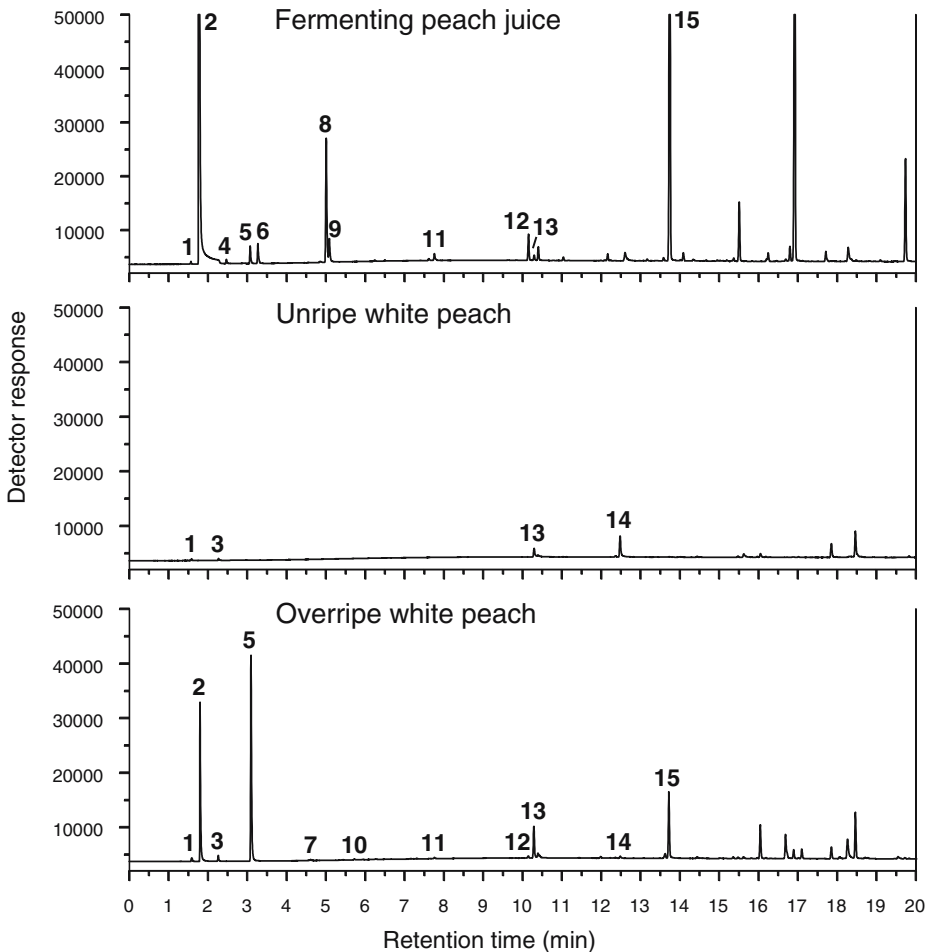


Fig. 1 SPME-GCs for three example sources of volatiles. Peak identifications as in Table 1

Clearly, a variety of volatile blends can be highly attractive to *C. davidsoni* (Fig. 1; Table 1). We used the fermenting juice as the primary model for developing a practical beetle attractant because canned peach juice was always available for laboratory work, whereas the fresh peaches were seasonal.

Effect of Fermentation Time on Activity and Chemistry of Fermented Juice

The chemical analyses and bioassay responses for fermenting peach juice of various ages are summarized in Table 2. Samples were generally similar with respect to volatile composition and bioassay activity, regardless of the length of time after inoculation, but the greatest activity and highest amounts of emitted esters and ethanol occurred after 1 to 2 days of fermentation. The peach juice used in the experiment showed some evidence of fermentation even before the addition of yeast, based on the amount of ethanol emitted, but no microbiological examination was made.

Synthetic mixtures that would mimic peach juice after 1 to 3 days of fermentation were prepared. There were at least 50 compounds emitted from fermented juice, but most of

Table 1 Emission rates of volatiles from fermented peach juice (1 d after inoculation), unripe peach, and overripe peach and corresponding bioassay activity

Compound/bioassay	Emission rate or index of activity		
	Fermented peach juice	Unripe peach	Overripe peach
Chemical analysis (emission rate in ng/min)			
1 Acetaldehyde	311	210	55
2 Ethanol	58,400		320
3 Methyl acetate		72	220
4 1-Propanol	38		
5 Ethyl acetate	210		2400
6 2-Methyl-1-propanol	92		
7 Propyl acetate			4.4
8 3-Methyl-1-butanol	206		
9 2-Methyl-1-butanol	37		
10 2-Methylpropyl acetate			1.7
11 3-Methylbutyl acetate	5		0.81
12 Ethyl hexanoate	5		0.41
13 Z-3-Hexenyl acetate	2	3.3	10
14 Methyl octanoate		1.7	0.15
15 Ethyl octanoate	20		1.8
Bioassay (IA)	164	26	204

these were present at extremely low levels and were not identified (see Fig. 1). Based on previous experience with other species (Smilanick et al., 1978; Lin and Phelan, 1991; Phelan and Lin, 1991; Bartelt and Zilkowski, 1998; Bartelt and Wicklow, 1999), we hypothesized that a blend of the more volatile and more abundant alcohols, aldehydes, and

Table 2 Emission rates of chemicals and bioassay activity of samples of peach juice after specified durations of fermentation

Compound/bioassay	Days of fermentation							
	0	1	2	3	4	5	6	7
Emission rate (ng/min)								
1 Acetaldehyde	300	620	800	1900	950	1300	1400	2300
2 Ethanol	3800	28,000	63,000	44,000	24,000	36,000	34,000	24,000
4 1-Propanol	4.0	22	31	22	12	21	19	14
5 Ethyl acetate	51	150	270	53	39	22	17	14
6 2-Methyl-1-propanol	7.6	87	150	110	61	100	77	56
8 3-Methyl-1-butanol	9.2	120	330	240	120	200	190	110
9 2-Methyl-1-butanol	3.0	36	92	71	40	66	56	36
11 3-Methylbutyl acetate	4.1	6.3	8.6	1.4	1.9	1.6	0.95	0.47
12 Ethyl hexanoate	0.58	8.2	9.3	2.9	2.9	2.1	2.0	0.82
13 Z-3-hexenyl acetate	4.4	2.6	1.6	0.34	–	0.19	0.19	–
15 Ethyl octanoate	0.13	8.0	15	6.4	2.4	4.6	4.2	1.3
Bioassay activity (IA)	66	157	162	132	119	101	136	126
Mean landings ^a	18.9c	45.0a	46.4a	37.7ab	34.2ab	29.0b	38.6ab	36.1ab

^a Means ($N = 5$) followed by same letter not significantly different (LSD, 0.05 level) after transformation to $\log(x + 1)$. Means for standard and control: 28.7b and 0.2d, respectively. Overall F test of transformed data: $F = 84.3$, $df = 9,36$, $P < 0.001$.

esters (a total of 10, see below) would be sufficient to account for the attraction of *C. davidsoni* to fermenting peach juice and that it would not be necessary to include all natural constituents.

Simulation of Fermenting Juice

Four simulations of fermenting peach juice are summarized in Table 3, with the natural juice and synthetic blend being matched as closely as possible with respect to 9 or 10 compounds and delivered at the 100% rate by the VDS. The bioassay comparisons were all favorable, and no differences between natural juice and synthetic blend were significant ($P > 0.6$).

A dose–response comparison was carried out for one simulation (set 3, see Fig. 2). The ANOVA model included terms for dose (five levels), volatile sample (two levels), dose-by-sample interaction, and replication of the experiment (“block”, four levels). Differences among the doses were dramatic ($F = 102$, $df = 4$ and 25 , $P < 0.001$), and response decreased smoothly as dose decreased. The responses to the synthetic blend were slightly lower than to the natural juice ($F = 4.54$, $df = 1$ and 25 , $P = 0.04$), but there was no good evidence that the lines in Fig. 2 were not parallel ($F = 0.88$ for the dose-by-sample interaction, $df = 4$ and 25 , $P = 0.49$). Two extreme data points (synthetic blend at 25% and 5% of maximum dose in the initial replication) were omitted from the analysis ($P < 0.005$ for outlier test).

The subtle difference between the curves for natural juice and synthetic blend was explored further with regression analysis, using $\log(x + 1)$ -transformed response data and \log -transformed doses. A single quadratic dose–response model was applied to both volatile samples, allowing, in addition, for differences among the four blocks ($R^2 = 0.927$ for this model, residual mean square = 0.0068). [The joint F statistic for the linear and

Table 3 Emission rates of compounds (ng/min) for four fermenting peach juice samples and the synthetic mixtures prepared to simulate them and corresponding bioassay comparisons

Compound/bioassay	Set 1 (1 d)		Set 2 (2 d)		Set 3 (3 d)		Set 4 (1 d)	
	Natural	Synthetic	Natural	Synthetic	Natural	Synthetic	Natural	Synthetic
1 Acetaldehyde ^a	310	260	380	320	850	690	220	180
2 Ethanol ^a	58,000	49,000	44,000	42,000	47,000	39,000	46,000	39,000
5 Ethyl acetate ^b	210	340	430	780	180	120	67	61
6 2-Methyl-1-propanol ^a	92	86	68	77	64	56	50	56
8 3-Methyl-1-butanol ^a	210	190	160	180	150	150	140	150
9 2-Methyl-1-butanol ^a	37	38	30	35	28	30	21	29
11 3-Methylbutyl acetate ^b	5.3	11	0.91	2.8	0.62	0.92	1.7	2.3
12 Ethyl hexanoate ^b	4.8	7.0	1.8	4.5	1.8	1.4	1.5	1.3
13 Z-3-hexenyl acetate ^b	1.6	2.9	0.31	1.1			0.50	0.52
15 Ethyl octanoate ^b	20	13	10	15	8.3	4.7	8.7	6.4
Mean landings ^c	97.2	94.3	62.5	62.2	67.2	64.5	55.7	55.6
<i>N</i>	6	6	6	6	6	6	4	4

^a Formulated in water solution.

^b Formulated in oil solution.

^c Within sets, means were not significantly different ($P > 0.6$). Paired t tests, using pooled error from whole experiment (18 df).

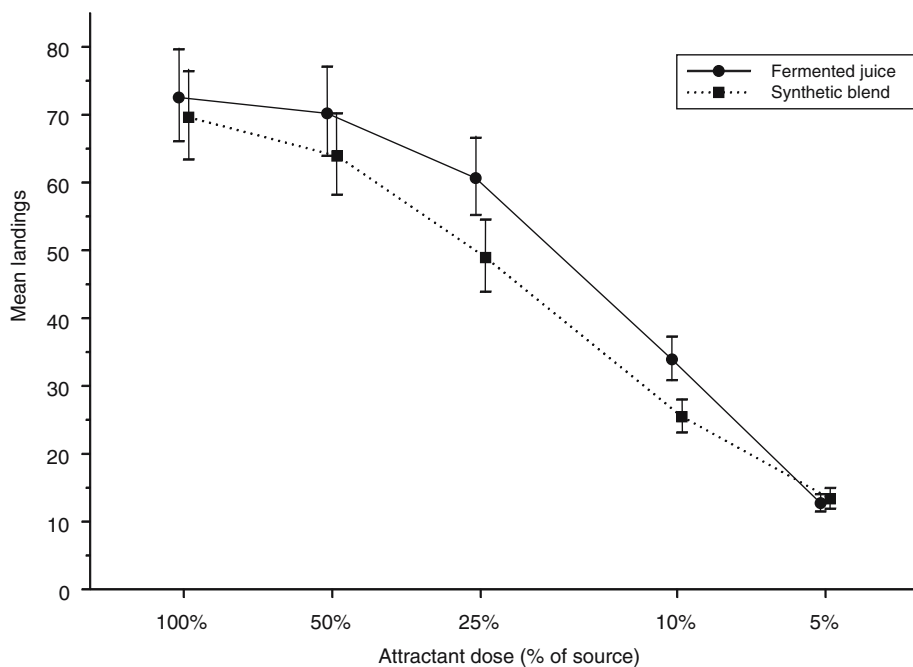


Fig. 2 Wind tunnel response to doses of a sample of fermenting peach juice and the synthetic blend prepared to simulate it. The various doses were generated by the VDS. Means ($N = 4$, except $N = 3$ for the synthetic blend at 25% and 5%) and standard errors (bars) were based on ANOVA after transformation of counts to $\log(x + 1)$, but the values were back transformed for presentation here. See text

quadratic terms, fitted last, was 198 ($df = 2$ and 32, $P < 0.001$), but subsequent addition of cubic and quartic terms gave no further significant improvement ($F = 2.06$, $df = 2$ and 30, $P = 0.15$). Then, the dose values for the synthetic blend were systematically adjusted until residual error for the model was minimized; conceptually, this process slid the curve for the synthetic blend in Fig. 2 along the X axis until it matched that for the natural juice as closely as possible. The minimum error was achieved when the doses for the synthetic blend were 86% of those for the natural juice (R^2 increased to 0.933 and residual mean square decreased to 0.0062, a residual error that was even smaller than in the initial ANOVA model, which was 0.0064). The calculated effective dose (86% of the natural juice) agreed remarkably well with the actual difference in total emission rates shown in Table 3 (83%), nicely accounting for the offset of the curves in Fig. 2.

More importantly, the well-behaved dose–response for set 3 suggested that the favorable bioassay comparison of natural and synthetic sources for all sets in Table 3 was genuine, rather than being an artifact of a “saturated” bioassay.

Importance of Components and Blend Optimization

Whereas the blend of 10 compounds was sufficient to account for the attractiveness of the fermenting juice, it was unknown if all 10 were necessary. Thus, the blend (specifically, set 4 in Table 3) was considered further in two portions, the five components formulated in water and the five formulated in oil. The GC traces of the natural model and the aqueous and oil synthetic formulations are shown in Fig. 3, and corresponding bioassay data are given in Table 4. The water formulation was strongly attractive by itself, but it was not as

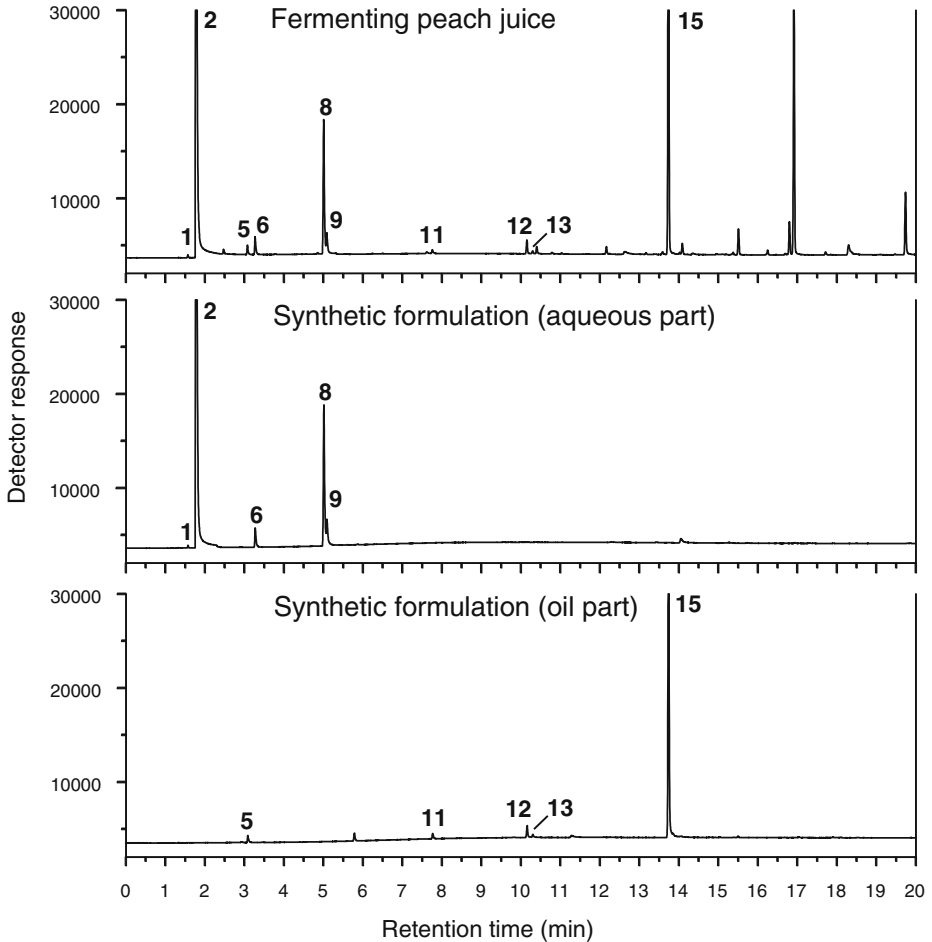


Fig. 3 SPME-GCs showing a sample of fermenting peach juice 1 day after inoculation and the synthetic formulations (aqueous part and oil part) that together simulate the natural sample. Peak identifications as in Table 1

attractive as the whole blend. The oil formulation was barely attractive on its own, but it synergized the aqueous formulation, and the synthetic combination was as attractive as the natural juice sample. The same trend was seen for all four simulation blends described in Table 3 (data not shown).

The water formulation was examined further by preparing corresponding samples that contained just acetaldehyde, ethanol, or the three higher alcohols (2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol; Fig. 4). When bioassayed individually, in pairs, or all together, it was clear that all three samples contributed to attraction (Table 5, upper portion). The best response was when the entire water formulation was reconstructed. However, no clear answer was obtained about the relative importance of the three higher alcohols (Table 5, lower portion). Adding any one of 2-methyl-1-propanol, 3-methyl-1-butanol, or 2-methyl-1-butanol to a blend of acetaldehyde plus ethanol elicited a response that was significantly greater than to just acetaldehyde plus ethanol. However, the response caused by adding all three at once was not obviously different from that caused by adding

Table 4 Bioassay comparison of peach juice (fermented for 1 d), the synthetic mixture prepared to simulate it (composed of water and oil formulations, combined by VDS), and the two individual formulations

Treatment	Mean landings (<i>N</i> = 4) ^a
Fermenting peach juice (1 d)	55.7a
Synthetic mixture (water and oil formulations)	55.6a
Water formulation ^b	36.4b
Oil formulation ^c	2.0c
Control	0.2d

^a Means followed by the same letter are not significantly different (LSD, 0.05 level); analysis after log(*x* + 1) transformation.

^b Includes acetaldehyde, ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol.

^c Includes ethyl acetate, 3-methylbutyl acetate, ethyl hexanoate, Z-3-hexenyl acetate, and ethyl octanoate.

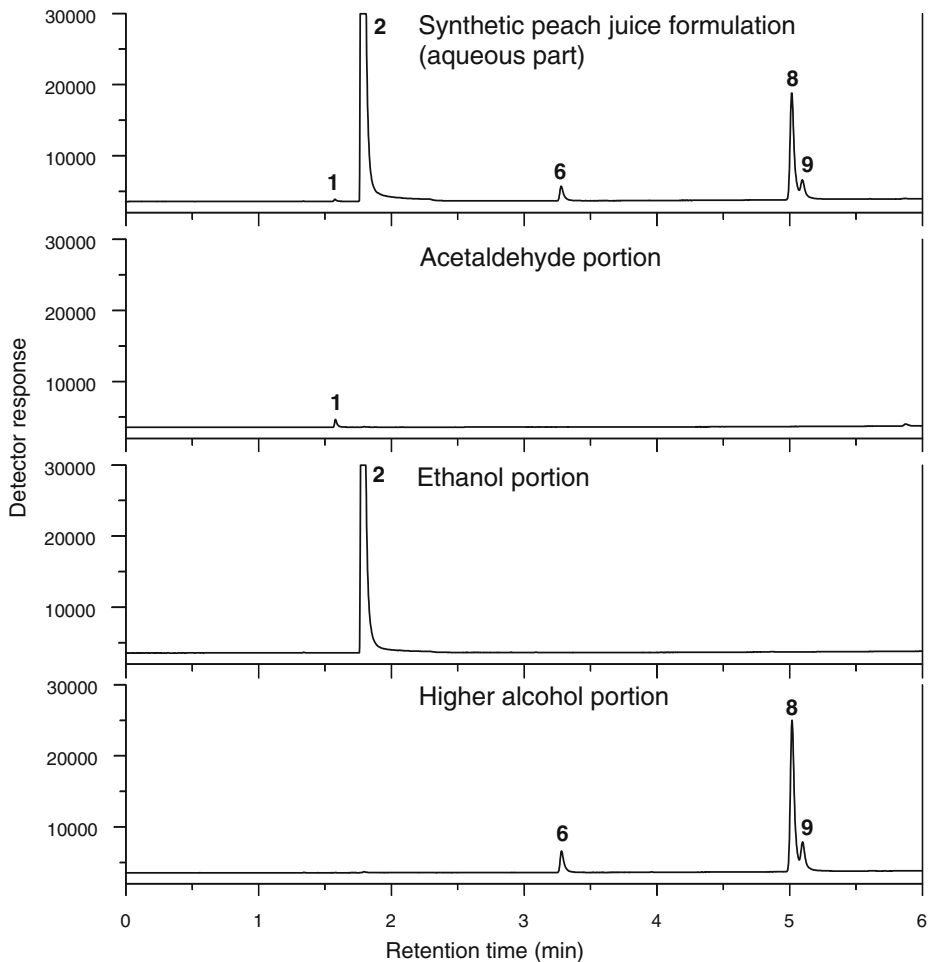


Fig. 4 SPME-GCs for synthetic blend of five compounds in water, based on a sample of fermenting peach juice, and three additional samples that together constitute this blend. Peak identifications as in Table 1

Table 5 Wind tunnel activity of the five components of the water solution

Treatment	Mean landings ^a
Experiment 1: Acetaldehyde vs. ethanol vs. three higher alcohols (<i>N</i> = 11)	
Control	0.0f
Acetaldehyde (Ald) ^b	0.6ef
Ethanol (Eth) ^c	3.4d
Higher alcohols (HiA) ^d	1.5e
Ald + Eth	14.3b
Ald + HiA	3.7d
Eth + HiA	7.4c
Ald + Eth + HiA	38.4a
Experiment 2: Addition of all higher alcohols to acetaldehyde + ethanol (<i>N</i> = 9)	
Ald + Eth	18.9b
Ald + Eth + HiA	43.6a
Experiment 3: Addition of 2-methyl-1-propanol to acetaldehyde + ethanol (<i>N</i> = 9)	
Ald + Eth	13.3b
Ald + Eth + 2-methyl-1-propanol ^e	32.7a
Experiment 4: Addition of 3-methyl-1-butanol to acetaldehyde + ethanol (<i>N</i> = 9)	
Ald + Eth	15.2b
Ald + Eth + 3-methyl-1-butanol ^f	29.3a
Experiment 5: Addition of 2-methyl-1-butanol to acetaldehyde + ethanol (<i>N</i> = 9)	
Ald + Eth	14.3b
Ald + Eth + 2-methyl-1-butanol ^g	35.7a

^a In each experiment, means followed by the same letter were not significantly different (LSD, 0.05).

^b Emission rate for acetaldehyde (**1**) 700 ng/min.

^c Emission rate for ethanol (**2**) 30,000 ng/min.

^d Higher alcohols = 2-methyl-1-propanol (**6**) + 3-methyl-1-butanol (**8**) + 2-methyl-1-butanol (**9**). Emission rates for **6**, **8**, and **9** were 83, 210, and 42 ng/min, respectively.

^e Emission rate for 2-methyl-1-propanol (**6**) 68 ng/min.

^f Emission rate for 3-methyl-1-butanol 200 (**8**) ng/min.

^g Emission rate for 2-methyl-1-butanol (**9**) 27 ng/min.

just one of them. Given that all three of the compounds would be inexpensive to use, we decided to retain all three in the final blend.

Finally, the oil-soluble components were considered. From Table 4, the nonpolar components from fermenting peach juice were of marginal importance, but such components were generally more abundant in overripe fruit (Table 1) and efforts to enhance the blend further were guided by this finding. Bioassays with additional esters are summarized in Table 6. Ethyl acetate was the most effective of the treatments, and this was added to the blend for field testing. Because ethyl acetate is a small, relatively polar ester, it is moderately soluble in water. Therefore, it was possible to formulate all six blend components in water solution. Figure 5 shows an SPME-GC analysis of the final aqueous blend, along with the measured emission rates and concentrations of components in the water.

The final six-component blend is chemically similar to the three-component bioassay standard that was (arbitrarily) used when the research began. The final blend is more attractive than the bioassay standard, calculated from comparisons in the tables, although it contains lower amounts of the three components possessed in common (ethanol, acetaldehyde, and ethyl acetate). The three additional, higher alcohols present in the final

Table 6 Activity of esters found in overripe peaches

Treatment	Mean landings ^a
Experiment 1: Screening of esters ($N = 9$)	
5-Component aqueous blend (5Aq) ^b	48.7c
5Aq + ethyl acetate (EtAc) ^c	96.3a
5Aq + 3 esters ^d	69.6b
5Aq + Z-3-hexenyl acetate ^e	53.6c
Experiment 2: Further testing of EtAc ($N = 8$)	
5Aq	73.2b
5Aq + EtAc ^c	119.0a

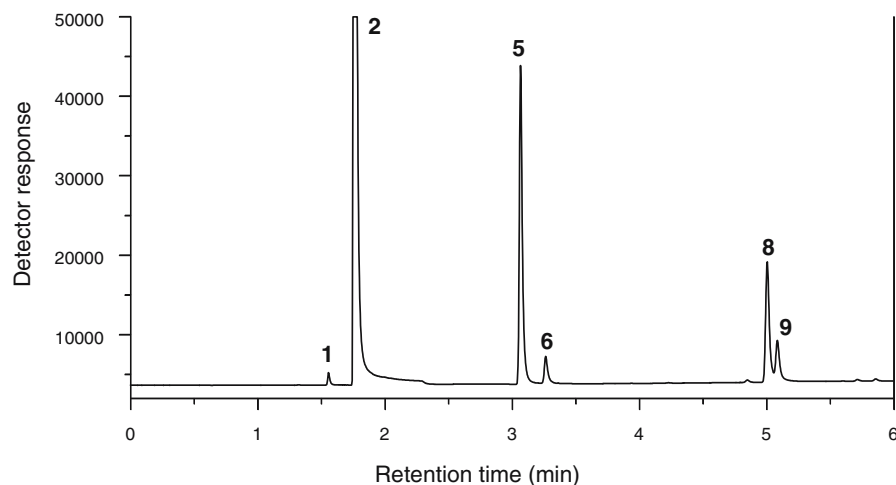
^a In each experiment, means followed by the same letter are not significantly different.

^b 5-Component aqueous formulation as in Table 5 (**1** + **2** + **6** + **8** + **9**).

^c Emission rate for ethyl acetate (**5**) 2200 ng/min (a typical rate for an overripe peach).

^d Emission rates for the three esters: propyl acetate (**7**) 76 ng/min, 2-methylpropyl acetate (**10**) 41 ng/min, and 3-methylbutyl acetate (**11**) 13 ng/min (about 10 times higher than for an overripe peach).

^e Emission rate for Z-3-hexenyl acetate (**13**) 1.6 ng/min (a typical rate for an overripe peach).



Properties of formulation for field

Chemical	Emission rate (ng/min)*	Concentration in solution (mg/ml)
1 Acetaldehyde	910	0.051
2 Ethanol	38,000	35.
5 Ethyl acetate	2700	0.094
6 2-Methyl-1-propanol	88	0.027
8 3-Methyl-1-butanol	130	0.060
9 2-Methyl-1-butanol	44	0.020

* For 10 ml solution under standard laboratory conditions

Fig. 5 SPME-GC of the final synthetic blend used for field tests and the corresponding compound emission rates and solution composition

blend (Fig. 5) clearly contribute to attractiveness, despite their low levels. Overall, the intense behavioral response to the minute amounts of airborne chemicals was remarkable.

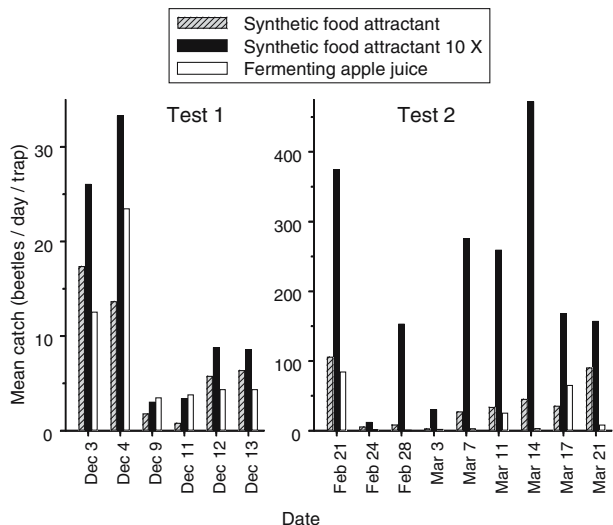
Field Study

Carpophilus davidsoni was the dominant species, accounting for >95% of the trap catches, and all results below refer to this species. In the first test (Fig. 6, left side), there were overall differences in trap catch among the three attractants ($F = 10.4$, $df = 2, 6$, $P = 0.011$). Catches for SFA10X (untransformed mean of 10.3 beetles per trap per day) were larger ($P < 0.05$) than those for SFA or FAJ (untransformed means of 5.6 and 7.0, respectively), but the SFA and FAJ were not significantly different from each other. From analysis of variance, the treatment-by-day interaction was significant ($F = 2.48$, $df = 10, 75$, $P = 0.013$), indicating that the ratios of catches among treatments were not statistically consistent from day to day. However, the interaction had a relatively minor effect on trends in Fig. 6 (e.g., SFA10X always had the highest or second highest trap catch). On two of the eight test dates (December 5 and 6), all trap catches were zero due to cold weather; these days were omitted from the analysis because they gave no information about treatment differences.

The results for the second test were more dramatic, both in terms of trap catch and treatment differences (Fig. 6, right side; for treatment effect, $F = 97.0$, $df = 2, 6$, $P < 0.001$). The overall means for SFA10X, SFA, and FAJ were 206.3, 37.7, and 18.3 beetles per trap per day, respectively, after back transformation, and all of the treatment differences were significant ($P < 0.001$). Once again, the treatment-by-day interaction was significant ($F = 6.22$, $df = 16, 120$, $P < 0.001$) but was minor relative to main treatment effects (e.g., SFA10X was superior during each interval of the experiment; see Fig. 6). Key results were that the synthetic blends were at least as effective as FAJ throughout, and that the higher dose of the synthetic attractant was more attractive. For the range of doses tested, no saturation or inhibitory effect was seen.

The differences in results between the two field tests remain unexplained. The beetle population was lower during the first test, which at least partially accounted for the smaller trap catches, and different weather conditions likely contributed as well. More

Fig. 6 Average number of *Carpophilus* beetle caught in traps baited with different co-attractants during two tests in orchards. Test 1 was before fruit coloring (December 2–13), and test 2 was after fruit harvest (February 19–March 21)



interesting was the dramatic preference for the high dose of synthetic attractant in the presence of a dense, highly responsive beetle population. The practical potential of the synthetic blend is clear.

Relationship to Previous and Future Research

The results were similar to conclusions for previously studied *Carpophilus* spp.. Qualitatively, the attractant blend for *C. davidsoni* from this study (Fig. 5) included many or all of the same compounds as blends developed previously for *C. hemipterus* (Smilanick et al., 1978; Phelan and Lin, 1991), *C. lugubris* (Lin and Phelan, 1991), and *C. humeralis* (Bartelt and Wicklow, 1999; Bartelt and Zilkowski, 1998). The common thread is a general responsiveness to blends of volatiles associated with fermenting fruit or food products, including ethanol, acetaldehyde, and various other alcohols and esters. Considerable plasticity is apparent in composition of attractive blends, but use of blends rather than single compounds is always advantageous. Further improvements in attractiveness are likely possible by adding still other components, by adjusting blend proportions, or by increasing overall dose. Research is continuing to incorporate the synthetic blend into the killing station concept for practical beetle control. A key remaining need is a more practical formulation method than the water solutions used here that will still allow the six components to be released at appropriate rates.

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Based on this study, a new attractant system for green lacewings is being developed for both domestic and international markets.

Keywords Hoverfly · Attractant · Pheromone · Iridodial · Methyl salicylate · 2-phenylethanol · Nepetalactone · Nepetalactol · 1-tridecene · GC–EAD · Field flight behavioral assay

Introduction

Lacewings (Chrysopidae) are important predators of aphids and other soft-bodied insects (New, 1975; Tauber et al., 2000). Furthermore, because of their commercial availability and resistance to insecticides, green lacewings are also among the most commonly released predators for augmentative biological control (Ridgway and Murphy, 1984; Tulisalo, 1984; Aldrich, 1999). However, methods are still needed to retain the predators near augmentation sites and/or to attract wild predators to target areas (Baker et al., 2003).

Lacewings are attracted to semiochemicals from different trophic levels, including host plant volatiles (Flint et al., 1979; Zhu et al., 1999, 2005; Hooper et al., 2002); herbivore-induced plant volatiles, such as methyl salicylate (MS; James, 2003a,b, 2006; James and Price, 2004); and sex pheromones of scale insects (Mendel et al., 2003) and aphids (Boo et al., 1998, 2003; Hooper et al., 2002). In particular, some lacewings are attracted to certain isomers of nepetalactone and nepetalactol, which are components of aphid pheromones and are also found in the catnip plant (Lamiaceae: *Nepeta cataria* L.; McElvain et al., 1941). Recently, we identified a male-produced pheromone from the goldeneyed lacewing, *Chrysopa oculata* Say, the first pheromone for any lacewing (Zhang et al., 2004). The synthetic pheromone, (1*R*,2*S*,5*R*,8*R*)-iridodial (IRI; Chauhan et al., 2004), outperformed all other reported attractants for the green lacewings in the genus *Chrysopa*, and has potential for manipulating lacewings for pesticide-free control of garden and agricultural pest insects (Zhang et al., 2004).

Chrysopa nigricornis Burmeister is a large green lacewing distributed throughout the United States (Agnew et al., 1981). Similar to *C. oculata*, both the adults and larvae are predacious. It is the most common green lacewing species in the U.S. Pacific Northwest and is a useful component of integrated pest management programs in tree and vine crops such as hops (James, 2003a). To develop a pheromone-based attractant for this beneficial insect, we carried out a series of experiments in 2004 that focused on (1) pheromone production and perception in *C. nigricornis* with gas chromatographic–electroantennographic detection (GC–EAD) and gas chromatographic (GC)–mass spectrometric analyses; (2) field testing the newly identified pheromone and other known lacewing semiochemicals, such as MS, *Z,E*-nepetalactone (*ZE*-lactone), *Z,E*-nepetalactol (*ZE*-lactol), 2-phenylethanol (2PE), and various combinations of these compounds in garden and orchard environments; and (3) dispenser technology, including dispenser types and the duration of attraction.

Methods and Materials

Adult Insects and Preparation of Extracts

Adult lacewings for GC–EAD and GC–mass spectrometric analyses were collected during summer 2004 from either sticky Delta traps or nonsticky grapefruit-shaped plastic traps in

Spokane, WA. Lacewings recovered quickly from sticky surfaces of the traps and were still viable for EAD recordings. *C. nigricornis*, *C. oculata*, *C. coloradensis* Banks, and the common green lacewing, *Chrysoperla plorabunda* (Fitch; = *carnea*) were collected for EAD recordings, but only male *C. nigricornis* were collected from nonsticky traps for dissection and GC–mass spectrometer. Adults of these lacewings were processed within 6 to 20 hr of capture for GC–EAD and GC–mass spectrometric analyses. Adult male *C. nigricornis* were anesthetized with CO₂, eviscerated under tap water, and their thoracic cuticle was separated from their abdominal cuticle so that each body portion could be extracted individually in 50 µl of methyl *tert*-butyl ether. No head extracts were made because no glands had been previously reported from the heads of lacewings. All extracts were kept at –20°C until they were analyzed.

Gas Chromatographic–EAD and GC–Mass Spectrometric Analyses

Lacewing extracts and chemical standards were analyzed in splitless mode with a Varian CP-3800 GC equipped with a polar column (CP-Wax 52CB; 1.0 µm film thickness, 30 m × 0.53 mm i.d., Varian, Inc., Middelburg, the Netherlands), and a 1:1 effluent splitter that allowed simultaneous flame ionization detection and EAD of the separated volatile compounds. Helium was used as the carrier gas, and the injector temperature was 220°C. The column temperature was 30°C for 2 min, rising to 240°C at 10°C/min, and then held for 10 min. The outlet for the EAD was held in a humidified airstream flowing at 0.5 m/sec over an antennal preparation. EAD recordings were made by using silver wire-glass capillary electrodes filled with Beadle-Ephrussi Ringer (Zhang et al., 2000) on freshly cut antennae. The antennal signals were stored and analyzed on a PC equipped with a serial IDAC interface box and the program EAD version 2.5 (Syntech, Hilversum, the Netherlands). Antennally active peaks in the lacewing extracts were identified with a GC–mass spectrometer (Varian CP-3800 GC coupled to a Varian Saturn 2000 mass selective detector) operated in split mode (1:10) with a CP-Wax 52CB column (0.25 µm film thickness, 60 m × 0.25 mm i.d., Varian, Inc.), programmed at 30°C for 2 min, increasing to 240°C at 10°C/min, and then held for 10 min. Compounds were identified by comparison of mass spectra and retention times to those of authentic standards. In addition, EAD responses from male antennae of the four species noted above were recorded to a synthetic mixture (100 ng/µl each) containing five *C. oculata*-produced compounds (1-tridecene, nonanal, nonanol, nonanoic acid, and IRI); three compounds associated with aphid prey [(1*R*,4*aS*,7*S*,7*aR*)-nepetalactol, (4*aS*,7*S*,7*aR*)-nepetalactone (both sex pheromone components), and (4*aS*,7*S*,7*aS*)-nepetalactone (non-pheromone compound)]; and three herbivore-induced plant volatiles [MS, (*Z*)-3-hexenyl acetate (Z3HA), and benzaldehyde (BA)].

Chemical Standards

1-Tridecene (97%), 1-nonanol (97%), skatole (98%), MS (99%), 2PE (99%), Z3HA (98%), and BA (99.5%) were obtained from Aldrich Chemical (Milwaukee, WI); nonanal (99%) and nonanoic acid (98%) were from Emery Industries (Cincinnati, OH). (4*aS*,7*S*,7*aR*)-Nepetalactone and (4*aS*,7*S*,7*aS*)-nepetalactone are often referred to as *Z,E*- and *E,Z*-nepetalactone, respectively, in the literature (e.g., Hooper et al., 2002). *Z,E*-lactone (98%), *E,Z*-nepetalactone (96%), (1*R*,4*aS*,7*S*,7*aR*)-nepetalactol [ca. 90% *Z,E*-isomer (Hooper et al., 2002) with ca. 2% impurity of iridodial isomers], and the lacewing pheromone, IRI [80%; with 20% of (1*R*,2*S*,5*R*,8*S*)-iridodial as an impurity] were isolated or synthesized as described by Chauhan et al. (2004).

Field Trapping

Field-trapping experiments were carried out from early May through the end of September 2004 in either garden or small orchard environments in Spokane by using Pherocon VI traps (Trécé, Inc., Adair, OK) with removable sticky inserts. Traps were hung 1.0 to 1.5 m above the ground on either garden stakes or the branches of cherry (*Prunus*)/apple (*Malus*) trees ca. 5 to 10 m apart within each trap line. For each trapping experiment, two sets of traps (each set contained all treatments) were deployed with treatments allocated randomly to their initial trap positions within a set. The treatments were then systematically rotated among trap positions within a set after each replicate so that treatments appeared at least once per location (Latin-square design; Byers, 1991). To minimize positional effects, lacewing collections and trap rotations were carried out when two or more lacewings were caught in any trap. Each replicate lasted several days to 1 wk, depending on lacewing flight activity. The sticky inserts were taken to the laboratory to record species, gender, and catch.

Experiment 1 (May 7–August 3, 2004) was conducted to determine potential response to the newly discovered lacewing pheromone [from *C. oculata* (Zhang et al., 2004) and *C. nigricornis* (this article)], IRI (5 mg loaded onto rubber septa inserted into a 1.5-ml open plastic centrifuge tube; release rate not determined because of the low quantities emitted and the difficulty of applying the gravimetric method), and three other reported lacewing kairomone attractants, MS [2 g in a 12-mil (≈ 0.31 mm) polyethylene (PE) bag, Associated Bag Company, Milwaukee, WI, USA; 40×50 mm, with felt; with a release rate of ca. 30 mg/day, measured gravimetrically at 20 to 23°C in the laboratory], Z3HA (2 g in 4-ml open glass vial with 1/2 cotton ball; ca. 25 mg/day, measured gravimetrically at 20°C to 23°C in the laboratory), and 2PE [2 g in a 3-mil (≈ 0.076 mm) PE bag (40×50 mm) with felt; ca. 5 mg/day], in a full factorial experiment design with a total of 16 treatments (i.e., all individual compounds and all binary, ternary, and quaternary combinations). Each compound was loaded in a separate dispenser; thus, combinations have higher total releases than the individual treatments. Trap set 1 was deployed at a blackberry field (ca. 0.2 ha) in the Strawberry Hill Farm owned by Mr. Fallstorm that was surrounded by mixed pine (*Pinus*) forests. Trap set 2 was set up in Christensen's Cherry Orchard (ca. 0.4 ha) with typical agricultural land surroundings.

Experiment 2 (May 7–July 7, 2004) was similar to experiment 1, but tested BA [2 g in a 12-mil (≈ 0.31 mm) PE bag (40×50 mm) with felt; ca. 35 mg/day] in addition to the previous four individual compounds, along with selected combinations for a total of eight treatments. This study occurred in an urban setting in two smaller residential gardens (Florionovich and Rosengrant) in Spokane Valley, WA (one set of traps in each garden).

Experiment 3 (July 28–August 24, 2004), with a total of six treatments, compared the responses to IRI with ZE-lactone, *Z,E*-nepetalactol, the combination of IRI with MS (5 mg of each compound loaded onto rubber septa), and a commercial product, Benallure™ (Gardens Alive Inc., Lawrenceburg, IN, USA). This study occurred at Strawberry Hill Farm described above.

Experiment 4 (June 22–July 27, 2004), with a total of six treatments, tested the aging effect of IRI (5 mg) on rubber septum dispensers (aged from 0 to 5 wk). The dispensers were aged at ca. 20°C to 23°C in the laboratory hood with mild airflow (ca. 0.3 m/sec); the study occurred at Strawberry Hill Farm described above.

Experiment 5 (August 5–September 30, 2004) with a total of seven treatments, tested the aging effect of the combination of IRI (5 mg) and MS (1.5 g) in 6-mil (≈ 0.16 mm) PE bags (20×50 mm; with felt; aged from 0 to 5 wk). Fresh IRI (5 mg) rubber septum dispenser

[0(RS)] was included as a positive control. The study occurred at Christensen's Cherry Orchard described above.

Experiment 6 (September 1–30, 2004), with a total of four treatments, tested the response to IRI (5 mg), MS (1.5 g), and their combinations, using PE bag type dispensers (6-mil \approx 0.16 mm; 20 \times 50 mm; with felt; ca. 50 mg/d release for MS; release for IRI unknown). Combinations included IRI + MS-1, where both compounds were loaded in one PE bag and IRI + MS-2, where the two compounds were loaded in separate PE bags. The study occurred at Christensen's Cherry Orchard described above.

Statistical Analysis

For each experiment, data from the two sets of traps were pooled for statistical analysis because no block effects were found. Because of heterogeneity of variances among treatments, trap catch data (number of lacewings caught/trap/wk) were analyzed using the non-parametric Kruskal–Wallis ANOVA on rank test, followed by the Student–Newman–Keuls all pairwise comparison to separate means (Zar, 1984). The total trap catches between *C. nigricornis* and *C. oculata* within each experiment were compared by the χ^2 goodness of fit test at $\alpha = 0.05$.

Results

Gas Chromatographic–EAD and GC–Mass Spectrometric Analyses

Gas chromatographic–EAD analyses of extracts of thoracic and abdominal cuticle of male *C. nigricornis* (both forms: the western form with the entire antenna pale-white; the eastern form with the basal third of antenna dark; Penny et al., 2000) indicated no EAD responses by male antennae to any peaks from abdominal extracts (Fig. 1c), whereas two peaks from thoracic extracts elicited significant antennal responses by males of both forms (Fig. 1c; females were not available for testing). The two EAD-active compounds were identified as 1-tridecene and IRI by comparison of the mass spectra and retention times to those of authentic standards (for details, see Zhang et al., 2004). Other major components from the thoracic extracts were (*E*)-5-undecene, a C_{13} -hydrocarbon with two double bonds, 2-methylpropanoic acid, *N*-3-methylbutylacetamide, 2-methyl-1-*H*-indole, and skatole (3-methyl-1-*H*-indole), all of which elicited no EAD response (Fig. 1). GC–EAD analyses with male *C. nigricornis* antennae using synthetic mixtures showed that nonanal, MS, and IRI elicited higher EAD responses than did nonanol, 1-tridecene, *ZE*-nepetalactone, *EZ*-nepetalactone, *ZE*-nepetalactol, BA, or 2PE (Table 1). A similar EAD response pattern was also found for male *C. coloradensis*; however, its antennal responses to IRI were relatively weak compared with those of *C. nigricornis*. Antennae of male *Cl. plorabunda* showed similar EAD responses to most of the compounds in the synthetic mixture, but were unresponsive to IRI and its (8*S*)-stereoisomer (Table 1).

Field-Trapping Experiments

At least four species of green lacewings, *C. nigricornis* (both western and eastern forms; >75%), *C. oculata* (ca. 24%), *C. coloradensis* (<2%), and *Cl. plorabunda* (rare) were caught in the various treatments of all experiments. In addition, other beneficial insects,

such as the brown lacewing, *Hemerobius ovalis* Carpenter (total catch, 10), the hoverfly, *Metasyrphus americanus* (Weidemann; total catch, 100), and a few convergent lady beetles, *Hippodamia convergens* (Guérin-Méneville), were captured in the traps.

In experiment 1 (at Strawberry Hill Farm and Christensen's Cherry Orchard), a total of 495 *C. nigricornis* males and 192 *C. oculata* males were caught. More *C. nigricornis* were captured than *C. oculata* ($\chi^2 = 134$, $df = 1$, $P < 0.001$). There was an effect of treatment for males of both species (*C. nigricornis*: $H = 215.6$, $df = 15$, $P < 0.001$; *C. oculata*: $H = 149.7$, $df = 15$, $P < 0.001$), and traps baited with IRI alone or combined with other test compounds caught more males of both species than did unbaited traps (Fig. 2). MS, Z3HA, and 2PE alone were inactive. No females of either species were captured.

In experiment 2 (Florianovich and Rosengrant residential gardens), a total of 10 *C. nigricornis* males and 106 *C. oculata* males were caught and there was a treatment effect for both species (*C. nigricornis*: $H = 19.8$, $df = 7$, $P = 0.006$; *C. oculata*: $H = 68.3$, $df = 7$, $P < 0.001$). Again, traps baited with IRI alone or its binary combination with MS or BA were attractive to males of both species, whereas MS, Z3HA, 2PE, and BA were inactive when presented alone (Fig. 3). In contrast to experiment 1, traps baited with IRI alone or mixtures containing IRI caught more *C. oculata* than *C. nigricornis*, which may have been because of the habitat differences between the experimental sites ($\chi^2 = 79.1$, $df = 1$, $P < 0.001$).

In experiment 3 (Strawberry Hill Farm), 155 *C. nigricornis* males and 48 *C. oculata* males were caught. More *C. nigricornis* were captured than *C. oculata* ($\chi^2 = 56.4$, $df = 1$, $P < 0.001$). There was a treatment effect for both species (*C. nigricornis*: $H = 56.4$, $df = 5$, $P < 0.001$; *C. oculata*: $H = 44.8$, $df = 5$, $P < 0.001$). IRI attracted more males of both lacewing species than did ZE-lactol (Fig. 4). The number of *C. nigricornis* males in ZE-

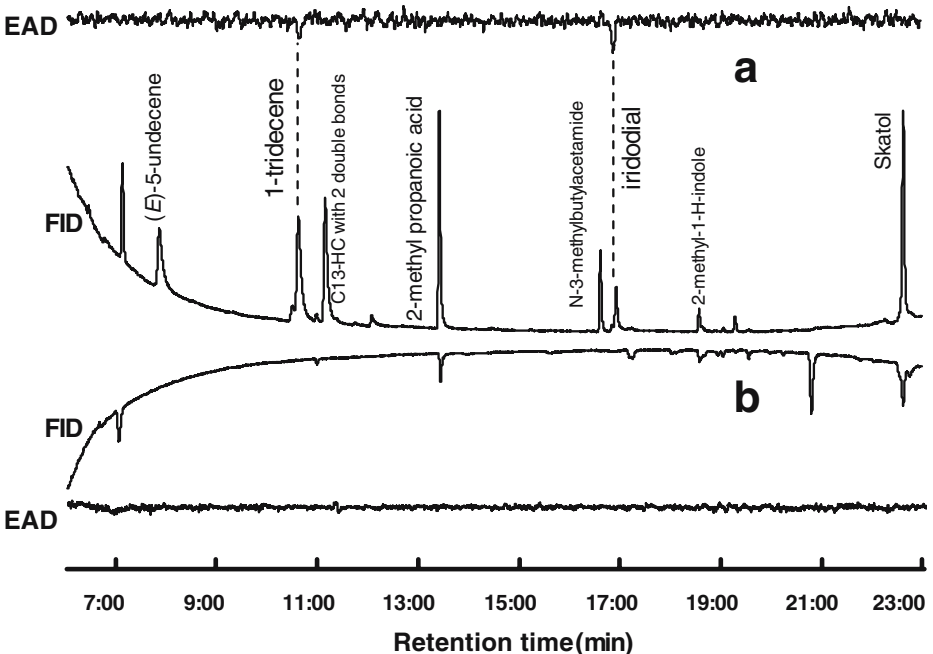


Fig. 1 Gas chromatographic–EAD responses of male *C. nigricornis* (western form) to (a) thoracic and (b) abdominal cuticle extracts of conspecific males

Table 1 Electroantennographic detection responses of male *Chrysopa* and *Chrysoperla* spp. to pheromone components and other semiochemicals

Chemicals	Natural sources	Species ^a						
		<i>C. oculata</i>	<i>C. nigricornis</i>	<i>C. coloradensis</i>	<i>C. quadripunctata</i>	<i>Cl. plorabunda</i>	<i>Cl. rufilabris</i>	
Tridecane	Unknown	•	•	•	•	•	•	•
1-Tridecene	Lacewing thorax	••	••	•	••	••	••	••
(Z)-3-hexenyl acetate	Plants; HIPV ^b	○	○	○	•	○	•	•
Nonanal	Lacewing abdomen/plants	••••	••••	••••	••••	••••	••••	••••
Benzaldehyde	Plants; HIPV	•	•	••	•	•	•	•
Nonanol	Lacewing abdominal cuticle	••	••	•	••	••	••	••
Methyl salicylate	HIPV	••	••	••••	••	••	••	••
(1R,2S,5R,8R)-iridodial	Lacewing abdominal cuticle/thorax	•••••	••••	•	••••	•	••••	○
(1R,2S,5R,8S)-iridodial	Unknown	••	••	^d	••	○	○	○
2-Phenylethanol	Plants	•	•	•	•	•	•	•
(4aS,7S,7aR)-Nepetalactone (ZE)	Catnip/aphid sex pheromone	•	•	^d	•	•	•	•
(4aS,7S,7aS)-Nepetalactone (EZ)	Catnip	••	••	•	•	•	•	•
(1R,4aS,7S,7aR)-nepetalactol (ZE)	Catnip/aphid sex pheromone	••	••	^d	••	••	••	••
Nonanoic acid	Lacewing abdominal cuticle	••	••	•	••	••	••	••
Skatole	Lacewing thorax	○	○	○	○	○	○	○
References ^e	1, 2	1, 2	2	2	1	2	2	1

^a • EAD active; ○ EAD inactive at the concentration (100 ng/μl) tested.

^b HIPV: herbivore-induced plant volatile.

^c Not tested yet.

^d Results not clear, more tests needed to confirm.

^e 1, Zhang et al. (2004); 2, this study.

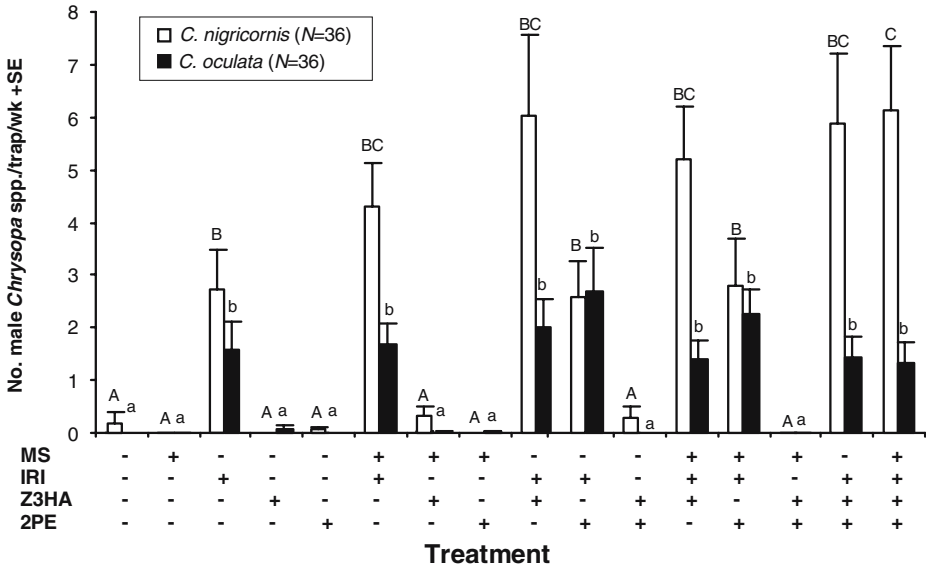


Fig. 2 Captures of male *C. nigricornis* and *C. oculata* in traps baited with MS, IRI, Z3HA, 2PE, and all possible binary, ternary, and quaternary combinations, Strawberry Hill Farm, and Christensen’s Cherry Orchard, Spokane, WA, May 7 to August 3, 2004. Bars with the same letter within the same species indicate means that are not significantly different ($P > 0.05$), Kruskal–Wallis ANOVA on ranks, followed by the Student–Newman–Keuls all pairwise comparison test

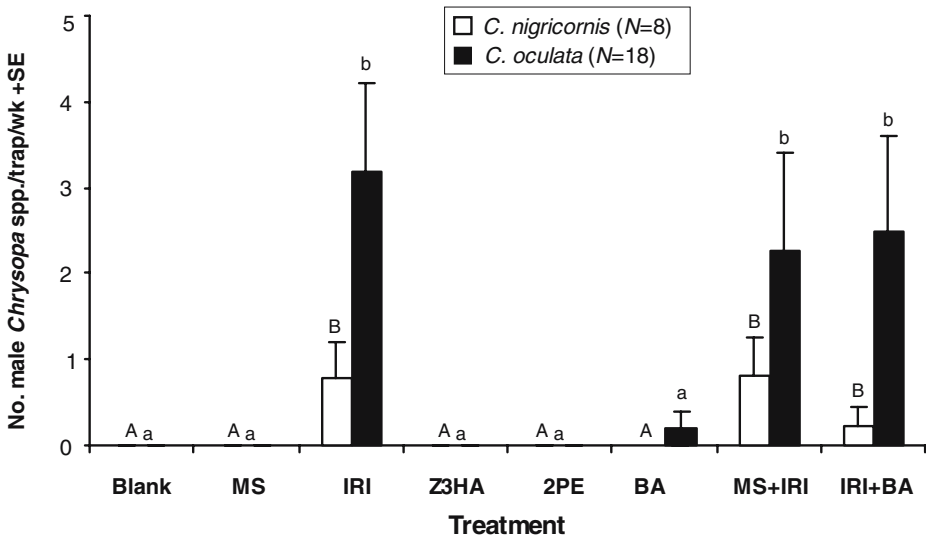


Fig. 3 Captures of male *C. nigricornis* and *C. oculata* in traps baited with MS, IRI, Z3HA, 2PE, BA, and selected binary combinations; at Florianovich and Rosengrant residential gardens, Spokane Valley, WA, May 7 to July 7, 2004. Bars with the same letter within the same species indicate means that are not significantly different ($P > 0.05$), Kruskal–Wallis ANOVA on ranks, followed by the Student–Newman–Keuls all pairwise comparison test

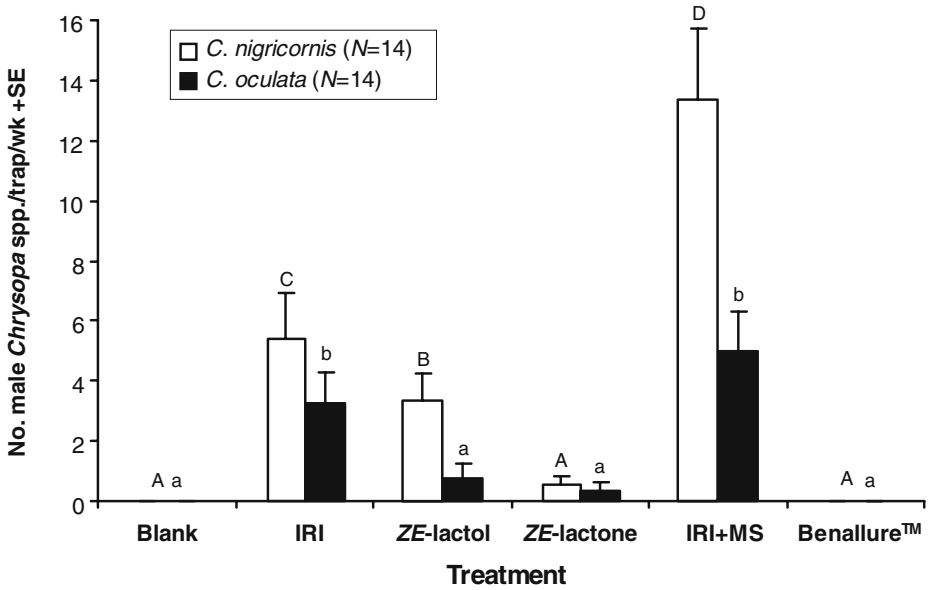


Fig. 4 Captures of male *C. nigricornis* and *C. oculata* in traps baited with IRI, ZE-lactol, ZE-lactone, IRI + MS, and the commercial lacewing/lady beetle attractant, Benallure™ at Strawberry Hill Farm, Spokane, WA, July 28 to August 24, 2004. Bars with the same letter within the same species indicate means that are not significantly different ($P > 0.05$), Kruskal–Wallis ANOVA on ranks, followed by the Student–Newman–Keuls all pairwise comparison test

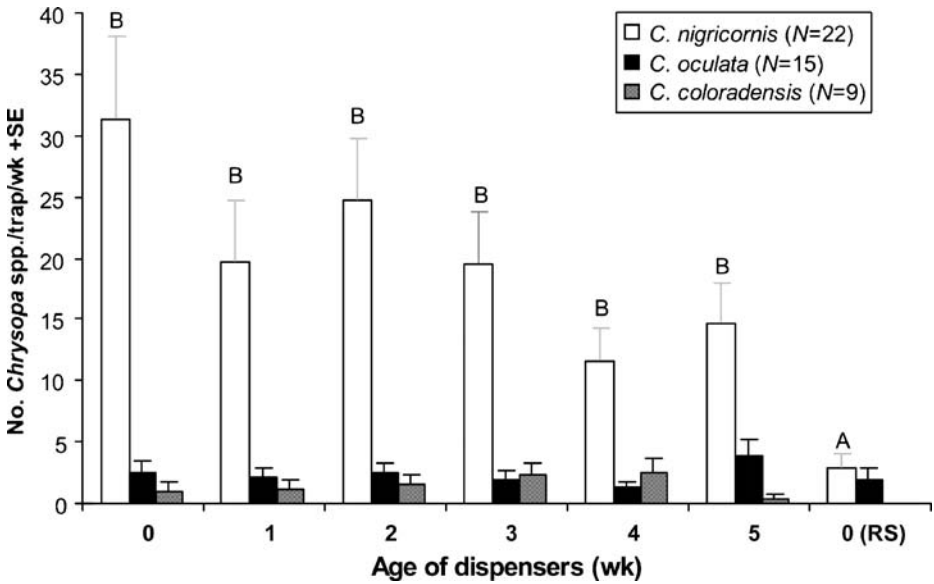


Fig. 5 Captures of *C. nigricornis*, *C. oculata*, and *C. coloradensis* in traps baited with aged PE bag dispensers loaded with 5 mg of IRI and 1.5 g of MS compared with the attraction to fresh rubber septa [0 (RS); loaded only with 5 mg IRI] at Christensen’s Cherry Orchard, Spokane, WA, August 5 to September 30, 2004. Bars with the same letter for *C. nigricornis* indicate means that are not significantly different ($P > 0.05$), Kruskal–Wallis ANOVA on ranks, followed by the Student–Newman–Keuls all pairwise comparison test. No further pairwise comparisons were made for *C. oculata* and *C. coloradensis* because no significant treatment effects were detected by ANOVA

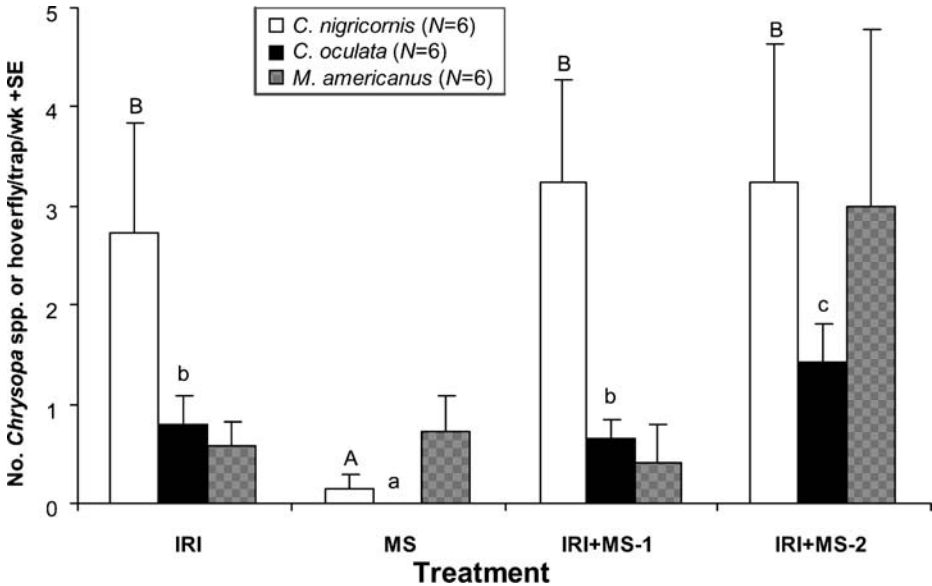


Fig. 6 Captures of *C. nigricornis*, *C. oculata*, and the hoverfly, *M. americanus*, in traps baited with IRI, MS, and their binary blends in PE bag dispensers (IRI + MS-1: in the same PE bag; IRI + MS-2: in two separate PE bags) at Christensen's Cherry Orchard, Spokane, WA, September 1 to 30, 2004. Bars with the same letter within the same species indicate means that are not significantly different ($P > 0.05$), Kruskal–Wallis ANOVA on ranks, followed by the Student–Newman–Keuls all pairwise comparison test. No further pairwise comparisons for *M. americanus* took place because no significant treatment effects were detected by the ANOVA

lactol-baited traps was higher than that in the unbaited traps. ZE-lactone and the commercial attractant, Benallure™, were both inactive. Interestingly, addition of MS to IRI increased the trap catches for *C. nigricornis*, but not for *C. oculata* (Fig. 4).

In experiment 4 (Strawberry Hill Farm), all traps baited with 5 mg of IRI on rubber septa aged from 0 to 5 wk caught males of both *C. nigricornis* (59) and *C. oculata* (32). More *C. nigricornis* were captured than *C. oculata* ($\chi^2 = 8.01$, $df = 1$, $P = 0.005$), but there was no treatment effect in the experiment for either species (*C. nigricornis*: $H = 7.9$, $df = 5$, $P = 0.16$; *C. oculata*: $H = 9.04$, $df = 5$, $P = 0.11$).

In experiment 5 (Christensen's Cherry Orchard), traps baited with variously aged PE bag dispensers containing 5 mg of IRI plus 1.5 g of MS caught a total of 1150 *C. nigricornis*, 109 *C. oculata* males, 28 *C. coloradensis* males, 57 *M. americanus*, and 26 *H. convergens*. The ratio of eastern/western forms of *C. nigricornis* in the traps was ca. 1:3. More *C. nigricornis* were captured than *C. oculata* ($\chi^2 = 861$, $df = 1$, $P < 0.001$). There was a treatment effect in the experiment for *C. nigricornis* ($H = 30.1$, $df = 6$, $P < 0.001$), but not for *C. oculata* ($H = 1.56$, $df = 6$, $P = 0.96$) or *C. coloradensis* ($H = 10.2$, $df = 6$, $P = 0.12$). All *C. nigricornis* captured during the August and early September were males; however, from mid to late September, 23 out of the 63 caught (mostly the western form) were females. Trap catches for *C. nigricornis* seemed to decrease with the lure age (Fig. 5), but this decline was not statistically significant. The 0- to 5-wk-old PE bag dispensers were significantly (five to six times) more attractive to *C. nigricornis* than were the fresh IRI rubber septa dispensers [0(RS)], although the total loading of IRI was the same. Thus, the PE bag dispensers loaded with IRI and MS should stay attractive for at least 5 wk in the field.

In experiment 6 (Christensen's Cherry Orchard), a total of 61 *C. nigricornis* (41 males and 20 females), 18 *C. ocellata* males, and 28 *M. americanus* were captured during September 2004. More *C. nigricornis* were captured than *C. ocellata* ($\chi^2 = 23.4$, $df = 1$, $P < 0.001$). There were treatment effects for *C. nigricornis* ($H = 7.8$, $df = 3$, $P = 0.049$) and *C. ocellata* ($H = 9.32$, $df = 3$, $P = 0.025$), but not for *M. americanus* ($H = 1.87$, $df = 3$, $P = 0.60$). IRI (5 mg in PE bag dispenser) alone or combined with MS in the same PE bag (IRI + MS-1) or in separate PE bags (IRI + MS-2) were significantly more attractive to *C. nigricornis* than was MS alone (Fig. 6). No synergistic effect on *C. nigricornis* and *M. americanus* was found between IRI and MS; however, the combination of these two compounds in separate PE bags caught significantly more *C. ocellata* than did the IRI or MS alone (Fig. 6). More work is needed to confirm the effects of MS (with or without IRI) on both lacewing species.

Discussion

(1R,2S,5R,8R)-Iridodial was recently discovered as a male-produced pheromone of the goldeneyed lacewing, *C. ocellata*, and is believed to be produced in elliptical glands abundantly distributed between the third and eighth abdominal sternites of the males (Zhang et al., 2004). Our current study indicates that IRI is also a male-produced (females were not available for chemical analysis) pheromone for the green lacewing, *C. nigricornis*. It elicited an EAD response, and attracted conspecific males into traps. It also attracted some females late in the season. In contrast to *C. ocellata*, IRI was detected in *C. nigricornis* males from thoracic extracts, rather than from abdominal cuticle extracts. Thus, we expect pheromone-producing glands in the thorax of this species. Another antennally active compound from thoracic extracts of *C. nigricornis* males, 1-tridecene, was not field tested in the current study because it was reported as one of the defensive compounds produced in the thoracic glands of *C. ocellata* and reduced the numbers of *C. ocellata* males captured in IRI-baited traps (Zhang et al., 2004).

In addition to the responses of *C. nigricornis* and *C. ocellata*, a few males of *C. quadripunctata* (Zhang et al., 2004) and *C. coloradensis* (this article) were captured in the pheromone-baited traps. It is not known if these two species also use IRI or its isomers as pheromone components. The low-trap catches might be a result of either extremely low populations in the test areas or missing pheromone component(s) in the treatments. Recently, males of a Eurasian green lacewing species, *C. septempunctata* Wesmael, were also found to be strongly attracted to synthetic IRI (Zhang et al., 2006). Two other common green lacewing species from the genus *Chrysoperla*, *Cl. plorabunda* (= *C. carnea*) and *Cl. rufilabris* Burmeister, did not respond to IRI antennally or behaviorally in this and in a previous study (Table 1; Zhang et al., 2004).

Methyl salicylate is reportedly an attractant for both sexes of *C. nigricornis* and *C. ocellata* (James, 2003a, 2006) along with several other beneficial insects (James and Price, 2004). James and Price (2004) also recently found evidence for recruitment and retention of beneficial insects in grapes and hops by using controlled-release dispensers of MS, in Prossor, WA. However, in our study, in Spokane, WA, ca. 300 km northeast of Prossor, MS was inactive alone, but in a few instances enhanced the responses of *Chrysopa* spp. to IRI. MS alone and its binary blend with IRI attracted another beneficial insect, the hoverfly, *M. americanus*. Surprisingly, the commercial lacewing/ladybeetle attractant, Benallure™, did not catch any lacewings or other beneficial insects in our experiments. ZE-lactol was slightly

attractive to *C. nigricornis* and *C. oculata*, which might be because of the presence of small amount of IRI as an impurity, whereas ZE-lactone was inactive at the release rate tested.

If, indeed, some sympatric *Chrysopa* species share IRI as a pheromone component, there might be cross-attraction between species, which might undermine species isolation. However, earlier studies on acoustical communication of green lacewings showed that chrysopids produce species-specific, low-frequency, substrate-borne vibrations that guide the conspecifics to one another on a plant (Henry, 1982). Comparative acoustical studies of *Chrysoperla* versus *Chrysopa* species indicated that *Chrysoperla* spp. [*C. rufilabris*, *C. carnea* (Stephens), and *C. downesi* (Smith)] are more dependent on acoustic signals for mating success than are *Chrysopa* spp. (*C. oculata* and *C. chi* Fitch; Henry, 1979, 1980a, b, c). Thus, species of *Chrysoperla* rely on acoustic communication with no obvious role for pheromones, whereas *Chrysopa* species communicate with pheromones at long range and, to a lesser extent than *Chrysoperla*, with species-specific acoustic signals at short range for courtship and isolation from sympatric species. Adults of *Chrysopa* (*sensu stricto*) are predacious, whereas adults of *Chrysoperla* are phytophagous (Principi and Canard, 1984), suggesting that predation in the adult stage somehow favors chemical communication or selects against communication by substrate vibration.

Surprisingly, no females of *C. oculata* were caught during any of our current tests nor in the experiments carried out by Zhang et al. (2004), although antennae from the females were as sensitive to IRI and other male-produced compounds as were the antennae of males (Zhang et al., 2004). Sweep netting and live insect counting at a soybean field in 2004 at Beltsville, MD, showed that IRI not only attracted *C. oculata* males, but also females to the pheromone-baited plots (Chauhan et al., unpublished data). However, unlike males, the females did not completely approach and enter the traps, possibly because females call males acoustically at close range or females require male-produced, species-specific acoustical signals for close-range communication. Similar to *C. oculata*, no females of *C. nigricornis* were captured in IRI-baited traps during the summer season; however, in the early fall (September), some *C. nigricornis* females were captured in IRI or IRI/MS-baited traps.

Our field testing indicated that the lacewing pheromone, IRI, loaded onto either rubber septa or its binary blend with MS in PE bags remained attractive for at least 5 wk in the field during the mid-summer season for both *C. nigricornis* and *C. oculata*. Synthetic lacewing pheromone IRI or its combination with MS (the herbivore-induced plant volatile) attracts and retains both male and female lacewings to treated areas, which will probably elevate the local lacewing population (Chauhan et al., unpublished data). Furthermore, the more males attracted to the treated area, the more natural pheromone would be released by males, further strengthening attraction and potentially enhancing biological control. Moreover, IRI and MS or their binary blend also attract many other beneficial insects, such as hoverflies, lady beetles, and predatory bugs (James and Price, 2004 and this article). Based on this study, a new lacewing attractant/trap system is being developed for both domestic and international markets. This product will have potential for manipulating natural or artificially augmented populations of lacewings and other beneficial insects to enhance biological control of garden, agricultural, and forest pest insects.

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stereomeric purity >94% showed the highest attractivity. The presence of 5% of the *Z,Z* isomer in the lure did not induce any synergistic or inhibitory effect, and the alcohol precursor of the pheromone was inactive. The results show that use of a stereomerically pure pheromone may not be necessary in pest control strategies.

Keywords Sex pheromone · Identification · Synthesis · Sweet potato weevil · *Cylas formicarius elegantulus* · Electrophysiological activity · Olfactometer bioassay · Field tests

Introduction

Sweet potato, *Ipomoea batatas* (L.) Lam., is one of the seven most important food crops worldwide, with an estimated annual production of 115 million metric tons (FAO, 1984). It is grown in more than 100 countries with 98% of the world production proceeding from developing countries (Jansson and Raman, 1991). One of the most important constraints that limit sweet potato production worldwide are the pre- and postharvest losses from insect feeding, especially the sweet potato weevils *Cylas formicarius* (Fabricius), *C. puncticollis* (Boheman), and *C. brunneus* (Fabricius) (Jansson and Raman, 1991). *C. formicarius* [= *C. f. elegantulus* (Summers)] abounds in more than 50 countries in tropical and subtropical regions and is the most devastating pest of sweet potato worldwide (Chalfant et al., 1990). In response to insect feeding, sweet potato tissue produces toxic sesquiterpenes that induce an extremely bitter taste in the roots, rendering it unfit for consumption (Akazawa et al., 1960; Sato et al., 1981). For this reason, even low population densities may cause devastating crop losses, i.e., yield losses of between 60% and 100% in many developing countries. Owing to the cryptic character of these weevils, insects are difficult to control with conventional insecticides, and growers in many countries have tried to reduce populations primarily by cultural practices and, more recently, through biological control (Jansson, 1991).

The sex pheromone of *C. f. elegantulus* was identified by Heath et al. (1986, 1988) as (*Z*)-3-dodecenyl (*E*)-2-butenolate (**1**) from a laboratory-reared colony. In this paper, we present the pheromone analysis of feral insects from Cuba, the first scanning electron microscopy (SEM) studies on insect antenna, new electrophysiological and behavioral responses to the pheromone in a double dual-choice olfactometer, and activity of several pheromone formulations in the field.

Methods and Materials

Insects

Weevils were collected in infested sweet potato fields near the Instituto de Investigaciones Fundamentales (INIFAT; Santiago de las Vegas, Cuba). They were sexed, fed with commercial sweet potato, and sent to Barcelona, Spain, for pheromone analysis and biological and electrophysiological activity. They were kept in a climatic chamber on a 16:8 L/D photoperiod.

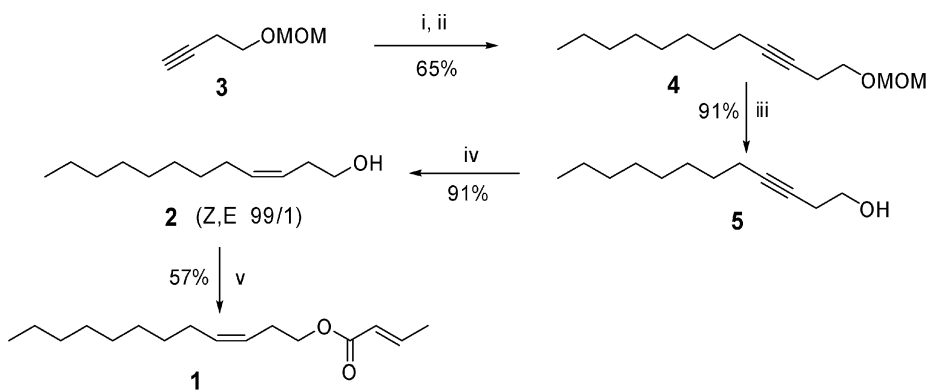
Solid Phase Microextraction

Commercial fibers of polydimethylsiloxane (7 and 100 μm thickness) (Supelco Inc., Bellefonte, PA, USA) were previously conditioned in a Carlo Erba 6000 gas chromatograph

at 250°C injector temperature for 1 hr. The fiber was introduced into a Teflon-coated screw-top glass vial containing batches of 26–90 virgin females. Volatiles emitted by the insects were adsorbed for 24 hr at room temperature and desorbed in the injector port of a ThermoQuest gas chromatograph coupled to a Trace MS ThermoQuest mass selective detector. The gas chromatography (GC) conditions used were injection in splitless mode on an HP-5 MS capillary column (30 m × 0.25 mm ID × 0.25 μm) at 60°C for 5 min, and then programmed to 230°C at 5°C/min. The GC-mass spectrometry (MS) was run under SIM mode of ions of m/z 166, 138, 96, 87, and 69.

Chemicals

Synthesis of pheromone **1** was carried out by esterification of (*Z*)-3-dodecen-1-ol (**2**), which was prepared from MOM-protected 3-butyn-1-ol (**3**) (Scheme 1). A solution of 4-methoxymethoxy-1-butyne (**3**) (0.5 g, 4.3 mmol) in anhydrous tetrahydrofuran (THF) (3.5 ml) was cooled to −20°C. Then, a freshly prepared 0.5 M solution of LDA (8.8 ml, 4.3 ml) was slowly added. The solution was stirred for 10 min at −20°C and 1 hr at room temperature. A solution of 1-bromooctane (0.83 g, 4.3 mmol) in a mixture of anhydrous THF (2 ml) and anhydrous 3,4,5,6-tetrahydro-2-(1*H*)-pyrimidinone (DMPU) (3 ml) was added, and the mixture stirred for 3 hr. THF was evaporated under vacuum, and the residue was poured over ice/water. Organic material was extracted with hexane, washed with water, and dried (Na₂SO₄). After evaporation of the solvent, the residue was purified by flash column chromatography over neutral alumina III to obtain the condensation product **4** (0.6 g, 65%) in pure form. ¹H NMR δ 4.65 (s, 2H, OCH₂O), 3.61 (t, $J = 6.9$ Hz, 2H, CH₂O), 3.37 (s, 3H, CH₃O), 2.46 (tt, $J = 6.9$, $J' = 2.4$ Hz, 2H, C ≡ CCH₂), 2.14 (tt, $J = 6.9$, $J' = 2.4$ Hz, 2H, CH₂C ≡ C), 1.46 (m, 2H, CH₂CH₂C ≡ C), 1.26 (br, 10H, 5CH₂), 0.87 (t, $J = 6.7$ Hz, 3H, CH₃) ppm. ¹³C NMR δ 95.5, 80.9, 77.3, 65.8, 54.5, 31.1, 28.5, 28.4, 28.3, 28.1, 21.9, 19.6, 17.9, 13.8 ppm. MS (EI) m/z (%): 226 (M⁺, 1). Elem. Anal. Calcd. for C₁₄H₂₆O₂: C, 74.28, H, 11.58; Found: C, 74.22, H, 11.56. A mixture of compound **4** (0.211 g, 0.93 mmol), MeOH (2 ml), and 12 M HCl (0.21 ml) was stirred at room temperature for 24 hr. The solvent was evaporated and



i: LDA/THF; ii: BrC₈/THF-DMPU; iii: HCl 12M/MeOH; iv: H₂, Lindlar cat/quinoline/hexane; v: crotonyl chloride/CH₂Cl₂

Scheme 1 Synthesis of pheromone **1** by esterification of (*Z*)-3-dodecen-1-ol (**2**)

the residue was extracted with CH_2Cl_2 and washed with NaHCO_3 sat solution. Usual workup and purification by flash column chromatography over neutral alumina III furnished compound **5** (0.153 g, 91%). IR ν 3340, 2220 cm^{-1} . ^1H NMR δ 3.67 (dt, $J = J' = 6.3$ Hz, 2H, CH_2O), 2.43 (tt, $J = 6.3$, $J' = 2.4$ Hz, 2H, $\text{C} \equiv \text{CCH}_2$), 2.15 (tt, $J = 7$, $J' = 2.4$ Hz, 2H, $\text{CH}_2\text{C} \equiv \text{C}$), 1.81 (t, $J = 6.3$ Hz, 1H, OH), 1.48 (m, 2H, $\text{CH}_2\text{CH}_2\text{C} \equiv \text{C}$), 1.26 (br, 10H, 5 CH_2), 0.88 (t, $J = 6.75$ Hz, 3H, CH_3) ppm. ^{13}C NMR δ 82.7, 76.2, 61.3, 31.8, 29.2, 29.1, 28.9, 28.8, 23.1, 22.6, 18.7, 14.05 ppm. MS (EI) m/z (%): 182 (M^+ , 2). Alkynol **5** (0.59 g, 3 mmol) was hydrogenated with Lindlar (Pd-CaCO_3) catalyst (20 mg) in hexane (3 ml) and two drops of quinoline at atmospheric pressure and room temperature. The reaction was monitored by GC with an HP-5 capillary column (0.33 μm , 25 m \times 0.20 mm ID). The crude was filtered through Celite, and after usual workup, compound **2** (0.54 g, 91%) was obtained with a stereomeric purity Z,E 99/1. IR ν 3334 cm^{-1} . ^1H NMR δ 5.56 (m, 1H, $\text{HC}=\text{}$), 5.35 (m, 1H, $=\text{CH}$), 3.64 (t, $J = 6.5$ Hz, 2H, CH_2O), 2.33 (c, $J = 6.5$ Hz, 2H, $=\text{CCH}_2$), 2.05 (c, $J = 6.6$ Hz, 2H, $\text{CH}_2\text{C}=\text{}$), 1.70 (s, 1 H, OH), 1.26 (br, 12H, 6 CH_2), 0.87 (t, 3H, $J = 6.75$ Hz, CH_3) ppm. ^{13}C NMR δ 133.6, 124.9, 62.3, 31.9, 30.8, 29.7, 29.5, 29.3, 29.2, 27.4, 22.7, 14.1 ppm. MS (EI) m/z (%): 184 (M^+ , 0.4), 166 ($\text{M}^+ - \text{H}_2\text{O}$, 12). A mixture of crotonyl chloride (0.19 ml, 2 mmol) and alcohol **2** (0.3 g, 1.6 mmol) was dissolved in CH_2Cl_2 (3 ml) and cooled to 0°C. Pyridine (0.16 ml, 2 mmol) in CH_2Cl_2 (3 ml) was added dropwise to the solution, and the mixture was stirred at 0°C for 1 hr (Heath et al., 1986). Usual workup and purification over flash column chromatography on silica gel yielded compound **1** (0.23 g, 57%), as a mixture of isomers (3*Z*)-**1** and (3*E*)-**1** with the *E* stereochemistry of the double bond at the acid side and the isomer containing the two double bonds with *Z* stereochemistry in 94:1:5 ratio, respectively. IR ν 1724, 1660, 968 cm^{-1} . ^1H NMR δ 6.97 (dq, $J = 15.6$, $J' = 6.9$ Hz, 1H, $\text{OCCH} = \text{CH}$), 5.84 (dq, $J = 15.6$, $J' = 1.75$ Hz, 1H, $\text{OCCH} = \text{CH}$), 5.50 (m, 1H, $\text{HC}=\text{}$), 5.35 (m, 1H, $=\text{CH}$), 4.12 (t, $J = 6.75$ Hz, 2H, CH_2O), 2.40 (m, 2H, $=\text{CCH}_2$), 2.03 (m, 2H, $\text{CH}_2\text{C}=\text{}$), 1.87 (dd, $J = 6.9$, $J' = 1.75$ Hz, 3H, $=\text{CCH}_3$), 1.26 (br, 12H, 6 CH_2), 0.88 (t, $J = 7.0$ Hz, 3H, CH_3) ppm. ^{13}C NMR δ 166.6, 144.6, 132.9, 124.3, 122.7, 63.7, 31.9, 29.6, 29.5, 29.3, 27.3, 26.9, 22.7, 18.0, 14.1 ppm; MS (EI) m/z (%): 166 ($\text{M}^+ - \text{CH}_3 - \text{CH} = \text{CH} - \text{COOH}$, 76).

For field tests, samples of 3-dodeceny (*E*)-2-butenate were prepared as mixtures of (3*Z*)- and (3*E*)-dodeceny crotonate in several ratios through Wittig reaction of 3-hydroxypropyltriphenylphosphorane with nonanal, or by reaction of methylenetriphenylphosphorane with ethylene oxide followed by metallation and Wittig reaction with nonanal (Maryanoff et al., 1985).

Scanning Electron Microscopy

The head of a freshly sacrificed weevil was cut, fixed on a stub with conductive glue, air-dried, and metalized. Observations were performed on a Jeol JSM 840 SEM at 5 eV.

Electrophysiological Activity

Electroantennogram (EAG) responses were recorded under standard techniques by using a pair of glass microelectrodes filled with Ringer's saline (Guerrero et al., 1986). The tip of the antenna club was cut off, the reference electrode was introduced into the base of the head, and the recording electrode was slipped into the open tip of the antenna. A flow of humidified air (900 ml/min) was continuously directed over the antenna, and olfactory stimuli were delivered by puffing air (300 ml/min) with a CS-1 stimulus generator (200 ms

duration; Syntech, Hilversum, The Netherlands) through a Pasteur pipette directly into the main stream of the air. The pipette contained a small filter paper on which several amounts (0.001–1 μg) of the pheromone were deposited. All experiments were performed with the synthetic pheromone **1** having the highest stereochemical purity (mixture of isomers *Z,E* and *E,E* in a 98:2 ratio). Control puffs (pure air) were intercalated between two consecutive pheromone stimuli to determine the real depolarizations to the attractant. Responses from the antenna were amplified (1000 \times), filtered (DC to 1 kHz) with an IDC 02 interface (Syntech), digitized on a PC, and analyzed with the EAG 2.6 program (Syntech).

Olfactometer Bioassay

For behavioral studies, a dual-choice olfactometer consisting of two methacrylate-based plates of 20 \times 20 cm adjusted to each other by four plastic screws was built. The upper plate contained a hole (diam 5.5 cm) in the center that allowed us to suck out the air of the system via an ordinary vacuum pump. The vacuum allowed volatiles from the vials to reach the test insects, which had been initially placed in groups of 4–5 in the center of the chamber. Each of the four olfactometer sides had an outlet with a Tygon[®] hose adjusted to a glass vial. Each vial contained a piece of paper impregnated with a bait (pheromone) or blank (hexane) so that the two baited vials were surrounded by two control vials in an alternate form. The number of insects that reached each arm of the olfactometer was counted, and the percentage of responses with regard to the total number of insects was calculated. The apparatus was rotated 90 $^\circ$ after each assay so that the vials occupied all four possible positions in each assay. By rotating the system, any photokinetic side effect induced by indirect light coming into the chamber was avoided. The experiments were performed in the dark, and the chamber was thoroughly cleaned with 96% ethanol after every assay. To establish the optimum response of males, 10 insects were released and their responses to 10 ng pheromone recorded at different times of the scotophase. Eight replicates were done for each assay. Responses of males of the 6th hr into the scotophase to several pheromone doses (0.1 to 10,000 ng) were also recorded, and 10 replicates with 10 insects/replicate were run for each dose. For comparison, the activity of a commercial sample (50 ng) was also determined (12 replicates with 10 insects/replicate).

Field Tests

Field experiments were conducted in 1-ha infested sweet potato fields at the Cuban Institute of Tropical Agricultural Research (Santiago de las Vegas, Cuba) (July–August 2000–2001). The traps were made with the materials available in the country. Two thick 50-cm-long iron bars supported an open square iron box at an approx. height of 45 cm from the floor. Two opposite sides of the box were covered with a plastic sheet impregnated with Tanglefoot[®]. The roof of the trap was cardboard covered with aluminum foil, from which a thin wire was suspended with a rubber septum (Aldrich, Milwaukee, WI, USA) loaded with 1 mg of the formulation. In the lower part of the trap and ca. 10 cm from the ground, another plastic sheet impregnated with Tanglefoot[®] helped to increase trap catch. The distance between the traps was 15 m. The insects caught were counted daily for 1 mo, and the traps were rotated once a week. Traps were randomly placed within the plots, and 3 replicates per formulation were considered. Septa loaded with hexane served as controls. For the tests, formulations were designated in terms of the stereochemical purity of the pheromone used; and thus, for instance, a formulation *Z,E/E,E* 95:5 refers to a bait containing a mixture of the two isomers (*Z,E*)-**1** and (*E,E*)-**1** (the letters refer in this order to the stereochemistry of the double bond at the alcohol side and the acid side, respectively) in 95:5 ratio.

Statistical Analysis

Behavioral data were analyzed by the χ^2 homogeneity test ($P < 0.05$). EAG depolarizations were analyzed by Student's t -test ($P < 0.05$). Field data were transformed ($\sqrt{x + 0.5}$) and analyzed for significance via Student's t -test ($P < 0.05$).

Results and Discussion

(*Z*)-3-Dodecenol (**2**) has been documented as a candidate trail-following semiochemical for several termite species (Argenti et al., 1994) as well as the major component of the trail-following pheromone of the fungus-growing termite *Macrotermes annandalei* (Peppuy et al., 2001). In an attempt to develop a simple procedure of preparation of (*Z*)-3-dodecenol (**2**) that could be adapted and scaled up in Cuba for larger treatments, a similar procedure to that described by Lo et al. (1992) was developed. In our case, we protected 3-butyn-1-ol as the corresponding MOM ether (**3**) instead of the tetrahydropyranyl (THP) derivative (Lo et al., 1992) since MOM compounds are simpler and provide NMR spectra that are easier to interpret than THP ethers. Therefore, MOM-protected butynol **3** was alkylated with 1-bromooctane in THF–DMPU in the presence of LDA [the alkylation reaction with sodamide as base in ammonia (Lo et al., 1992) failed in our hands] to provide acetylenic derivative **4** in 65% yield. When MeLi or *n*-BuLi was used as base, 1-dodecen-3-yne was obtained as by-

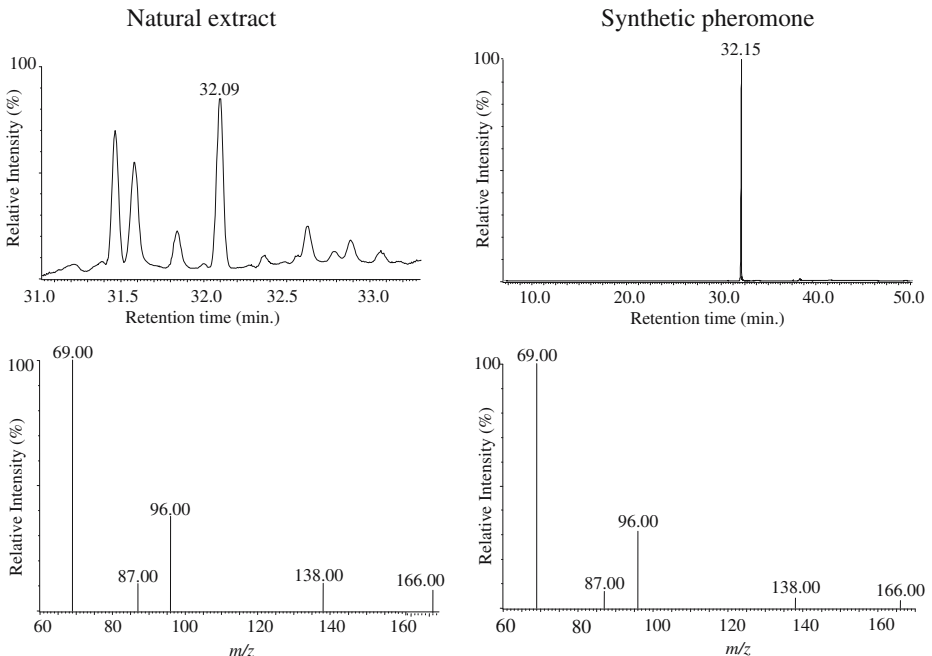


Fig. 1 Partial GC-MS profile of a SPME volatile extract of 90 virgin females of *Cylas formicarius elegantulus* (left) compared with the synthetic pheromone (right). The mass spectrum was run in SIM mode of selected ions (see text). GC conditions: injection in “splitless” mode at 60°C for 5 min and then program to 230°C at 5°C/min

product in 10 – 30% yield. This compound has not hitherto been described in such a process, but a similar case was reported when the THP-protected 1-butynol was subjected to alkylation for the synthesis of the sex pheromone of the tomato pest *Scrobipalpaloides absoluta* (Svátos et al., 1993). Hydrolysis of the MOM group followed by partial hydrogenation with Lindlar catalyst/quinoline furnished dodecenol **2** in good overall yield (53.8%) and excellent

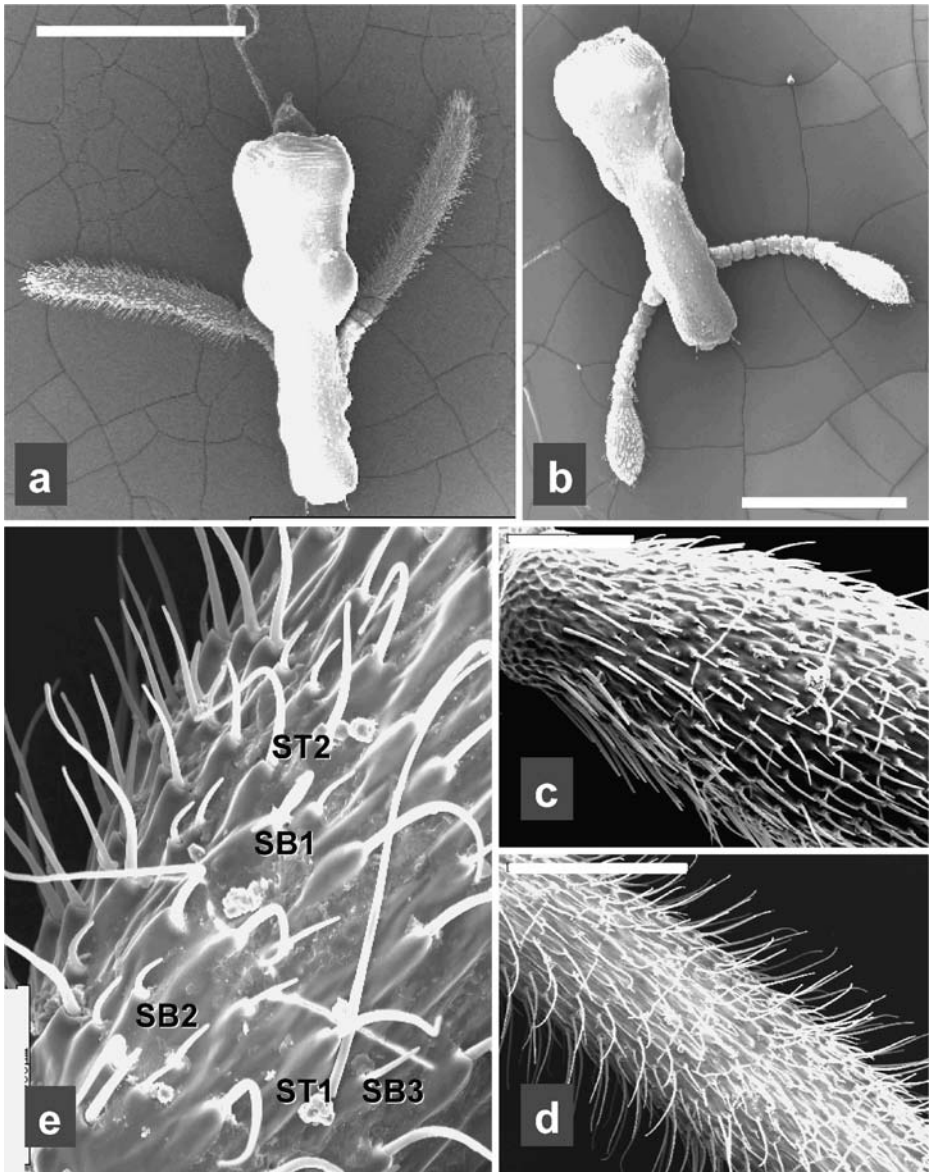


Fig. 2 Morphology of *Cylas formicarius elegantulus* antennae. (a) SEM micrograph of a male head. (b) SEM micrograph of a female head. (c) SEM view of a distal subsegment of a female antenna. (d) SEM view of a distal subsegment of a male antenna. (e) High magnification of the club surface of a male antenna showing different types of sensilla. ST1: Sensilla trichoidea type 1; ST2: sensilla trichoidea type 2; SB1: sensilla basiconica type 1; SB2: sensilla basiconica type 2; SB3: sensilla basiconica type 3

stereochemistry (*Z/E* 99:1). Esterification of alcohol **2** with crotonyl chloride/pyridine in CH_2Cl_2 (Heath et al., 1986) afforded the expected pheromone **1** in 57% yield.

Characterization of the Pheromone

Because of the expected low contents/release of pheromone in sweet potato weevil females of other origins (Heath et al., 1986), solid-phase microextraction (SPME) was considered for identifying the pheromone. The first report of the utilization of SPME in pheromone analysis was in monitoring the diel rhythm of emission of airborne volatiles from the sugar cane weevil *Metamasius hemipterus* (Malosse et al., 1995). Since then, other examples of applications have been reported (Frérot et al., 1997; Rochat et al., 2000; Peppuy et al., 2001).

In our case, analysis of an extract of volatiles from 90 virgin females, adsorbed for 24 hr on a 100 μM fiber, by GC-MS under SIM conditions of ions of *m/z* 166, 138, 96, 87, 69 confirmed the presence of the pheromone **1** when compared with an authentic sample (Fig. 1). The selected ions had been previously noted as major or characteristic fragments in the ms of the synthetic pheromone (Lo et al., 1992). The amount of pheromone present in the volatile extract was estimated as 1.8 ng, i.e., 20 pg of pheromone emitted per female every 24 hr. Trials to detect the pheromone by rubbing the syringe over the abdomen of virgin females were unsuccessful. Likewise, an SPME extract of 30 female volatiles gave no response when using the male antenna as detector [GC-electroantennographic detection

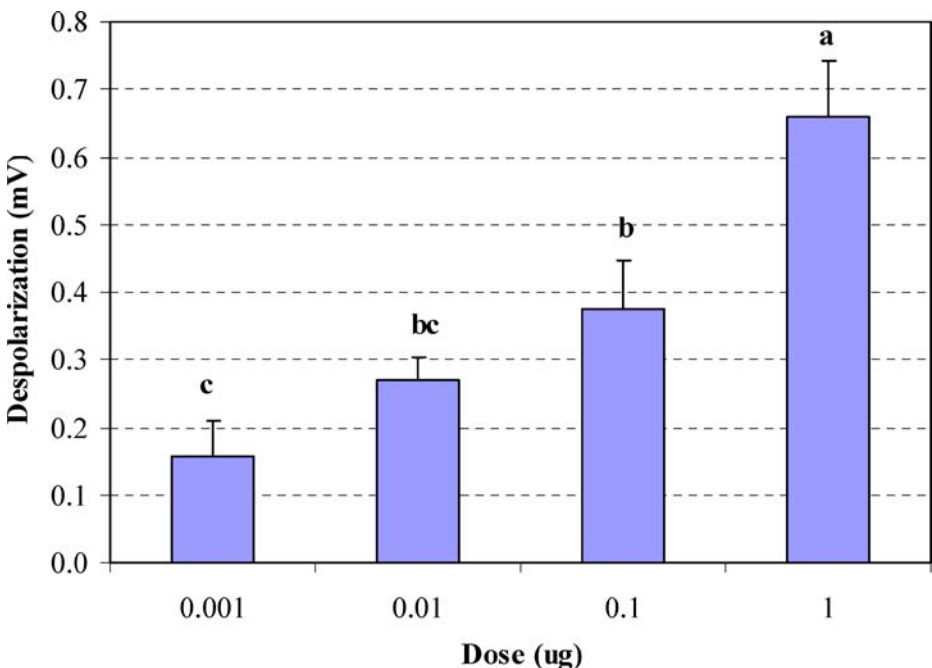


Fig. 3 Dose-response depolarizations of *Cylas formicarius elegantulus* male antennae to several amounts of pheromone **1**. Error bars are standard errors. Bars followed by the same letter are not significantly different (Student's *t*-test, $P < 0.05$)

(EAD)], probably as a result of the high threshold and low EAG amplitude shown by this insect.

Electrophysiology

Male and female antennae comprise a scape, a pedicel, and a flagellum with nine subsegments. The first eight subsegments of the flagellum are short, whereas the last one is considerably larger. This last distal subsegment is club shaped in females and elongated in males. The following types of sensilla were found on the male distal flagellum segment: sensilla trichoidea type 1 (ST1) as long hairs of 100–150 μm; sensilla trichoidea type 2 (ST2) as short hairs of 50–60 μm; sensilla basiconica type 1 (SB1) as thick pegs of 20–25 μm; sensilla basiconica type 2 (SB2) as curved pegs of 10–15 μm, and sensilla basiconica type 3 (SB3) as thin and straight short pegs of 15–20 μm (Fig. 2). The same types were observed on female antennae, but ST1 are less abundant than on male antennae. Sensilla chaetica were also found on the first eight flagellum subsegments in both sexes. Although we did not run single sensillum recordings to confirm that sensilla trichoidea house the olfactory receptor neurons for pheromone detection, we may assume that ST1 are probably the most likely sites for pheromone detection for the following reasons: (1) the pheromone is emitted by females; (2) only males are attracted by the pheromone both in the laboratory and in the field; (3) male antennae show sensitivity to the pheromone on EAG but not female antennae; and (4) ST1 are more abundant in male antennae.

EAG responses of a male antenna to the synthetic pheromone 1 were generally small, as expected from such a type of antenna, but typical depolarizations of 0.6–0.8 mV were nevertheless obtained with puffs on 1 μg of pheromone. A dose–response plot with puffs on 0.001–1 μg of pheromone is shown (Fig. 3), with higher doses inducing higher

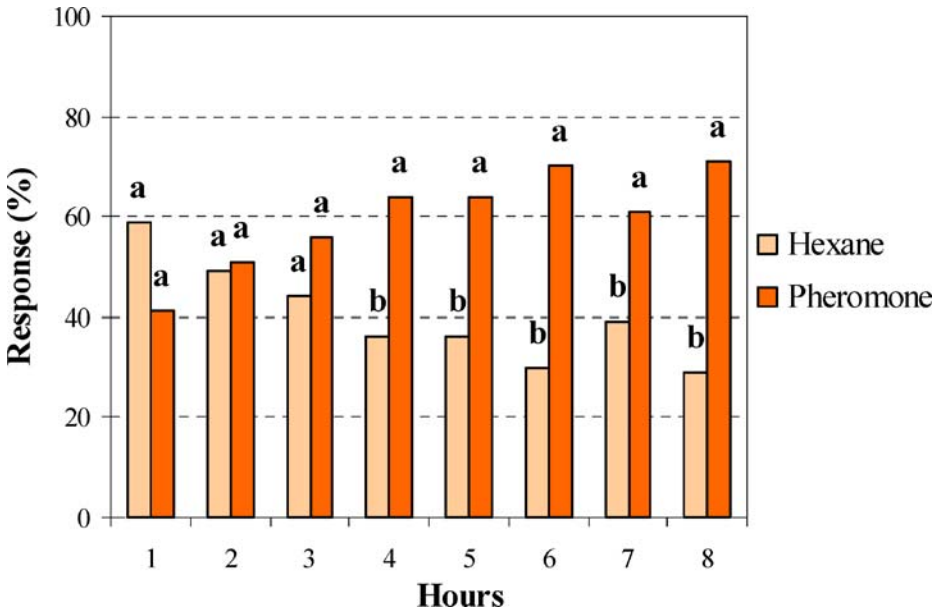


Fig. 4 Responses of *Cylas formicarius elegantulus* males to 10 ng of synthetic pheromone 1 at different times of the scotophase compared with control (hexane). Bars followed by the same letter are not significantly different (χ^2 homogeneity test, $P < 0.05$)

depolarizations as expected. Female antennae did not show any response even at a 100- μ g dose.

To determine the specificity of the crotonate ester on the antennal response, the formate, acetate, propionate, and butyrate esters of (*Z*)-3-dodecenol were prepared by standard methods (not shown), and their possible electrophysiological activity was tested. None of the analogues elicited EAG responses from male antennae, which demonstrates the key role played by the crotonate function on pheromone activity. In this regard, negligible attractant activity in the field by the butyrate ester has also been reported (Heath et al., 1991).

Behavior

To assess the behavioral response of males to the synthetic pheromone, we developed a double dual-choice olfactometer based on a four-armed bioassay designed by Vet et al. (1983) to measure olfactory responses of hymenopterous parasitoids. Preliminary experiments showed that the insects made the best choice between pheromone and blank vials after exposure for 3 min. We found that the difference in response to pheromone and control generally increased with the time of the scotophase, being highest at the 8th hr (70% of insects walked to the pheromone vials vs. 30% that moved towards control vials) (Fig. 4). Nevertheless, the responses were significantly different from the 4th hr, indicating that the maximum activity of males was between the 4th and the 8th hr of scotophase.

As regards the effect of pheromone dose, low amounts of pheromone (0.1–1 ng) induced no behavioral preference for any particular arm of the olfactometer. However, higher doses, particularly 50–1000 ng, elicited significantly higher responses (70–75%) on males orienting to the two sources (Fig. 5). Higher doses (10,000 ng) inhibited male response. The

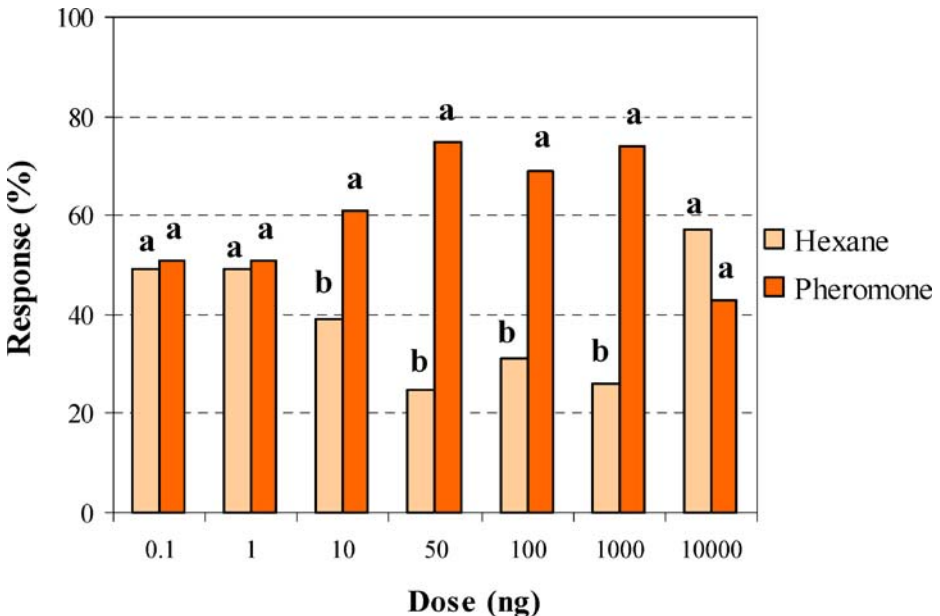


Fig. 5 Responses of *Cylas formicarius elegantulus* males to several pheromone doses at the 6th hr of the scotophase compared with control (hexane). Bars followed by the same letter are not significantly different (χ^2 homogeneity test, $P < 0.05$)

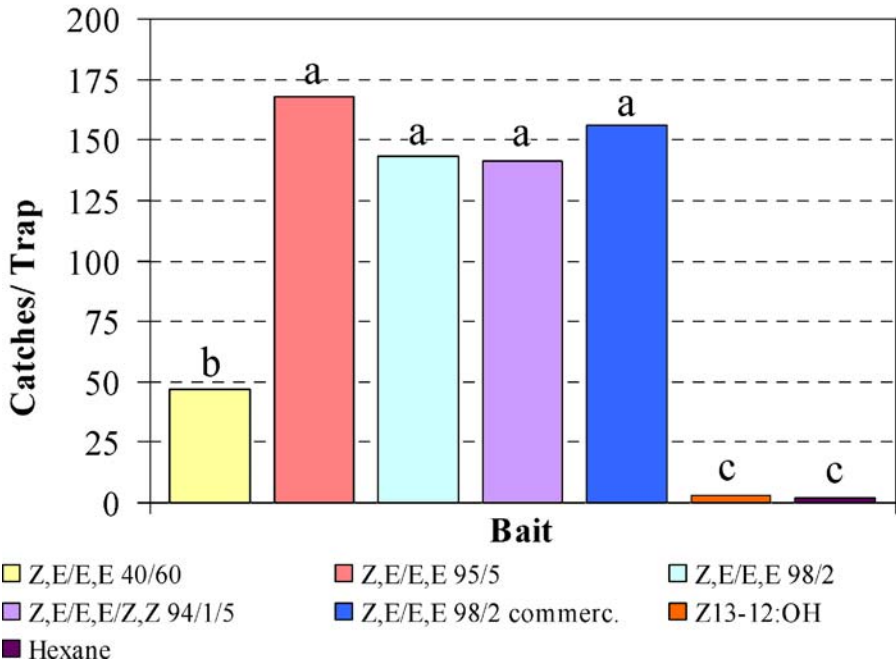
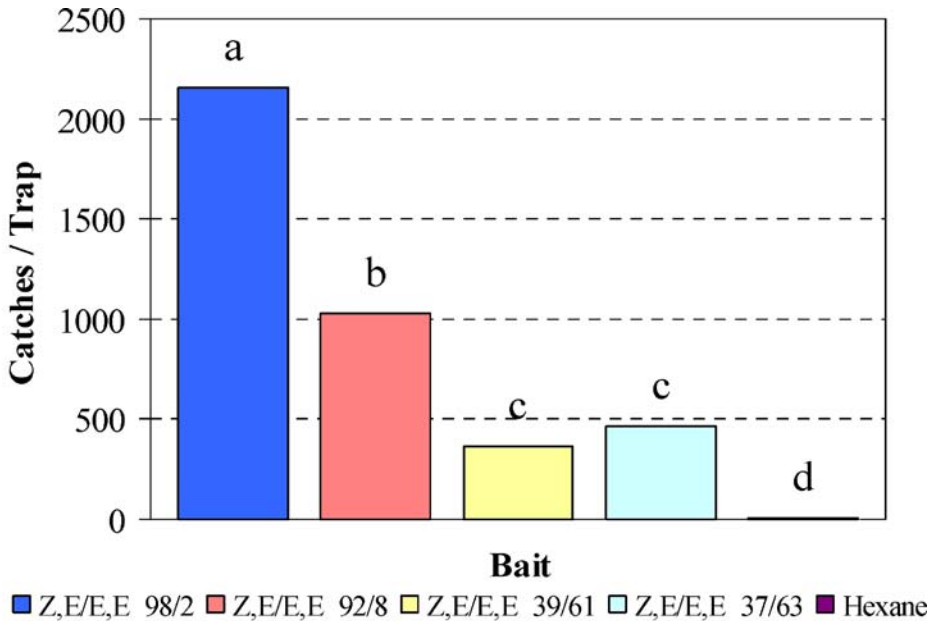


Fig. 6 Number of *Cylas formicarius elegantulus* males caught in traps baited with different pheromone formulations. Bars containing the same letter are not significantly different according to the Student's *t*-test ($P < 0.05$). Tests were performed in infested sweet potato fields in Santiago de las Vegas (Cuba) (July–August 2000, 2001)

commercial pheromone (Pherobank, Wageningen, The Netherlands) induced males at the same level of preference (70%) vs. control as our synthetic material (results not shown).

One drawback of the system could be the confined space in which insects are exposed to stimuli. This can be offset by using a sufficient number of individuals and replicates to obtain statistically significant responses. In addition, although in our olfactometer insects had to choose between two pheromone vials and two blanks (50% choice); in principle, our system could also allow them to discriminate between one, two, or three odors simultaneously. Moreover, the setup could determine the effect of the stimulus (attraction or repellency).

Field Tests

A number of reports on the attractiveness of the pheromone in the field have been described (Talekar and Lee, 1989; Jansson et al., 1990, 1992; Mason et al., 1990; Yasuda et al., 1992; Pillai et al., 1994). Here, we studied the effect of the stereomeric purity of the pheromone. We found in the first-year experiments that the formulation of the highest stereochemical purity was the most effective. The commercial sample (mixture of isomers *Z,E* and *E,E* in a 98:2 ratio) caught more males than the formulation with the same mixture of isomers in a 92:8 ratio, which in turn was more active than formulations with a blend of isomers in ca. 40:60 ratio (Fig. 6).

In the following year, although formulations containing the *Z,E* isomer in the range 94–98% were as active as the commercial pheromone, the bait containing a blend of the *Z,E* and *E,E* isomers in a 40:60 ratio was again inferior. In both experiments, as noted previously by other authors, a clear correlation between the content of the *Z,E* isomer with activity was apparent. The pheromone sample containing 5% of the *Z,Z* isomer induced no synergistic or inhibitory effect, and the alcohol precursor of the pheromone was inactive. These results contrast with those of Jansson et al. (1992) who reported that pheromone purities between 75% and 98% did not consistently affect weevil collections in traps, although some significant effects of purity were observed when trap counts were lower. In line with our results, Yasuda et al. (1992) reported that the activity of the synthetic pheromone was not inhibited by the addition of up to 10% of the *E,E* isomer. We suggest that the use of a stereochemically pure pheromone may not be necessary for future pest control strategies, and that formulations containing the active *Z,E* isomer with 94–95% stereomeric purity may be preferable owing to their easier availability and lower cost.

In summary, the sex pheromone of feral sweet potato weevil of a Cuban population has been confirmed as (*Z*)-3-dodecenyl (*E*)-2-butenate, as previously reported from a laboratory-reared colony (Heath et al., 1986). The synthetic compound elicited electrophysiological and behavioral activity in the laboratory and was highly attractive in the field, highlighting its potential utilization for pest control on the island. In this context, it is worth noting that the utilization of the pheromone in an IPM strategy in India has resulted in a considerable reduction of damage, leading to an increased production of marketable tubers by 53% (Pillai et al., 1993).

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Introduction

The pine weevil, *Hylobius abietis* (L.) (Coleoptera: Curculionide), is a severe pest of newly planted forest trees in large parts of Europe and Asia (Långström and Day, 2004). Adult weevils girdle and kill planted conifer seedlings by feeding on the bark of the stem (Day et al., 2004). If no countermeasures are taken, this frequently results in more than 80% seedling mortality in the first two years after planting (Örlander and Nilsson, 1999; Petersson and Örlander, 2003). The *H. abietis* problem is generally managed by treatment of seedlings in the plant nursery with a relatively persistent insecticide (Långström and Day, 2004). However, several European countries currently strive to abandon this insecticide usage. Novel ways to handle the *H. abietis* problem are, therefore, urgently needed.

H. abietis appears to avoid feeding on root bark close to where their eggs have been laid, thus indicating the presence of a deterrent substance that may be useful in conifer seedling protection (Nordlander et al., 2000; Bylund et al., 2004). Furthermore, antifeedant activity has been demonstrated in a methanol extract of female feces, which are placed over the egg during oviposition (Nordlander et al., 2000; Borg-Karlson et al., 2006). For identification of the active compounds, a feces extract was fractionated and the fractions were bioassayed by using both sexes of *H. abietis* (Borg-Karlson et al., 2006). In the most active fraction, oxygenated aromatic compounds, presumably originating from lignin, were identified. These and a number of structurally related compounds had an antifeedant effect when tested separately.

Among other substances, benzoic acid derivatives were found in the active extract fractions (Borg-Karlson et al., 2006). Initial work with this group of substances shows that *H. abietis* responds selectively to variations in the chemical structure of the isomers tested, and that the biological activity is related to the functional groups present and to the positions of the substituents on the aromatic ring. Similarly, the 10 isomers of methyl hydroxy-methoxybenzoate differ considerably in their antifeedant effects on *H. abietis* (Legrand et al., 2004). This emphasizes the importance of investigating the various isomers of potential antifeedants.

The rationale for research on structure–activity relationships is derived from studies in which the correlations between biological activity of plant growth regulators and chemical structure were analyzed (Hansch et al., 1963). The original approach was based on the assumption that chemical properties of the analogs are optimized for the compound to enter a cell and reach receptors where biological responses are triggered. The methodology has been applied for insecticides, (Hansch and Fujita, 1964), photosynthesis inhibitors (Hansch and Deutsch, 1966), and drug design (Topliss, 1972). Several structure–activity studies on insect antifeedants have previously been reported (e.g., Luteijn and DeGroot, 1981; Fischer et al., 1990; Ley et al., 1991; Luthria et al., 1993; Morimoto et al., 1999).

Antifeedant effects observed with *H. abietis* may result from activation of a number of receptors. Therefore, new analogs may result in increased antifeedant activity via one receptor, but may counteract the effect via another receptor. However, large differences in activity between close structural analogs show that this approach can be used to find promising antifeedants.

With the practical application that we have in mind (protection of conifer seedlings), we are not only interested in finding the most active chemical structure, but also in minimizing the cost of treatment. For example, we may find a commercially available analog having a lower biological activity than the most active substance, but available at a lower price. Specific properties of the compounds may be crucial when the compound is applied in the field (e.g., the melting point may be important for successful application and adherence to

the plant), or lower volatility may be necessary for persistence of the protective effect. An antifeedant compound might be physiologically detrimental to the seedling, either by penetrating through the bark or by being taken up by the roots (e.g., if the compound is leaking out from the formulation in which the compound is attached to the plant). Given these potential pitfalls, it is crucial to have more than one candidate antifeedant compound. It is also possible that two or more compounds may combine synergistically.

The potential of using antifeedants to protect seedlings against *H. abietis* damage has been previously demonstrated in field tests with methyl 3,5-dimethoxybenzoate (Nordlander et al., 2000) and with ethyl 2,3-dibromo-3-phenyl-propanoate, a substance identified in the bark of lodgepole pine, *Pinus contorta* Douglas ex Loudon (Bratt et al., 2001). In contrast, more volatile olfactory repellents, e.g., the monoterpenoid carvone, have provided poor protective effects against *H. abietis* damage in field tests (Schlyter et al., 2004), notwithstanding the strong antifeedant effect found in laboratory bioassays (Salom et al., 1994; Klepzig and Schlyter, 1999). In general, we hypothesize that the results of laboratory antifeedant assays should predict the results of field assays, but for use in the field, suitable dispenser matrices need to be developed to formulate the antifeedant for optimal activity. This is a complex issue that is beyond the scope of this paper.

This study was aimed at increasing our understanding of the physicochemical properties responsible for the antifeedant effects of benzoic acid derivatives on *H. abietis*. We also hoped to optimize any such effects in order to facilitate the development of an efficient method of protecting conifer seedlings from feeding damage by *H. abietis*. To these ends, we tested 55 structurally related compounds for antifeedant effects against *H. abietis* in a laboratory bioassay.

Methods and Materials

Collection and Maintenance of *H. abietis*

Both sexes of *H. abietis* were collected during spring migration at a sawmill in southern Sweden, where they landed in large numbers in response to massive emissions of attractive conifer volatiles. After collection, *H. abietis* were stored in darkness at 10°C and provided with fresh Scots pine, *Pinus sylvestris* L., branches or stems with tender bark as food. These storage conditions interrupted the reproductive development of the weevils, so that females did not begin to oviposit until about 1 week after they had been transferred to the experimental conditions, i.e., a light regime of 18:6 hr (L/D) at 22°C. This transfer was made at least 10 days before *H. abietis* was used in the following bioassay.

Feeding Bioassays

The compounds were tested on *H. abietis* by using a two-choice laboratory bioassay (Bratt et al., 2001). Fresh pieces of *P. sylvestris* twigs (50 mm long, 15 mm diam) were split, and each half (=test twig) was wrapped in aluminum foil. In each test twig, two sharp-edged metal rings (5 mm diam) were punched 25 mm apart through the foil and into the bark. The rings and the pieces of aluminum foil inside them were removed. The thin outer layers of corky bark inside the two circular areas on the surface of the twig were also carefully removed with a scalpel. Thereafter, new rings were fitted into the bark around the two exposed areas, and 100 µl of a 50-mM methanol solution of the compound to be tested were applied on the bark in one of the two rings. In the other ring, 100 µl of pure methanol

were added for control. When the solvent enclosed by the metal rings had evaporated and/or absorbed into the wood, the metal rings were removed. Each test twig was placed on moistened filter paper in a 142-mm-diam Petri dish, with one weevil in each dish for 24 hours (Fig. 1). The assay was replicated 20 times for both females and males. Weevils were all in the reproductive phase of their life cycle and were starved for 24 hours before the test period. Each weevil was used only once. Bioassays were conducted under a light regime of 18:6 (L/D) at 22°C. The amount of feeding on the treatment and control area of each test twig was recorded at the end of the bioassay. There was generally no significant difference in response between the sexes, and the data presented were therefore pooled.

The effects of the various treatments are described by two variants of the antifeedant index (AFI) (Blaney et al., 1984): $100 \times (C - T)/(C + T)$. (1) In AFIa, C represents the mean area of the control surfaces consumed and T represents the mean area of the treated surfaces consumed. (2) In AFIn, C represents the number of the control surfaces with feeding scars and T represents the number of the treated surfaces with feeding scars. Hence, AFIa is a measure that captures the reduction in feeding, whereas AFIn is a measure of complete inhibition of the initiation of feeding on the treated area. The two indices were fairly well correlated, but AFIa tended to be higher than AFIn because the antifeedant substances generally affected both the initiation of feeding and the amount of plant material consumed if feeding had started. For both indices, positive values (up to a maximum of 100) reflect an antifeedant effect, whereas negative values (down to a minimum of -100) indicate a stimulant effect. Statistical differences in feeding/no feeding between treatment and control were tested for each substance with Fisher's exact test of a 2×2 table: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Test Compounds

The origins of the compounds tested are given in Tables 1, 2, 3, and 4. Final purities of all compounds ranged from 96% to 99%, and, if necessary, compounds were purified by preparative chromatography (Baeckström et al., 1987) or flash chromatography on silica gel (Merck 60, 0.040–0.063 mm; Merck, Darmstadt, Germany). The compounds were synthesized from their corresponding carboxylic acids by method A, B, or C (see [Synthesis](#) below). Isopropyl benzoate (entry 27, Table 2) was obtained by method D, a transesterification using alkaline conditions (see [Synthesis](#) below). As can be seen in the



Fig. 1 *Hylobius abietis* on test twig with exposed treatment and control areas

Table 1 Effect of functional group (in benzoic and acetic acid derivatives) on antifeedant activity for the pine weevil, *Hylobius abietis*

Entry	Structural formula	Compound	AFIa	Rank AFIa	AFIn	Rank AFIn	Fisher's test
1		3,4-Methylenedioxybenzoic acid ^a	14	15	11	12	ns
2		Methyl 3,4-methylenedioxybenzoate ^b	57	11	25	11	**
3		2-Hydroxy-5-methoxybenzoic acid ^c	17	14	2	16	ns
4		Methyl 2-hydroxy-5-methoxybenzoate ^c	74	10	56	9	***
5		2-Hydroxy-3-methoxybenzoic acid ^c	22	12	3	15	ns
6		Methyl 2-hydroxy-3-methoxybenzoate ^a	95	3	85	1	***
7		3,4-Dimethoxybenzoic acid ^c	7	17	2	16	ns
8		Methyl 3,4-dimethoxybenzoate ^c	81	7	66	5	***
9		(4-Hydroxy-3-methoxyphenyl)acetic acid ^d	10	16	5	14	ns
10		Methyl (4-hydroxy-3-methoxyphenyl)acetate ^b	21	13	9	13	ns
11		3,5-Dimethoxyphenylacetic acid ^c	1	18	-4	19	ns
12		Methyl (3,5-dimethoxyphenyl)acetate ^b	96	1	84	2	***
13		Methyl (2,5-dimethoxyphenyl)acetate ^b	96	2	77	4	***
14		Methyl (2,4-Dimethoxyphenyl)acetate ^b	88	5	65	6	***
15		3,5-Dimethoxybenzoic acid ^c	-4	19	2	16	ns
16		Methyl 3,5-dimethoxybenzoate ^c	94	4	82	3	***
17		<i>N</i> - Ethyl 3,5-dimethoxybenzamide ^e	86	6	63	7	***
18		<i>S</i> -Ethyl 3,4-dimethoxybenzothioate ^f	74	9	57	8	***
19		3,5-Dimethoxyphenylmethanol ^a	75	8	47	10	***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

^a Purchased from Sigma - Aldrich (Stockholm, Sweden).

^b Prepared from the corresponding carboxylic acids by refluxing in the alcohol with H_2SO_4 as a catalyst.

^c Purchased from Lancaster Synthesis Co. (Lancashire, UK).

^d Obtained from previous work by H. Erdtman and T. Norin at the Dep. of Organic Chemistry, KTH (Stockholm, Sweden).

^e Prepared by stirring 3,5-dimethoxybenzoyl chloride in a solution of 70% ethylamine in water.

^f Prepared from the corresponding carboxylic acids by reaction with DCC and DMAP and the alcohol or thiol in CH_2Cl_2 .

Table 2 Effect of benzoic ester parent alcohol moiety on antifeedant activity for the pine weevil, *Hylobius abietis*

Entry	Structural formula	Compound	AFIa	Rank AFIa	AFIn	Rank AFIn	Fisher's test
20		Dodecyl 3,4-dimethoxybenzoate ^a	23	8	14	8	ns
21		Methyl 3,4-dimethoxybenzoate ^b	81	5	66	5	***
22		2-Methoxy-4-(2-propenyl)phenyl 3,5-dimethoxybenzoate ^c	17	9	6	9	ns
23		3-(3,4-Dimethoxyphenyl)-prop-1-yl 3,5-dimethoxybenzoate ^c	41	7	22	7	**
24		(3E)-Hexen-1-yl 3,5-dimethoxybenzoate ^c	62	6	37	6	***
25		2,2,2-Trifluoroethyl 3,5-dimethoxybenzoate ^c	86	4	72	4	***
26		Methyl 3,5-dimethoxybenzoate ^b	94	3	82	3	***
27		Isopropyl 2,4-methoxybenzoate ^d	96	2	95	1	***
28		Methyl 2,4-dimethoxybenzoate ^e	99	1	95	1	***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

^a Prepared from the corresponding carboxylic acids by refluxing in the alcohol with H_2SO_4 as a catalyst.

^b Purchased from Lancaster Synthesis Co.

^c Prepared from the corresponding carboxylic acids by reaction with DCC and DMAP and the alcohol in CH_2Cl_2 .

^d Prepared by a transesterification procedure using alkaline conditions.

^e Purchased from Sigma - Aldrich.

footnotes for Tables 1, 2, 3, and 4, the synthesis of a few compounds was reported in a previous paper (Legrand et al., 2004); a few compounds were obtained from previous work by H. Erdtman and T. Norin at the Department of Organic Chemistry, KTH, Stockholm, Sweden. The other test compounds were purchased from commercial vendors [Sigma-Aldrich (Stockholm, Sweden) or Lancaster Synthesis (Lancashire, UK)].

Synthesis

Commercial benzoic acids were either esterified by using methods A or B, or converted to amides by using method C.

Table 3 Effect of aromatic ring substituents of methyl benzoates on antifeedant activity for the pine weevil, *Hylobius abietis*

Entry	Structural formula	Compound	AFIa	Rank AFIa	AFIn	Rank AFIn	Fisher's test
29		Methyl 2-hydroxybenzoate ^a	21	22	13	17	*
30		Methyl 4-hydroxybenzoate ^a	34	18	26	11	**
31		Methyl 2-methoxybenzoate ^a	80	5	51	6	***
32		Methyl 3-methoxybenzoate ^a	89	3	65	4	***
33		Methyl 4-methoxybenzoate ^a	54	10	44	7	***
34		Methyl 4-octylbenzoate ^b	35	16	11	19	ns
35		Methyl 2,4-dihydroxy-3,6-dimethylbenzoate ^b	31	20	3	22	ns
36		Methyl 2,4-dihydroxybenzoate ^a	46	14	8	20	ns
37		Methyl 4-hydroxy-2-methoxybenzoate ^c	35	16	4	21	ns
38		Methyl 2-hydroxy-4-methoxybenzoate ^a	60	8	52	5	***
39		Methyl 2,4-dimethoxybenzoate ^d	99	1	95	1	***
40		Methyl 3,4-dihydroxybenzoate ^a	-7	23	2	23	ns
41		Methyl 4-hydroxy-3-methoxybenzoate ^d	53	12	22	14	*
42		Methyl 3-hydroxy-4-methoxybenzoate ^d	65	6	32	9	***
43		Methyl 3,4-dimethoxybenzoate ^a	81	4	66	3	***
44		Methyl 3,4-methylenedioxybenzoate ^b	57	9	25	13	**
45		Methyl 3-chloro-4-methoxybenzoate ^b	36	15	16	16	*
46		Methyl 3,5-dihydroxybenzoate ^a	23	21	13	17	ns
47		Methyl 3-hydroxy-5-methoxybenzoate ^c	54	10	26	11	***
48		Methyl 3,5-dimethoxybenzoate ^a	94	2	82	2	***
49		Methyl 3,5-dibromobenzoate ^b	50	13	36	8	***
50		Methyl 3,5-dinitrobenzoate ^b	34	18	22	14	**
51		Methyl 3,5-dimethylbenzoate ^b	61	7	32	9	**

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.^a Purchased from Lancaster Synthesis Co.^b Prepared from the corresponding carboxylic acids by refluxing in the alcohol with H_2SO_4 as a catalyst.^c Synthesis was reported in Legrand et al. (2004).^d Purchased from Sigma-Aldrich.

Table 4 Effect of aromatic ring substituent pattern of methyl benzoates on antifeedant activity for the pine weevil, *Hylobius abietis*

Entry	Structural formula	Compound	AFIa	Rank AFIa	AFIn	Rank AFIn	Fisher's test
52		Methyl 2-hydroxy-3-methoxybenzoate ^a	95	2	85	2	***
53		Methyl 2-hydroxy-6-methoxybenzoate ^b	73	8	54	8	***
54		Methyl 2-hydroxy-4-methoxybenzoate ^c	60	11	52	9	***
55		Methyl 2-hydroxy-5-methoxybenzoate ^a	74	7	56	6	***
56		Methyl 5-hydroxy-2-methoxybenzoate ^b	-3	20	-3	20	ns
57		Methyl 3-hydroxy-5-methoxybenzoate ^b	54	13	26	12	***
58		Methyl 4-hydroxy-2-methoxybenzoate ^b	35	17	4	19	ns
59		Methyl 3-hydroxy-2-methoxybenzoate ^b	75	6	35	10	***
60		Methyl 3-hydroxy-4-methoxybenzoate ^a	65	10	32	11	***
61		Methyl 4-hydroxy-3-methoxybenzoate ^a	53	15	22	13	*
62		Methyl 3,5-dimethoxybenzoate ^c	94	3	82	3	***
63		Methyl 2,4-dimethoxybenzoate ^a	99	1	95	1	***
64		Methyl 2,5-dimethoxybenzoate ^d	89	4	77	4	***
65		Methyl 3,4-dimethoxybenzoate ^c	81	5	66	5	***
66		Methyl 2,3-dimethoxybenzoate ^e	73	8	55	7	***
67		Methyl 2,6-dimethoxybenzoate ^a	51	16	10	16	ns
68		Methyl 2,4,6-trimethoxybenzoate ^a	55	12	21	14	ns
69		Methyl 2,3,4-trimethoxybenzoate ^d	54	13	21	14	ns
70		Methyl 3,4,5-trimethoxybenzoate ^d	32	18	8	17	ns
71		Methyl 4-hydroxy-3,5-dimethoxybenzoate ^d	10	19	5	18	ns

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

^a Purchased from Sigma - Aldrich.

^b Synthesis was reported in Legrand et al. (2004).

^c Purchased from Lancaster Synthesis Co.

^d Prepared from the corresponding carboxylic acids by refluxing in the alcohol with H_2SO_4 as a catalyst.

^e Prepared from the corresponding carboxylic acids by reaction with DCC and DMAP and the alcohol in CH_2Cl_2 .

Method A Esters were prepared from the corresponding carboxylic acids by refluxing in the alcohol with H_2SO_4 as a catalyst. For a typical procedure: methyl 2,3,4-trimethoxybenzoate (Table 4, entry 69). 2,3,4-Trimethoxybenzoic acid (500 mg, 2.36 mmol) was dissolved in methanol (20 ml), and a few drops of H_2SO_4 were slowly added to the stirred reaction mixture. The mixture was heated, and refluxed until the reaction was completed by thin layer chromatography. After cooling, solvent was removed under reduced pressure (10–20 mm Hg). The residue was taken up in CH_2Cl_2 , and washed twice with brine. The organic layer was dried over MgSO_4 , and the solvent was evaporated, leaving methyl 2,3,4-trimethoxybenzoate as a colorless oil (450 mg, 84%).

Method B Esters were prepared from the corresponding carboxylic acids (1.5 eq.) by reaction with 1.5 eq. dicyclohexylcarbodiimide (DCC) and 0.1 eq. *N,N*-dimethylaminopyridine (DMAP) and the alcohol or thiol in CH_2Cl_2 . For a typical procedure: (3*E*)-hexen-1-yl 3,5-dimethoxybenzoate (Table 2, entry 24). 3,5-Dimethoxybenzoic acid (545 mg, 3.00 mmol) was dissolved in a solution of CH_2Cl_2 (5 ml) containing DCC (618 mg, 3.00 mmol) and DMAP (24 mg, 0.2 mmol). (3*E*)-Hexen-1-ol (200 mg, 2 mmol) was added to the reaction mixture, which was then stirred overnight at room temperature. The white precipitate was filtered off and the solvent evaporated. The crude product was purified by liquid chromatography on silica gel by using an eluting gradient of EtOAc in hexane (1.25%, 2.5%, 5.0%, 7.5%, 10.0%, 15.0%, 20.0%, 30.0%, 50.0%, and 80.0% via a constant volume mixing chamber) to give (3*E*)-hexen-1-yl 3,5-dimethoxybenzoate (262 mg, 50%).

Method C *N*-Ethyl 3,5-dimethoxybenzamide (Table 1, entry 17) was prepared by stirring 3,5-dimethoxybenzoyl chloride (300 mg, 1.50 mmol) in a solution of 70% ethylamine in water (10 ml). After stirring overnight, the crystals were filtered off, and subjected to chromatography. The product amide was analyzed by NMR and the yield was 111 mg (35%).

Method D An example of the transesterification procedure is given for isopropyl 2,4-dimethoxybenzoate (Table 2, entry 27). Sodium (0.1 g, 4.3 mmol) was dissolved in 7.5 ml isopropanol, and a solution of methyl 2,4-dimethoxybenzoate (403 mg, 2.06 mmol) in 10 ml isopropanol was added. The reaction mixture was stirred at room temperature overnight. Then, 20 ml of EtOAc were added, and the organic phase obtained was washed twice with H_2O and once with a saturated NH_4Cl solution. The organic phase was dried over magnesium sulfate and evaporated to give isopropyl 2,4-dimethoxybenzoate (220 mg, 48%) as a yellow oil.

Method E Methyl 2-hydroxy-6-methoxybenzoate (Table 4, entry 53) and methyl 3-hydroxy-5-methoxybenzoate (Table 4, entry 57) were synthesized from the commercially available symmetric 2,6-dihydroxy- and 3,5-dihydroxybenzoic acids (Legrand et al., 2004). The acids were esterified and then *O*-monomethylated by using methyl iodide in the presence of a weak base (K_2CO_3) in MeOH. Analytical data from these two benzoates were identical with those previously reported (Hoffmann and Pete, 2001; Kühnert and Maier, 2002). The syntheses of the methyl hydroxy-methoxybenzoates (Table 4, entries 56, 58, and 59) were executed by a regioselective protection-deprotection synthetic sequence (Legrand et al., 2004).

Results and Discussion

When interpreting our results, we have specifically focused on the importance of four types of structural features for antifeedant activities: (1) the functional groups (Table 1); (2) the carbon chain length of the parent alcohol moieties in benzoic esters (Table 2); (3) the

structures of the substituents on the aromatic rings (Table 3); (4) the patterns of substituents (Table 4). Antifeedant activities (AFIa and AFIn values) were compiled and compared for these four categories of structural features with the aim of demonstrating the effect of each structural feature by itself (Tables 1, 2, 3, 4).

Effect of Functional Group on Antifeedant Activity (Table 1)

Functional groups of benzoic acid derivatives were important for antifeedant activity. Benzoic acids *per se* seemed to have weak activity or none at all, whereas the corresponding methyl esters generally were highly active (compare entries 1 vs. 2, 3 vs. 4, 5 vs. 6, 7 vs. 8, and 15 vs. 16). Because some methyl phenylacetates (entries 9–14) also possess antifeedant activities, the carbonyl moiety does not need to be directly attached to the aromatic ring. These examples show that aromatic carboxylic acids are relatively poor antifeedants in comparison with their ester analogs. For example, both 3,5- and 2,5-dimethoxy isomers of methyl phenylacetate are good antifeedants (entries 12–13), whereas 3,5-dimethoxyphenylacetic acid (entry 11) is not (Table 1). Test results of the secondary benzamide (entry 17), thioester (entry 18), and benzyl alcohol (entry 19) show that the functional group does not need to be an ester moiety for a compound to have antifeedant activity.

Effect of Benzoic Ester Parent Alcohol Moiety on Antifeedant Activity (Table 2)

Esters with a short alkyl chain from the parent alcohol had relatively high antifeedant activities (entries 21, 25–28), whereas esters with bulkier substituents from the parent alcohol had relatively low activities (entries 20, 22–24).

Effect of Aromatic Ring Substituents on Antifeedant Activity (Table 3)

Comparisons of monosubstituted benzoates revealed that monomethoxylated benzoates were generally better antifeedants than the corresponding monohydroxylated benzoates (Table 3, entries 29 and 30 vs. entries 31 and 33). A methyl benzoate substituted with a long alkyl chain (e.g., entry 34) showed no significant antifeedant activity.

Comparison of disubstituted benzoates revealed that methoxy-substituted benzoates generally give better antifeedants than hydroxy-substituted benzoates (Table 3, entries 36 vs. 39; 40 vs. 43; 46 vs. 48). The hydroxy–methoxy analogs (Table 3, entries 38, 41, 42, and 47) were relatively good, in contrast to the corresponding dihydroxybenzoates (Table 3, entries 36, 40, and 46), which were not significantly active. Dimethoxy-substituted benzoates (entries 39, 43, and 48) possessed some of the highest antifeedant activities among substances tested. The connection of two adjacent oxygen atoms via a methylene group did not yield high antifeedant activity (entry 44).

Relative to the dimethoxy analog, the activities of methyl benzoates were lower when they were substituted with strong electron-withdrawing substituents like the nitro group (Table 3, entry 50 vs. 48). Halogen substituents (bromo or chlorine) also resulted in lower antifeedant capacity of methyl benzoates (e.g., a dibromo derivative, entry 49 or a monochlorobenzoate, entry 45). The result with the lipophilic 3,5-dimethyl analog (entry 51) discouraged us from further testing of even more hydrophobic analogs.

Effect of Aromatic Ring Substituent Pattern on Antifeedant Activity (Table 4)

Given that monomethoxylated benzoates were some of the most efficacious antifeedants, we evaluated the effect of the position of the methoxy groups on the activity. Derivatives with the methoxy group in the *meta*- or *ortho* positions are superior to those with the methoxy group in the *para* position (Table 3, entries 31–33). Hydroxy–methoxybenzoates with hydroxy groups in *ortho* positions (Table 4, entries 52–55) had moderate to high antifeedant activities, and methyl 2-hydroxy-3-methoxybenzoate showed the highest activity in this class. We hypothesize that this may be correlated to hydrogen bonding between the *ortho*-hydroxy and adjacent carbonyl group. The other hydroxy–methoxybenzoates (entries 56–61), showed no, low, or moderate antifeedant activities. The results of the assays of these compounds did not indicate an optimal substitution pattern, although the weevils exhibited a remarkable selectivity in their responses to close structural analogs. For example, there was almost no antifeedant activity from the 5-hydroxy-2-methoxy analog (entry 56), whereas the 2-hydroxy-5-methoxy analog (entry 55) was highly active. This also supports the hypothesis that hydrogen bonding between *ortho*-hydroxy and carbonyl groups is important for antifeedant activity.

The activities of benzoates with two dimethoxy groups (entries 63–67) vary but are generally high, with the exception of the 2,6-analog (entry 67). The somewhat higher antifeedant activity of the 2,4-analog (entry 63), compared with the 3,5-dimethoxy analogue (entry 62) in the 50-mM concentration was consistent even when tested at lower concentrations (25 and 5 mM; unpublished results). An attempt to find a compound with an even higher activity than that of methyl 2,4-dimethoxybenzoate (entry 63) by adding a third methoxy substituent in position 6 failed completely (entry 68). Other derivatives with a third methoxy or hydroxy group also showed no significant antifeedant effect (entries 69–71). Some aromatic compounds related to the ones tested here are emitted by sporulating fruiting bodies of tree-decaying fungi, e.g., anisole, benzaldehyde, methylanisate, and methyl 4-methoxyphenylacetate (Rösecke et al., 2000; Rösecke and König, 2000). The ecological significance of these substances to *H. abietis* females may be that they indicate that the host is infested with fungi and in a state of decay, thus making it unsuitable for oviposition (von Sydow, 1993). If compounds such as anisole and methyl (4-methoxyphenyl)acetate were tested, the response might be similar to that of the deterring signals from substances in *H. abietis* feces [i.e., methyl (2,4-dimethoxyphenyl)acetate (Table 1, entry 14), or 3-methylanisole, AF1a 35, AF1n 23, which was tested in Borg-Karlson et al., 2006].

In summary, This study has revealed the following structural criteria for benzoic acid derivatives that have high antifeedant activity against *H. abietis*:

1. The functional group of the benzoic acid derivative is apparently not critical as long as it is not a –COOH group.
2. The carbon chain of the parent alcohol in the ester derivative must be short.
3. The optimal substituents are methoxy groups. Longer alkoxy groups do not result in more effective antifeedants. Nitro and hydroxy groups are seemingly too polar, whereas halogens and methyl groups are apparently too lipophilic to be effective.
4. Two substituent patterns seem to give optimal antifeedant effects. First, in hydroxy–methoxy derivatives, the hydroxy group should be situated in the *ortho* position and adjacent to the carbonyl group (Table 4, entries 52–61). Second, all dimethoxy derivatives, except the 2,6-dimethoxy derivative, possess good antifeedant activities. Inductive or resonance effects are apparently not important for antifeedant activity, as both the *ortho*–*para*-2,4-dimethoxy analog (entry 63) and the *meta*-3,5-dimethoxy

analog (entry 62) had high activity relative to most other structures tested. No substituent pattern can be declared to be optimal.

Additionally, esters or amides formed from a short parent alcohol and with two small alkoxy substituents on the aromatic ring lead to high antifeedant activity for *H. abietis*. Long carbon chains in the substituents or from the parent alcohol portion of the ester reduce the antifeedant effect and hydroxy substituents reduce the activity except when the hydroxy group is in the *ortho* position.

Several benzoic acid derivatives had strong antifeedant effects against *H. abietis* in the laboratory feeding tests. Five compounds tended to have at least as high, or even higher, antifeedant activity than methyl 3,5-dimethoxybenzoate, which previously had been identified as a potent antifeedant in laboratory and field tests (Nordlander et al., 2000). These new, highly effective antifeedants are methyl 2,4-dimethoxybenzoate, isopropyl 2,4-dimethoxybenzoate, methyl 2-hydroxy-3-methoxybenzoate, methyl (3,5-dimethoxyphenyl) acetate, and methyl (2,5-dimethoxyphenyl)acetate. Further tests at lower concentrations are needed to evaluate their relative potentials as *H. abietis* antifeedants. Field assays measuring volatility, stability, and physiological effects on seedlings will be necessary to assess the usefulness of these five antifeedants for seedling protection.

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estimated at about 50% in males and 70% in females. Newly eclosed adults did not produce volatile chemicals until 2 d after molting.

Keywords *Lygus hesperus* · Heteroptera · Miridae · (*E*)-4-oxo-2-hexenal · Hexyl butyrate · (*E*)-2-hexenyl butyrate · *Pogonomyrmex rugosus* · *Solenopsis xyloni* · Formicidae · Hymenoptera · Initial discharge percentage · Defensive secretion

Introduction

Plant bugs of the genus *Lygus* feed on meristematic tissues of plants, causing shedding of buds, blooms, seeds, and fruit (Debolt and Patana, 1985). There are at least nine species of *Lygus* bugs of economic importance in the northern hemisphere (Kelton, 1975), of which *Lygus hesperus* Knight is the most abundant in western North America (Clancy and Pierce, 1966; Kelton, 1975). The species is polyphagous, damaging cotton, alfalfa, and strawberries, and it is found on over 100 species of plants in 24 families (Scott, 1977; Blackmer et al., 2004). Several species in the genus, including *L. hesperus*, use a sex pheromone released by the female (Scales, 1968; Strong et al., 1970; Graham, 1987, 1988; McLaughlin, 1996). Several research groups have tried to identify the sex pheromone components of *L. hesperus* and *Lygus lineolaris* (prevalent in eastern United States) so far without success (Aldrich et al., 1988; Ho and Millar, 2002; Wardle et al., 2003).

Aldrich et al. (1988) extracted metathoracic scent glands and trapped airborne volatiles from *L. hesperus* and the sibling species *L. lineolaris* and found similar ratios of hexyl and (*E*)-2-hexenyl butyrate in males and females. (*E*)-4-Oxo-2-hexenal was also detected in glands of male *L. hesperus* and in both sexes of *L. lineolaris*. More recently, Ho and Millar (2002) found significant quantities of hexyl butyrate, (*E*)-2-hexenyl butyrate, (*E*)-2-hexenal, and 1-hexanol in solvent extracts of *L. hesperus* glands. These compounds, as well as lower amounts of additional components, were also found in aerations of the macerated adults. Although the sexes differed somewhat in the ratios of some components, each sex had all of the components. Field testing of all binary combinations of 16 components detected in aerations failed to attract either sex. Electroantennographic responses of each sex to 15 of the compounds indicated that both sexes can detect a wide range of compounds, including those from the macerated adults. Ho and Millar (2002) did not ascribe any biological function to the volatiles other than to suggest that one or more could be components of a sex pheromone blend.

Wardle et al. (2003) investigated volatile emissions from the sibling species *L. lineolaris* with the same goal of identifying a sex pheromone. They found that both sexes had large amounts of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-2-hexenal, with considerably more (*E*)-4-oxo-2-hexenal than in *L. hesperus* (compared with $\leq 1\%$ by Ho and Millar, 2002) as collected by Porapak Q traps. The adults were crowded together in a mass at the bottom of a glass tube to induce high rates of volatile release. Wardle et al. (2003) suggested that the volatiles may act as an alarm pheromone or as chemical defenses against predators; however, no experiments were carried out to test these hypotheses.

The objectives of this study were to (1) induce releases of volatiles from both sexes of *L. hesperus* and identify the compounds and their relative amounts, (2) compare these to amounts in solvent extracts of individual insects, and (3) test volatile compounds for intraspecific alarm functions and for defensive functions against two species of ants. A further objective was to look for compounds unique to females that might be sex pheromone components.

Methods and Materials

Induced Release of Defensive Chemicals from Adult Tarnished Plant Bugs and Behavioral Effects

Unless otherwise stated, *L. hesperus* (collected from Maricopa, AZ, USA) was reared in the laboratory on green beans and synthetic diet (Rodriguez-Saona et al., 2002; Blackmer et al., 2004). Sexes were separated by the presence or absence of an ovipositor groove. Volatile chemicals from male and female *L. hesperus* were collected from the headspace by solid-phase microextraction (SPME). Individual *L. hesperus* were chilled so that they did not release volatiles under stress when transferred to 2-ml glass vials with Teflon liners with a 0.5-mm hole, allowing insertion of the SPME needle. A 65- μ m Carbowax-divinylbenzene SPME fiber (Supelco, Bellefonte, PA, USA) was inserted into the vials for 10 min to collect background volatiles that were then desorbed in the GC–MS injection port at 250°C for 5 min. In some experiments, a vial was opened and forceps were used to grab the leg of an adult for about 10 sec (molestation) to induce defensive emissions, the vial was recapped, and volatiles were collected for 10 or 120 sec by SPME.

Gas chromatography–MS analysis of volatiles collected on SPME fibers was carried out with a Varian 3900 GC with a 30 m \times 0.25 mm ID column coated with 0.25 μ m CP-Sil 8 CB (Varian CP5860). Helium carrier gas was programmed for constant flow (1.2 ml/min). The injection at 250°C was splitless for 5 min (then split 20:1), whereas the oven/column temperature was held at 40°C during the same period before increasing at 5°C/min to 180°C. Thereafter, temperature increased at 20°C/min to 250°C and was held for 5 min. Compounds in GC effluents were analyzed with a Varian Saturn 2100D MS using the NIST02 and Wiley7 spectral libraries and comparison to commercial standards (all >98% purity, Aldrich Chemical Co. and Bedoukian Research, Danbury CT, USA), except (*E*)-4-oxo-2-hexenal (>98%, synthesized by H.D. Pierce Jr. and provided by R. Gries, Burnaby, B.C., Canada) and pentyl butyrate (previously identified by Ho and Millar, 2002).

In bioassay experiments, harvester ants, *Pogonomyrmex rugosus* Emery, and native fire ants, *Solenopsis xyloni* McCook, from Phoenix, AZ, were placed in 2-ml vials with an adult bug until the ant initiated an attack, whereupon SPME collection was performed for 1–2 min. The behavioral and lethal effects of saturated vapors of hexyl butyrate (50 μ l in 125-ml closed bottle) or 0.5 μ l direct applications of hexyl butyrate on *P. rugosus* were observed every 30 min for 1 min over an 8-hr period. Adults of *L. hesperus* grasped with forceps were presented within a few millimeters of resting *L. hesperus* nymphs and adults for several seconds to determine if any alarm behavior could be induced.

Quantification of Volatile Chemicals in Adult Tarnished Plant Bugs

Adult *L. hesperus* reared in the laboratory or collected on September 8, 2004, in Maricopa, AZ, from the field (wild) were extracted individually, with 100 μ l pentane with an internal standard of ethyl heptanoate (1 ng/ μ l, Aldrich). Adults were cooled to 3°C and dropped into solvent contained in a 100- μ l conical vial and crushed with a blunt, nickel-plated tapestry needle (Prym-Dritz Corp., Spartanburg, SC, USA). The solvent was immediately sucked off the mashed insect and placed in a new vial and analyzed within a few days by GC–MS. Leaving the crushed cadaver in solvent caused degradation of (*E*)-4-oxo-2-hexenal (see Results). The metathoracic glands of three 10-d-old individuals of each sex were dissected under water and extracted as above. The column and temperature program used for analysis

of solvent extracts were the same as for SPME above, but the flow was splitless for 0.75 min before being split 60:1 for 4.25 min (thereafter 20:1) and was maintained an additional 10 min at 250°C.

For quantification of various volatile chemicals in solvent extracts of *L. hesperus*, the total ion chromatogram peak areas at the respective retention times were compared with areas of the internal standard (ethyl heptanoate) and adjusted for the MS response factor sensitivities of identified compounds. The MS chromatogram areas of the three major compounds, hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal from adults were usually large and needed correction for nonlinear MS responses. This was carried out by multiplying the peak areas by a correction function (*Y*) before comparison to the internal standard. This function was determined by GC–MS for a concentration curve of each compound (0.1, 1, 10, 100, 1000 ng/μl) and regressing *X* (observed MS peak area at each amount) with *Y* (predicted peak area from linear extrapolation of the area for 1 ng times the number of nanograms injected divided by *X*) to obtain a best-fitting function (e.g., for hexyl butyrate: $Y = 0.862 + 0.0002593X^{0.5}$, $R^2 = 0.995$). Differences between means of chemical amounts were analyzed by *t*-tests, and linear and nonlinear regressions were performed on scatter plots of individual *L. hesperus* weights or adult age after last instar ecdysis vs. amounts of volatile compounds (Sokal and Rohlf, 1995; Statistica 5.1 by StatSoft, Inc.).

To determine the amounts of volatile compounds in each sex as they matured sexually (Strong et al., 1970), a large cohort of newly emerged adults from the laboratory colony were separated by sex and kept separately until extraction. In other experiments, adults of both sexes were kept together for known times until extraction. Individual weights of 16 males and 16 females from the laboratory colony were taken on a Mettler balance (precision of 0.1 mg) and each was extracted to determine if volatile compound amounts varied with weight.

Initial Discharge Percentage of Volatile Chemicals

The initial discharge percentage (IDP) is defined as the percentage of volatile chemical secretion that is discharged from an insect due to an initial molestation stimulus. Because extraction destroys the insects, a group of adults was split and individuals of one cohort were extracted for volatiles to obtain a mean (\bar{A}), and this was compared with the mean of the other cohort (\bar{B}) after they had discharged chemicals due to molestation. SPME collection (2 min, described above) and GC–MS were used to verify whether members of group B released volatiles before their extraction. After SPME, the molested adults were removed from the 2-ml vial and aired out for 12 min before extraction. The volatile secretion was defined as the sum of amounts of hexyl butyrate and (*E*)-2-hexenyl butyrate because the latter did not fully separate by GC on the column used. Because age plays a role in amounts of volatiles, 10-d-old males and females of the two cohorts were extracted to determine the IDP for volatile compounds:

$$\text{IDP} = \frac{\bar{A} - \bar{B}}{\bar{A}} \cdot 100 \quad (1)$$

Amounts of Volatile Compounds and their Cross-Correlations

In addition to the three major volatiles, six additional compounds were commonly identified in adults older than 2 d of age. Correlation matrices (Statistica) were carried out

on the quantified amounts of the nine compounds in individual males and females from the three major experiments (maturation, IDP, and weight variation).

Results

Induced Release of Volatiles from Adult Tarnished Plant Bugs and Behavioral Effects

Resting *L. hesperus* adults did not release SPME-detectable amounts of any volatiles in 10-min periods ($N = 6$). However, briefly grabbing one of the legs of an adult bug with fine forceps induced the immediate discharge of volatile compounds as determined by SPME and GC–MS ($N = 8$ of each sex, IPD experiment). Relative amounts of the compounds hexyl butyrate, (*E*)-4-oxo-2-hexenal, and (*E*)-2-hexenyl butyrate released were estimated from SPME peak areas corrected for MS responses and normalized, yielding $100 \pm 13\%$ ($\pm SE$), $44 \pm 9\%$, and $4 \pm 0.5\%$, respectively, from females and $83 \pm 18\%$, $37 \pm 9\%$, and $3 \pm 0.5\%$ from males.

Pogonomyrmex rugosus ($N = 4$) and *S. xyloni* ($N = 3$) ants placed individually with a bug in 2-ml vials usually spent a few minutes in searching/escape movements before opening their mandibles and grasping the bug in an attack. Immediately after this, the ant jerked its head away and violently began running. Thereafter, SPME for 10 sec showed high concentrations of the volatile components of the adult bug (similar to grabbing with forceps above) as well as smaller amounts of an alarm pheromone from *P. rugosus* (4-methyl-3-heptanone; McGurk et al., 1966). The ants and *L. hesperus* were removed from the vials after SPME and none appeared adversely affected. However, one *P. rugosus* after being irritated by the defensive secretion repeatedly attacked and killed the bug, and then the ant's movements became spasmodic. This ant was removed a few minutes later and recovered but had permanent damage to its antennae as evidenced by lack of movement. Two *S. xyloni* ants that attacked *L. hesperus* were left in the vials and died within 4 hr, whereas the adult bugs survived for more than 15 hr.

Pogonomyrmex rugosus ants exposed singly in 125-ml bottles to saturated vapors of hexyl butyrate for 8 hr showed no behavioral or toxic effects compared with a control group in similar bottles ($N = 6$ each group). However, topical application of $0.5 \mu\text{l}$ of neat hexyl butyrate caused violent escape behaviors by the ant that within seconds turned into spasms ($N = 6$). All treated ants recovered apparently normal motor behavior within an hour. None of the *L. hesperus* adults ($N = 5$) held by forceps and presented within a few millimeters of resting nymphs and adults elicited any movement or alarm behavior ($N = 8$ for each group for each held adult). All five forceps-held adults discharged average amounts of butyrates as indicated by SPME and solvent extraction.

Quantification of Volatiles in Adult Tarnished Plant Bugs

When adult *L. hesperus* were placed in pentane, they released a fluid as seen by refractive changes. (*E*)-4-Oxo-2-hexenal amounts (Y) declined rapidly with time (X) in the solvent extracts (e.g., $13.9 \mu\text{g}$ initially, 19% left after 75 min, 0.4% left after 4 hr) when the crushed cadaver remained in the solvent ($Y = e^{3-0.24\sqrt{x}}$, $R^2 > 0.99$, $N = 4$). This also caused a more gradual loss over several weeks of hexyl butyrate and (*E*)-2-hexenyl butyrate (as well as the internal standard). The loss of (*E*)-4-oxo-2-hexenal occurred rapidly in pentane as well as in diethyl ether and acetone. In the same cadaver extract, γ -caprolactone

[5-ethylidihydro-2-(3*H*)-furanone] was not detected initially but accumulated over time ($Y = -0.002 + 0.00085X$, $R^2 > 0.99$, $N = 4$; e.g., 0.22 μg after 4 hr, 1.23 μg after 24 hr). In contrast, if the solvent extract was sucked off the macerated bug and placed in a new vial, then (*E*)-4-oxo-2-hexenal and the esters were stable in solvent for weeks and γ -caprolactone did not accumulate. Using the decanting method, sample extracts were usually analyzed within a day or two of preparation to ensure the accuracy of quantification. The solvent extraction was efficient because a second extraction with 100 μl pentane performed on a few individuals recovered <1% of the material recovered in the first extraction.

Logarithmic regression of amounts of (*E*)-4-oxo-2-hexenal produced in *L. hesperus* of different adult ages showed nearly identical curves for males and females (female: $Y = 1.19 + 1.54\ln X$, $R^2 = 0.72$, $P < 0.001$, $N = 13$; male $Y = 1.15 + 1.56\ln X$, $R^2 = 0.69$, $P < 0.001$, $N = 13$; Fig. 1). Pooling data of adults aged 8 to 19 d gave a mean of 5.6 μg per male and 5.1 μg per female of (*E*)-4-oxo-2-hexenal (not significantly different, $t = -0.39$, $df = 39$, $P = 0.70$). However, females produced more hexyl butyrate ($Y = 4 + 6.64\ln X$, $R^2 = 0.67$, $P < 0.001$) and (*E*)-2-hexenyl butyrate ($Y = 0.44 + 0.58\ln X$, $R^2 = 0.77$, $P < 0.001$) than males ($Y = 3.3 + 3.52\ln X$, $R^2 = 0.77$, $P < 0.001$; $Y = 0.29 + 0.20\ln X$, $R^2 = 0.58$, $P = 0.003$, respectively). Pooling data from days 8 to 19 gave a mean of 22 μg hexyl butyrate per female, significantly more than the 13 μg per male ($t = 2.39$, $df = 39$, $P = 0.02$). Females had a mean of 1.9 μg (*E*)-2-hexenyl butyrate, significantly more than the 0.8 μg in males ($t = 3.77$, $df = 39$, $P < 0.001$).

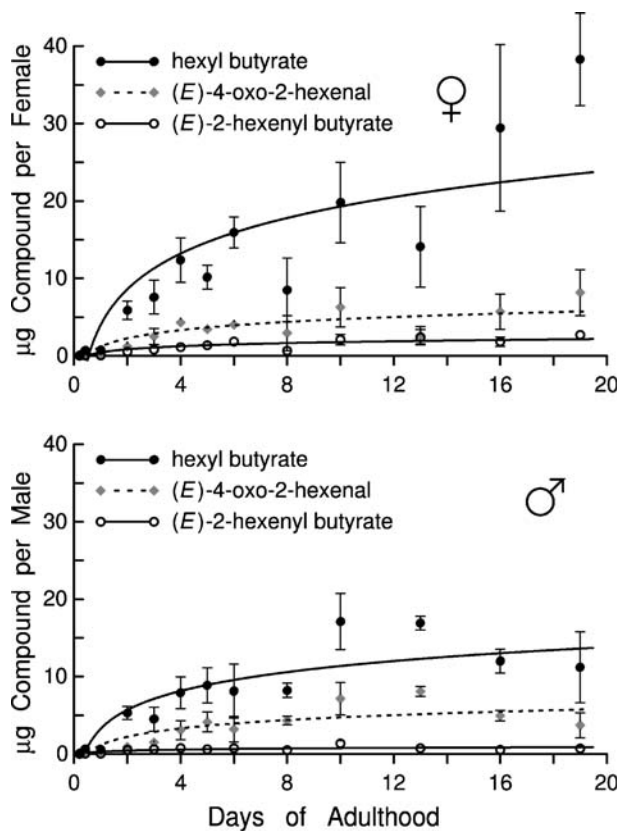
The amounts of (*E*)-4-oxo-2-hexenal and the pooled butyrates [hexyl and (*E*)-2-hexenyl butyrate] increased in relation to fresh weight of females reared in the laboratory (Fig. 2). In wild females, linear regression gave positive slopes but none were significant ($P > 0.05$), although hexyl butyrate had an $R^2 = 0.63$ ($N = 6$). In laboratory females, only the slope of (*E*)-4-oxo-2-hexenal was significant ($P = 0.01$) with an $R^2 = 0.37$. Combining the two butyrates (Fig. 2), because they did not separate fully by GC, gave a regression for laboratory females of $Y = -9.47 + 1.44X$, $R^2 = 0.18$, $N = 16$, suggesting a weak correlation and positive slope, although not significant ($P = 0.1$). The amounts of the volatile compounds in males showed no relationship with weight and had a high variation similar to females (Fig. 2). All regressions of male weight and amounts of volatiles (wild $N = 10$, laboratory $N = 16$) gave R^2 values of < 0.01, and none of the slopes were significantly different from 0 (Fig. 2).

No significant differences in body weight were found between laboratory and wild males, but the mean weight of laboratory females was greater than wild females ($t = 3.84$, $df = 20$, $P = 0.001$; Table 1). Laboratory females weighed more than laboratory males ($t = 15.19$, $df = 30$, $P < 0.001$) and wild females weighed more than wild males ($t = 4.93$, $df = 14$, $P < 0.001$). No significant differences in the amounts of (*E*)-4-oxo-2-hexenal, hexyl butyrate, and (*E*)-2-hexenyl butyrate were found between sexes of laboratory or wild adults (Table 1, Fig. 2), or between laboratory and wild adults of the same sex, except for wild males that had less (*E*)-4-oxo-2-hexenal than laboratory males ($t = 2.39$, $df = 24$, $P = 0.025$). Metathoracic glands dissected from laboratory males and females had comparable amounts of volatiles to those in whole bodies from other experiments (Tables 1 and 2).

Initial Discharge Percentage of Volatiles

Females had an IDP of 84% for (*E*)-4-oxo-2-hexenal and 59% for the combination of hexyl butyrate and (*E*)-2-hexenyl butyrate (Fig. 3). Males had an IDP of 66% for (*E*)-4-oxo-2-

Fig. 1 Amount of volatile compounds in male and female *L. hesperus* in relation to age in days after their final ecdysis (laboratory colony, March 2003). Each point and brackets represent a mean \pm SE ($N = 4$)

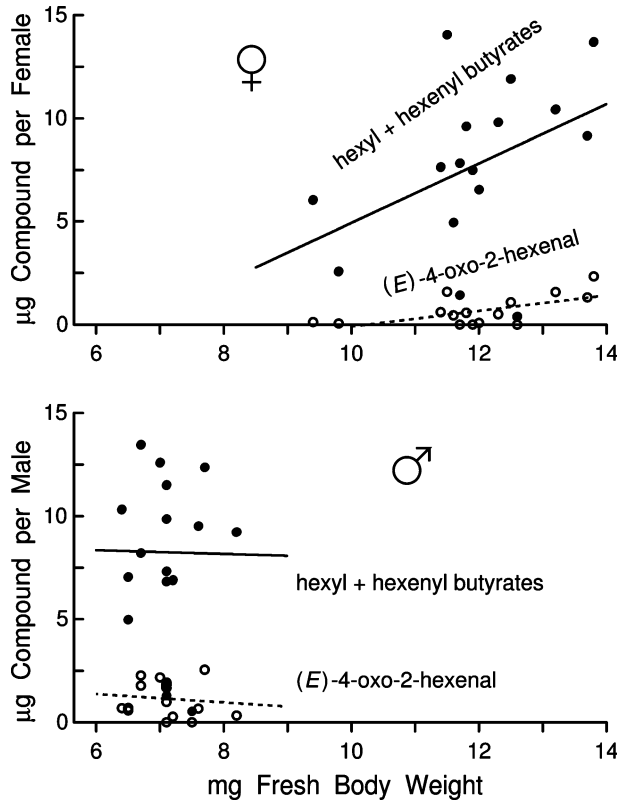


hexenal and 32% for hexyl butyrate plus (E)-2-hexenyl butyrate. Molested/discharged males and females contained lower amounts of the major volatiles than the nonmolested cohort, but only (E)-2-hexenyl butyrate in molested males ($t = 2.97$, $df = 14$, $P = 0.010$), and only hexyl butyrate in molested females ($t = 2.44$, $df = 14$, $P = 0.028$) were significantly less. Of the six minor constituents, most were less in molested males: (E)-2-hexenal (20%, as percentage of unmolested in Table 2, IDP experiment), 1-hexanol (45%), compound X (49%), pentyl butyrate (35%), and (Z)-3-hexenyl butyrate (50%). However, levels of hexyl acetate (130%) were higher in molested males. Compound X eluted at 12.15 min and had MS major fragments (m/z) of 56, 57 (base), 85, and 86. In females, compound X (25%), pentyl butyrate (36%), (Z)-3-hexenyl butyrate (30%), and hexyl acetate (70%) were reduced after molestation, whereas (E)-2-hexenal (237%) and 1-hexanol (234%) increased. However, of the minor constituents, only pentyl butyrate and (Z)-3-hexenyl butyrate were significantly less after discharge in both sexes (all $P < 0.01$).

Amounts of Volatiles and their Cross-Correlations

In addition to the three major volatiles presented earlier, six additional minor compounds were quantified in adults (8 d or older) from the maturation, IDP, and weight experiments

Fig. 2 Amounts of volatile compounds in relation to fresh body weight of 10-d-old adult male and female *L. hesperus* reared in the laboratory (August 2004, $N = 16$ each sex)



presented above to investigate biosynthetic aspects (Table 2). As adults aged, the ratios of the six minor components to the three major ones remained relatively constant (data not shown). No compounds were detected in 5-hr-old adults, but by 10 hr of age, (*E*)-2-hexenal, 1-hexanol, (*E*)-4-oxo-2-hexenal, hexyl acetate, (*Z*)-3-hexenyl butyrate, hexyl butyrate, and (*E*)-2-hexenyl butyrate were evident in small amounts. By 1 d of age, in addition to these compounds, males had pentyl butyrate and an unidentified compound X,

Table 1 Mean body weights and mean amounts of the major volatile components of laboratory and wild *L. hesperus* adults, and mean component amounts in methathoracic glands of laboratory adults

Source	Mean body weight (mg \pm SE)	(<i>E</i>)-4-Oxo-2-hexenal ($\mu\text{g} \pm$ SE)	Hexyl butyrate ($\mu\text{g} \pm$ SE)	(<i>E</i>)-2-Hexenyl butyrate ($\mu\text{g} \pm$ SE)
Laboratory male ($N = 16$)	7.09 \pm 0.12	1.15 \pm 0.21	7.84 \pm 0.90	0.40 \pm 0.05
Laboratory female ($N = 16$)	11.93 \pm 0.29	0.64 \pm 0.18	7.22 \pm 0.95	0.50 \pm 0.08
Wild male ($N = 10$)	7.45 \pm 0.23	0.41 \pm 0.19	5.16 \pm 1.17	0.47 \pm 0.13
Wild female ($N = 6$)	9.77 \pm 0.48	0.90 \pm 0.44	9.61 \pm 2.47	0.61 \pm 0.16
Male gland ($N = 3$)	–	2.62 \pm 1.06	5.83 \pm 1.47	0.56 \pm 0.17
Female gland ($N = 3$)	–	2.59 \pm 0.48	6.22 \pm 1.13	0.59 \pm 0.16

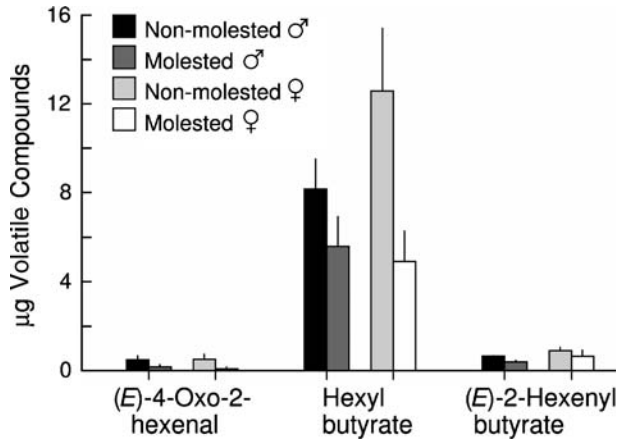
Table 2 Mean amounts of volatile chemicals in NG (\pm SE) found in male and female adults reared in the laboratory (listed in order of Gc retention times)

Chemical ^a	Maturation experiment 8- to 19-d-old adults		IDP experiment 10-d-old adults		Weight experiment 10-d-old adults		Grand total	
	Males (N = 21)	Females (N = 20)	Males (N = 8)	Females (N = 8)	Males (N = 16)	Females (N = 16)	Males (N = 45)	Females (N = 44)
(E)-2-Hexenal	79 \pm 39 ^{**b}	439 \pm 70	101 \pm 38	54 \pm 22	67 \pm 23	101 \pm 22	78 \pm 42 ^{**}	246 \pm 85
1-Hexanol	38 \pm 13 ^{**}	153 \pm 33	65 \pm 27	48 \pm 9	29 \pm 5	41 \pm 7	39 \pm 16 ^{**}	93 \pm 34
Compound X	19 \pm 2	18 \pm 4	45 \pm 10	43 \pm 15	57 \pm 7	44 \pm 9	37 \pm 8	32 \pm 10
(E)-4-Oxo-2-hexenal	5607 \pm 617	5146 \pm 1037	483 \pm 193	508 \pm 232	1154 \pm 215	644 \pm 181	3113 \pm 929	2666 \pm 1178
Hexyl acetate	4 \pm 1	6 \pm 2	3 \pm 1	12 \pm 5	3 \pm 1	5 \pm 2	3 \pm 1 [*]	7 \pm 3
Pentyl butyrate	5 \pm 1	6 \pm 1	7 \pm 1	14 \pm 4	6 \pm 1	6 \pm 1	6 \pm 1	8 \pm 2
(Z)-3-Hexenyl butyrate	13 \pm 1	17 \pm 2	18 \pm 3	26 \pm 4	14 \pm 1	14 \pm 2	14 \pm 2	17 \pm 3
Hexyl butyrate	13,034 \pm 1304 [*]	22,027 \pm 3612	8163 \pm 1342	12,577 \pm 2821	7845 \pm 897	7223 \pm 951	10323 \pm 1632 [*]	14925 \pm 4048
(E)-2-Hexenyl butyrate	753 \pm 121 ^{**}	1929 \pm 294	648 \pm 28	897 \pm 149	404 \pm 45	498 \pm 75	610 \pm 126 ^{**}	1221 \pm 343

^a Chemicals listed in order of retention time, 8, 8.8, 12.15, 12.5, 14.2, 17, 19.8, 20.05, and 20.15 min, respectively.

^b Asterisks in male columns indicate mean was significantly different from corresponding female mean (*t*-test, **P* < 0.05 or ***P* < 0.01).

Fig. 3 Mean amounts of volatile compounds in cohort groups of male and female *L. hesperus* after being molested (grasping a leg in forceps) to induce discharge of volatiles as detected by SPME, or not molested (control). Lines above bars represent +SE (bugs from laboratory colony, June 2004, $N = 8$ for each bar)



whereas by day 2, both sexes had all compounds in Table 2. A comparison of means of the nine compounds in adult males vs. females indicated no significant differences between the sexes for compound X, (*E*)-4-oxo-2-hexenal, pentyl butyrate, and (*Z*)-3-hexenyl butyrate. Significant differences were found for (*E*)-2-hexenal, 1-hexanol, hexyl acetate, hexyl butyrate, and (*E*)-2-hexenyl butyrate, with females having more of each of these compounds on average (Table 2).

The correlation matrices of nine compounds in the defensive secretion of males and females (Table 3) gave a higher number of significant correlations ($P < 0.05$) among 36 possible pairings for each sex than expected of a null hypothesis. Of these pairings, 12 were significant for males (33%) and 19 for females (53%), much higher than the expected two correlations for each sex based on a 5% level of significance. All of the significant correlations were positive, suggesting that many of the nine compounds were involved in biosynthesis of the defensive secretion. There were 25 corresponding correlations for males and females that were similar in regard to significance (69.4%) and 11 cases where the significance levels differed between the sexes. The two major butyrates and (*E*)-4-oxo-2-hexenal were significantly correlated between themselves in each sex. By pooling males and females, significant positive correlations were found between all possible comparisons of 1-hexanol, (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, hexyl butyrate, and (*E*)-2-hexenyl butyrate (data not shown).

Discussion

Wardle et al. (2003) suggested that the function of hexyl butyrate and other volatiles released by agitated *L. lineolaris* could be either as an alarm pheromone or as defensive allomones, but no experiments were carried out with predators. In the present study, alarm behavior was not elicited in nymphs or adults of *L. hesperus* when presented with conspecifics releasing defensive secretions. Alarm pheromones are likely to evolve only when related individuals live in aggregations, such as with social insects or aphid colonies (Byers, 2005). It is possible that unrelated individuals could exploit defensive emissions of others as a warning of predatory danger, but this apparently has not evolved in *L. hesperus*. I also exposed adults of *L. hesperus* to two native species of ants found in fields near infestations of the bug. The adult bugs attacked by ants released a volatile secretion composed mainly of hexyl butyrate, (*E*)-4-oxo-2-hexenal, and (*E*)-2-hexenyl butyrate that

Table 3 Correlation matrix of nine *L. hesperus* volatile compounds in each sex

Compounds ^a	(E)-2-Hexenal	1-Hexanol	Compound X	(E)-4-Oxo-2-hexenal	Hexyl acetate	Pentyl butyrate	(Z)-3-Hexenyl butyrate	Hexyl butyrate
Males								
1-Hexanol	0.66* ^b							
Unknown X	0.02	0.08						
(E)-4-Oxo-2-hexenal	-0.14 ^c	-0.09 ^c	-0.24					
Hexyl acetate	0.44* ^c	0.15	-0.04	0.04				
Pentyl butyrate	-0.18	-0.08	0.29 ^c	0.15	0.09			
(Z)-3-Hexenyl butyrate	-0.08 ^c	-0.16	0.40*	0.24 ^c	0.12	0.75*		
Hexyl butyrate	-0.04 ^c	0.09 ^c	-0.00	0.81*	0.15	0.50*	0.55*	
(E)-2-Hexenyl butyrate	0.48*	0.44*	-0.08	0.47*	0.33* ^c	0.21 ^c	0.18 ^c	0.60*
Females								
1-Hexanol	0.47*							
Unknown X	-0.02	-0.11						
(E)-4-Oxo-2-hexenal	0.80* ^c	0.48* ^c	0.10					
Hexyl acetate	0.03 ^c	-0.03	0.20	0.20				
Pentyl butyrate	0.13	-0.03	0.62* ^c	0.23	0.07			
(Z)-3-Hexenyl butyrate	0.36* ^c	0.07	0.58*	0.44* ^c	0.22	0.87*		
Hexyl butyrate	0.75* ^c	0.44* ^c	0.21	0.85*	0.09	0.45*	0.67*	
(E)-2-Hexenyl butyrate	0.87*	0.35*	0.04	0.74*	0.05 ^c	0.33* ^c	0.52* ^c	0.80*

^a $N = 45$ males, 44 females.

^b Correlations with an asterisk are significant at $P < 0.05$.

^c Sexes were different in terms of significance.

apparently caused agitated movements and avoidance by the predator. These reactions were probably the result of direct contact with the secretion because application of hexyl butyrate (at high doses) elicited violent reactions, whereas ants showed no behavioral changes or toxicological effects during 8-hr exposure to saturated vapors of the compound. Stink bugs (Heteroptera: Pentatomidae) release similar volatile compounds such as (E)-2-decenyl acetate and (E)-2-decenal that deter feeding by birds and lizards (Krall et al., 1999).

Wardle et al. (2003) collected volatiles from disturbed *L. lineolaris* females for 1 hr on Porapak Q and determined that they released 23.6 μg of hexyl butyrate, 8 μg of (E)-2-hexenyl butyrate, 1.8 μg of (E)-4-oxo-2-hexenal, 0.7 μg of (E)-2-hexenal, and 0.4 μg of 1-

hexanol per bug-hour (100%, 34%, 7.5%, 3.2%, 1.7%, respectively). These compounds were also released in the present study from *L. hesperus*, but because the methods of the two studies differ, the compound ratios are not comparable. In this study, SPME exposure for 2 min was used to collect compounds in the vapor phase in which the more volatile (*E*)-hexenal, 1-hexanol, and (*E*)-4-oxo-2-hexenal should have volatilized more completely from the insect surface in the first few minutes after discharge than less volatile components such as hexyl butyrate and (*E*)-2-hexenyl butyrate. Thus, it seems more appropriate to compare the extracted amounts of females in this study (100%, 8%, 18%, 2%, 1%, respectively; Table 2) and assume they are discharged in the same proportions as would be collected by absorbent over a 1-hr period. Ho and Millar (2002) found all of the major and minor compounds (Aldrich et al., 1988) in aerations of macerated females as those presented here but in somewhat different ratios (100%, 4.5%, 1%, 10%, and 10%), possibly caused by degradation of some components during the 22-hr aerations. They also showed that heptyl butyrate placed on bugs was hydrolyzed to 1-heptanol. Significant positive correlations among many of the defensive components (Table 3) suggest that they are involved in biosynthesis and hydrolysis in an equilibrium that could be altered during aeration of macerated bugs (Ho and Millar, 2002) or even during solvent extractions if the bug remains to provide catalytic sites. The present experiments show that quantification is optimized by immediate decanting of solvent from the macerated bug followed by analysis within 2 d.

The most significant difference between studies was the low percentage of (*E*)-4-oxo-2-hexenal (1.2% in females and 0.05% in males) found by Ho and Millar (2002) compared with the 18% and 30% in the present study (Table 2). Also, they did not detect this compound in pentane extracts of metathoracic glands, rather only hexyl butyrate, (*E*)-2-hexenyl butyrate, (*E*)-2-hexenal, and 1-hexanol. However, in gland extracts of both sexes, the author found large amounts (0.5 to 6 μg) of (*E*)-4-oxo-2-hexenal, hexyl butyrate, and (*E*)-2-hexenyl butyrate with small amounts (4 to 30 ng) of 1-hexanol, compound X, pentyl butyrate, and (*Z*)-3-hexenyl butyrate. Earlier, Aldrich et al. (1988) found small amounts of (*E*)-4-oxo-2-hexenal in female metathoracic glands but detected none in males. Again, the most probable explanation for the discrepancy is that (*E*)-4-oxo-2-hexenal breaks down rapidly in solvent when the macerated gland or whole bug is present. Therefore, (*E*)-4-oxo-2-hexenal seems to be more important to the blend of defensive components than suggested from earlier data (Aldrich et al., 1988; Ho and Millar, 2002). Studies of semiochemical identification could miss important compounds if catalytic sites in the biological material remain active in solvent to alter chemical structures before analyses are carried out. Conversely, generation of artifacts through prolonged exposure of extracts to tissues, such as the production of gamma-caprolactone in this study, may complicate analyses.

Ho and Millar (2002) did not ascribe any biological function to the volatiles released by *L. hesperus* other than to suggest that one or more could serve as components of a sex or aggregation pheromone. However, they did not find any differences between the sexes that would suggest sex pheromone components nor were any two-way blends attractive in the field. The present study did not find any significant differences between the sexes either, but does demonstrate the defensive role of the secretions. The volatile components in recently eclosed adults are negligible but then increase with age, in agreement with results for hexyl butyrate in Ho and Millar (2002, their Fig. 1). Several studies (Scales, 1968; Strong et al., 1970; Graham, 1987, 1988; McLaughlin, 1996) provided convincing evidence that virgin females are attractive to males, but identification of *L. hesperus* sex pheromone components has so far been elusive for a number of possible reasons as discussed in Ho and Millar (2002).

The quantities of the hexyl and (*E*)-2-hexenyl butyrates and (*E*)-4-oxo-2-hexenal increased with an increase in weight of laboratory-reared females (Fig. 2). These 10-d-old females had mated with males and were producing eggs as observed during maceration in solvent. In contrast, male size did not appear to affect compound amounts (Fig. 2). It is unknown why young adults contain no defensive allomones for about 1–2 d after molting (Fig. 1) and, thus, are more vulnerable to predators than adults. Resources are probably needed more for maturation processes during this time than for biosynthesis of allomones.

Relatively few studies have reported increased semiochemical content as a function of body weight or size. Amounts of aggregation pheromone components of a bark beetle (Birgersson et al., 1988) and male butterfly courtship pheromone components (Sappington and Taylor, 1990) were not correlated with weight or size (0–3% of variation explained). Pureswaran and Borden (2003) found no relationship between body size and aggregation pheromone components of mountain pine beetles but did find a positive relationship for antiaggregation components. Byers (2005) found that as first instar to adult cotton aphid weights increased over a 60-fold range, the amounts of alarm pheromone, (*E*)- β -farnesene, found in all stages were positively related (66% of the variation in pheromone amounts explained by increases in weight). *Lygus hesperus* produces microgram quantities of defensive allomones compared with nanogram amounts of pheromones in bark beetles and aphids (Birgersson et al., 1988; Pureswaran and Borden, 2003; Byers, 2005). Thus, results for *L. hesperus* suggest that in insects producing large amounts of defensive allomones, the costs can be enough to manifest a positive relationship with size even in adults that vary less in weight compared with all developmental stages (e.g., aphids).

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Keywords Chemical defense · Allomones · *Mantella* · Chemical ecology · Temporal variation · Geographic variation · Habitat disturbance · Ant alkaloids · Alkaloid profiles · Poison frogs

Abbreviations

TAS	transcutaneous amphibian stimulator
Ampa	Ampasimpotsy
Vato	Vatoharanana
Saha	Sahavondrona
Vohi	Vohiparara
AMNH	American Museum of Natural History
UADAB	University of Antananarivo Department of Animal Biology
RA	relative abundance in EI mode
SVL	snout–vent length

Introduction

Frogs that are able to sequester alkaloids into skin from arthropod prey possess an important evolutionary advantage; they not only afford themselves chemical protection without expending energy to produce such defensive compounds, but also exploit a nutrition niche that other species may reject as bitterly distasteful and dangerous (dietary data not shown). Such ingested alkaloids are known nematocides, insecticides (including antimosquito), and neurotoxins, and provide these frogs with protection against a wide range of possible foes (Daly et al., 1999, 2005 and references therein). More than 800 lipophilic alkaloids from 24 classes, designated in boldface by molecular weight and distinguishing letter (e.g., **251O**; Fig. 1) (Daly et al., 2005), have been documented in certain frog genera from five families. Most genera within these families do not have alkaloids, and thus the traits for alkaloid sequestration and storage in skin glands, along with specialization on ants, has likely arisen multiple times in evolution (Clark et al., 2005 and references therein).

Such poison frogs include *Mantella* of Madagascar (Mantellidae), *Dendrobates*, *Epipedobates*, and *Phyllobates* of Central and South America (Dendrobatidae), *Melanophryniscus* of South America (Bufonidae), *Pseudophyrne* and, to some extent, *Geocrinia* of Australia (Myobatrachidae; see Smith et al., 2002), and to a lesser extent, one *Limnonectes* species of Thailand (Ranidae; see Daly et al., 2004, 2005). Multiple feeding experiments with certain captive-born poison frogs revealed that: (1) dietary alkaloids are usually sequestered unchanged; (2) some chemical modifications of ingested alkaloids exist; and (3) sequestered alkaloids are retained in frogs' skin glands for several years (Smith et al., 2002; Daly et al., 1997, 2003). Many of these "poison frog alkaloids" are also known from tropical and/or temperate ants, mites (Takada et al., 2005), millipedes, beetles, and plants (reviewed in Eisner et al., 1978; King and Meinwald, 1996; Leclercq et al., 2000; Daly et al., 2005; Laurent et al., 2005). In Madagascar, a siphonotid millipede and three ant subfamilies, some of which were confirmed frog prey, contained alkaloids in common with sympatric *Mantella* (Clark et al., 2005); however, putative sources of some of these same alkaloids occur in different ant subfamilies in the Neotropics (see Jones et al., 1999). Further related research on occurrence and pharmacology of alkaloids in poison frogs and their prey is reviewed in Daly et al. (1999, 2005), Saporito et al. (2004), Clark et al. (2005) and Daly (2005).

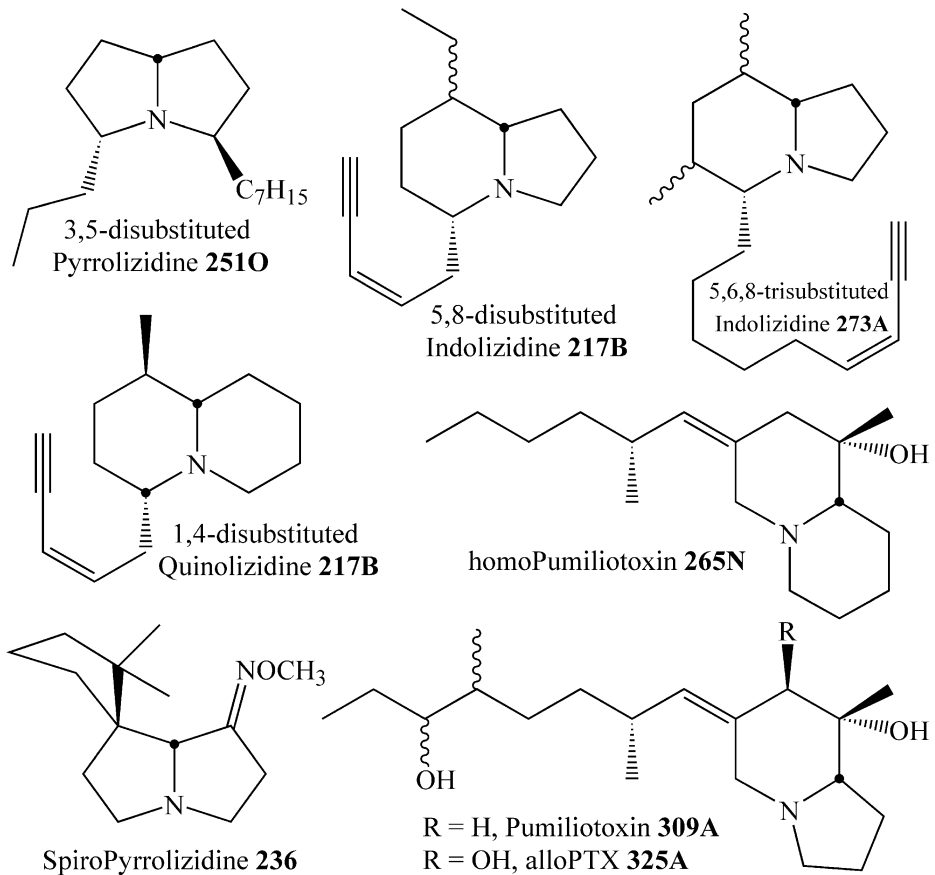


Fig. 1 Representative structures of select minor and major alkaloids detected in *Mantella* frogs of the Ranomafana region, suspected or confirmed to be of arthropod origin

Daly (1982; see Table 22) and his colleagues (Myers and Daly, 1980; Daly et al., 1990, 1992, 1994a, b, 1996, 2002) have demonstrated that the alkaloid content of pooled frog skins vary by population/locality. Myers et al. (1995) reported variability between frogs, with two individual *Dendrobates granuliferus* skins at each of three locales in Costa Rica; also, at one of these locales two individual and one 30-skin samples of microsympatric *D. pumilio* tentatively revealed that the two species are similar in alkaloid content. Saporito et al. (2006) also discuss variation in alkaloids recovered from 70 individual *D. pumilio* skins. Mebs et al. (2005) made methanolic extracts from 81 individual *Melanophryniscus montevidensis* toads from Uruguay, to reveal by quantitative gas chromatography-mass spectrometry (GC-MS) that there was variation among individuals and populations in the amount of both pumiliotoxin **251D** and the nonalkaloid phenolic hydroquinone.

We previously reported that within a locality, sympatric individuals of two *Mantella* species had similar alkaloid profiles (see supporting information online and Table 1 in Clark et al. 2005). Here, we explore patterns of variation for >80 alkaloids detected in individual *Mantella* poison frogs. Individual variation within or among poison frog species

of a region may reveal important aspects of community ecology; thus, we report the detailed alkaloid content (profiles) of 22 individual *Mantella* frogs of three species sampled from four localities in the Ranomafana region of southeastern Madagascar (Fig. 2), alongside data on frog size and “snapshot” prey consumption. Alkaloid profiles from *M. baroni* collected at two of these localities in 1989 and 1993 are compared to frogs from the 2003 expedition.

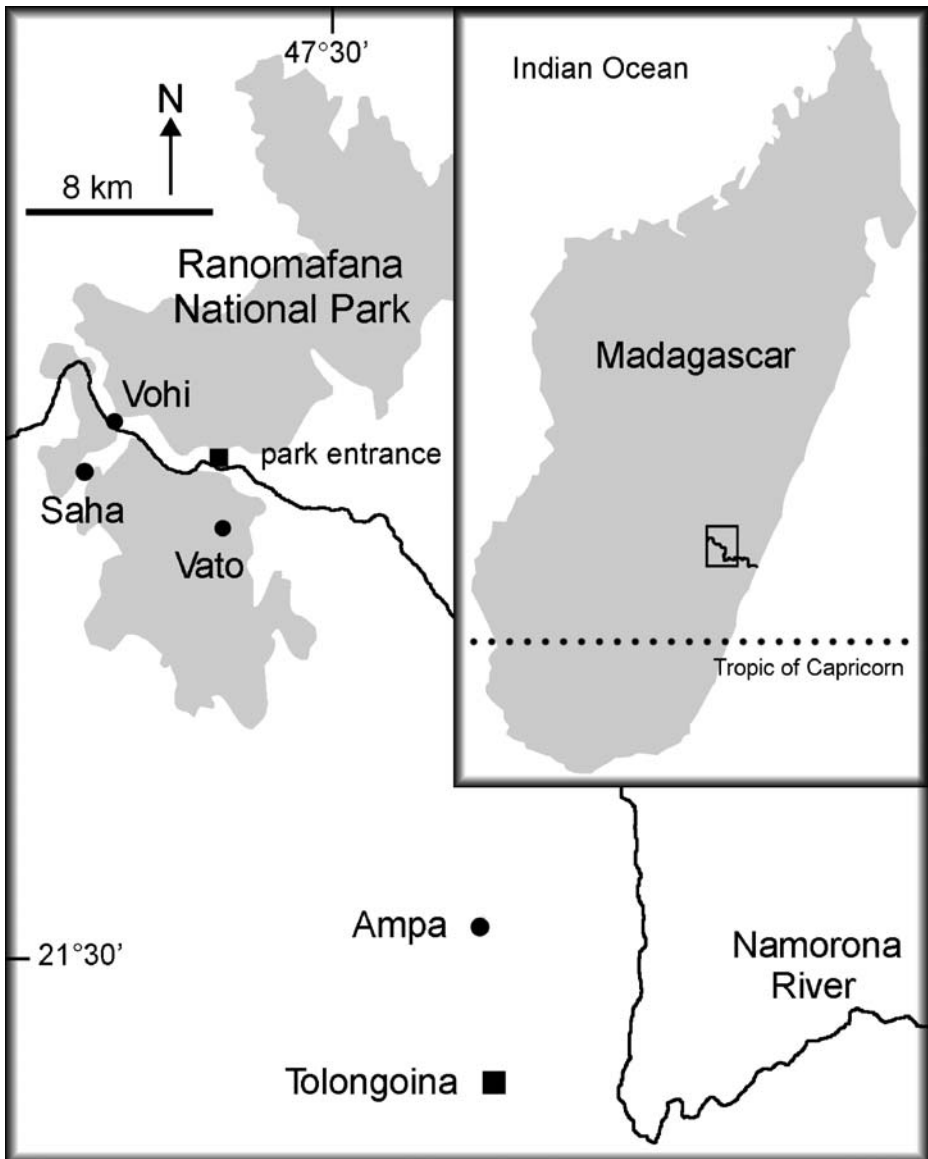


Fig. 2 *Mantella* individuals were sampled in and near Ranomafana National Park at riparian locales indicated by solid circles (after map in Bradt, 1999)

Methods and Materials

Field Collections

Materials and methods are described in detail in Clark et al. (2005). Briefly, riparian *Mantella madagascariensis*, *M. baroni*, and *M. bernhardi* were collected in March and April 2003 at localities in moist forests of the Ranomafana region in southeastern Madagascar (Fig. 2). Localities included Ampasimpotsy (Ampa, disturbed forest fragments, 550 m; 21° 28.796'S, 47°33.424'E), Vatoharanana (Vato, old-growth primary forest, 1100 m; 21°17.444' S, 47°25.569'E), Sahavondrona (Saha, disturbed roadside forest, 1100 m; 21°15.450'S, 47° 21.609'E), and both sides of the Kidonavo stream at Vohiparara (Vohi 1 and 2, disturbed roadside forest, 1100 m; 21°13.587'S, 47°22.193'E). Ampa is 25–34 km away from the other three localities, which are clustered 4–9 km from one another within Ranomafana National Park (Fig. 2). In addition, temporal variations in the alkaloid content of *M. baroni* were assessed with samples collected at Vato (December 1989, 10 combined skins) and Saha (January 1993, 17 combined skins); these two samples were provided by John W. Daly (National Institutes of Health), and previously described in Daly et al. (1996).

Sample Preparation and Analyses by Gas Chromatography-Mass Spectrometry

Eighteen *Mantella* frogs were skinned into methanol, and alkaloid fractions were made following the methods of Daly et al. (1994b). These and other frog voucher specimens were deposited and the stomach contents removed at the American Museum of Natural History (AMNH, A168355–A168393) and the University of Antananarivo, Department of Animal Biology (UADAB #1–8). Additional exudates were collected from four live frogs by using a TAS (purchased from Jacqueline Grant, and described in Grant and Land, 2002) and a methanol-laced Kim-wipe; these frogs were rinsed in water and released unharmed. Skin alkaloid fractions and TAS extracts were individually analyzed for alkaloids on a Micromass™ GC-time of flight (TOF)-MS in CI (with 100% NH₃) and EI modes with a ramp of 10°C/min from 100 to 280°C, with oven maintained at 100°C for the first 5 min and at 280°C for the final 5 min. The MS libraries of Daly et al. (1999, 2005) were used to identify over 80 known alkaloids in *Mantella* sampled at Ranomafana in 2003, and the two *M. baroni* standards of Daly et al. (1996) provided retention times of known alkaloids for comparison. Based only upon their relative abundance (RA) in EI mode, these alkaloids are reported in Table 1 as trace (RA < 10%), minor (10% < RA < 70%), or major alkaloids (RA > 70%; see chromatograms in Supplementary Information); although not absolutely quantitative, any possible ionization biases should be consistent among all samples tested.

Statistical Analyses

Statistics were performed with SPSS v. 13. A *t*-test was used to compare the mean alkaloid diversity of Vohi *M. baroni* sampled by TAS vs. full-skin extraction, and to compare means of both frog size [snout–vent length (SVL)] and number of prey between *M. bernhardi* and *M. baroni*. A linear regression was used to evaluate the relationship between frog size and number of prey within the Vohi locality. A *partial correlation* for all *Mantella*,

Table 1 Alkaloids detected in *Mantella* frog individuals^a of the Ranomafana region, southeastern Madagascar

Locality	<i>M. baroni</i>										<i>M. bernhardi</i>							Family ^b					
	<i>M. md.</i>					<i>M. baroni</i>					<i>M. bernhardi</i>												
Alkaloid class	MW code	U1	U2	82	L2	L3	L4	U3	U4	U5	U6	D ^a	87	88	89	90	91	92	93	U8	U7	Daly et al. ^b	
Polyzomanine	151B	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	M, D
Unclass	155	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D
Nicotine	162	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	M, D
Precococinelline	193C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	M, D, B
3,5-I	195B	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D, B
Pyr	197B	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	D
Pip	197E	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	D
5,8-I	203A	-	-	-	-	-	-	-	⊕	-	-	+	+	+	-	-	-	-	-	-	-	-	M, D
5,8-I	205A	-	-	-	-	-	-	⊕	⊕	-	-	+	+	+	-	-	-	-	-	-	-	-	M, D
5,8-I	207A	-	-	-	-	-	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	M, D, B
Tricyclic	207J	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	M, D
Unclass	207N	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D
5,8-I	209B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D
5,8-I	209I	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	M, D
1,4-Q	217A	-	⊕	⊕	+	⊕	+	⊕	⊕	+	+	⊗	⊗	⊗	⊗	+	⊕	-	⊕	⊕	⊕	⊕	M
5,8-I	217B	+	⊕	⊕	+	+	+	⊕	⊕	⊕	⊕	⊗	⊗	⊗	⊗	+	-	+	⊕	⊕	⊕	⊕	M, D
5,8-I	217B'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	⊗	⊕	⊕	M, D?
5,8-I	217B''	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D?
5,8-I	219F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	⊕	⊕	⊕	⊕	⊕	M, D
5,8-I	219L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M
5,8-I	221I	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	⊕	⊕	⊕	⊕	⊕	M
5,8-I	221I'	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	×
SpiroP	222	-	-	-	-	-	-	-	⊕	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D
3,5-P	223B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	⊕	⊕	⊕	⊕	⊕	M, D, B
Izidine	223C	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	M, D
3,5-P	223H	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	M, D, B

Table 1 (continued)

Locality	<i>M. baroni</i>										<i>M. bernhardi</i>							Family ^b									
	Vohiparara 1					Vohiparara 2					<i>M. md.</i>		<i>M. baroni</i>						<i>M. bernhardi</i>								
Alkaloid class	MW	code	U1	U2	82	L2	L3	L4	U3	83	U4	D ^a	84	57	U5	U6	D ^a	87	88	89	90	91	92	93	U8	U7	Daly et al. ^b
SpiroP	252A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D
Izidine	255B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	⊕	-	-	-	-	-	M, D
1,4-Q	257D	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	⊕	⊕	-	-	-	-	-	-	M, D
Tricyclic	261C	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M
Unclass	265F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M
hPTX	265N	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗	+	-	⊕	-	-	-	-	-	-	-	-	-	-	M, D, B
PTX	267C	-	-	-	-	-	-	-	-	-	⊗	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D, B, Myo
Unclass	271B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	M, D
3,5-I	271F	-	-	-	-	-	-	-	-	-	-	-	⊕	-	-	-	-	-	-	-	-	-	-	-	-	-	M
5,6,8-I	273A	⊗	⊗	⊗	⊗	⊕	⊕	⊕	⊕	⊕	-	-	⊕	+	-	+	-	-	-	-	-	-	-	-	-	-	M
5,6,8-I	273A'	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	M
3,5-I	275C	+	+	+	+	⊕	⊕	⊕	⊕	⊕	+	+	+	+	-	-	-	+	⊕	⊕	⊕	-	-	-	-	-	M, D
3,5-I	275C'	+	⊕	⊕	⊕	⊕	⊗	⊗	⊗	⊕	⊕	⊕	⊕	⊕	+	-	-	⊕	⊕	⊕	-	-	-	-	-	-	M, D
5,6,8-I	275E	-	-	-	-	-	-	-	-	⊕	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D
DeoxyPTX	281G	-	-	-	-	-	-	-	-	-	-	-	⊕	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D
DeoxyPTX	291E	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	⊕	+	-	-	-	-	-	-	-	M
PTX	291G	-	-	-	-	-	-	-	-	⊕	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D, B
DeoxyPTX	293B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	M
5,6,8-I	293C	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M, D
DeoxyPTX	293D	⊕	⊕	+	-	-	-	-	-	-	-	-	⊕	-	-	-	-	+	+	⊕	-	-	-	-	-	-	M
PTX	305B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M
PTX A	307A	-	-	-	-	-	-	-	-	-	-	-	⊗	-	-	-	-	+	-	-	-	-	-	-	-	-	M, D
PTX	307D	-	-	-	-	-	-	-	-	-	⊕	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D
PTX	307E''	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	M, D
PTX	307E'''	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	⊕	⊕	-	-	-	-	-	-	M, D
PTX	307G	⊕	⊗	⊗	⊗	-	-	+	⊕	⊗	+	+	+	⊕	⊕	⊕	⊕	⊗	⊕	⊕	-	-	-	-	-	-	M, D

controlled for by locality, was used to evaluate the relationship of SVL with both the number of prey and individual frog skin-alkaloid diversity. One-way analysis of variance (ANOVA) was used to compare mean alkaloid diversity per frog among all four localities, among the three localities of constant (1100 m) elevation, and between frogs of the two elevation groups. *K*-Means cluster analysis was performed on 22 *Mantella* frogs by using all alkaloids requesting four clusters, and was followed by a cross-tabulation between the cluster membership and the localities.

Results and Discussion

Variation in Alkaloid Content of Individual Frogs, within and among Localities

Cluster analysis revealed that the three *M. baroni* and 1989 standard from Vato represent a distinct group based on the specific alkaloid content of the resident individual frogs (see Cluster groups at bottom of Table 1), indicating that frogs within a locality are more similar to one another compared to frogs from different localities. Seven of nine frogs from both sides of the Kidonavo stream at Vohi clustered in another group of individuals, indicating little variation at this fine spatial scale (Table 1). The other two Vohi frogs clustered in a third unique group, demonstrating little difference in alkaloid profiles of microsympatric *Mantella* species. Frog individuals from the two most disturbed locales clustered exclusively in another group, including all four frogs from moderately disturbed Saha and all six frogs from the disturbed forest fragments of Ampa; these two localities also had the lowest mean number of alkaloids per frog and the six individuals with the fewest alkaloids (Table 1).

Out of the 91 alkaloids in Table 1, 26 were detected in only a single frog individual, 16 were detected in multiple frogs from a single locality, and 11 were detected in multiple individuals from two localities. Vohi 1 and 2 are separated only by the 2- to 4-m-wide Kidonavo stream, and frogs from these sites contained many alkaloids in common (e.g., **207N**, **251D**, **265N**, **273A**); 21 alkaloids were present on both sides of the Kidonavo stream, and the other 38 were only detected in frogs from either Vohi 1 or Vohi 2 (Table 1; of these 38 alkaloids, 25 were found only in a single frog). Of the 91 alkaloids, 26 were detected in only one of the various 22 frogs sampled, and may represent rare prey. The fact that Vohi samples contain 25 of these 26 unique (in one of 22 frogs) alkaloids suggests this intermediately disturbed site may have an elevated number of rare arthropod-source species. Alkaloids appearing in more than one frog that are limited to one locality include: **217B** and **223B** at Ampa; **151B**, **195B**, **207N**, **251R**, and **275E** at Vohi; **273A'** at Saha; and **207J**, **209I**, **223C**, **223M**, **243D**, **255B**, **307F''**, and **307F'''** at Vato. These results also suggest that frogs within a locality are more similar in terms of alkaloid content compared to frogs from different localities. Alkaloids that appear in the majority of frogs from only two localities in 2003 include **203A**, **205A**, **207A**, **219F**, **221I**, **223H''**, **241F**, **245C**, **265N**, **291E**, and **293D** (Table 1).

The distribution and amount of particular alkaloids in individual *Mantella* frogs should reflect how abundant the leaf-litter source arthropod is in the environment. Although frog sequestration efficiency varies among alkaloid classes (see Daly et al., 2003), such sequestration biases should be constant among *Mantella* sampled in this study. For example, alkaloids such as **251O** and **217B** were found in all or most of the 22 individuals (Table 1, Fig. 1); these alkaloids are likely common in frogs because the source arthropod is particularly abundant in the region, so it was not surprising that the endemic ant source species for these common alkaloids was discovered (Clark et al., 2005). As-yet-undetermined arthropod sources for **217A**, **233A**, and **325A** are also likely widely distrib-

uted across the entire region, whereas sources of **231A**, **275C**, and **307G** appear limited to localities within Ranomafana Park boundaries (see Table 1). Ampa is relatively far from the three 1100-m localities and has an elevation of only 550 m. This suggests that the arthropod sources of **231A**, **275C**, and **307G** could be limited to higher elevations. In fact, previous work indicates ant species assemblages and diversity do vary with elevation (Fisher, 1998).

Ants were the most numerous taxon in *Mantella* stomach samples (>67%), and individuals of the same ant species were often collected in the same stomach sample (Table 4 of Clark et al., 2005; unpublished data of V.C.C. and B.L.F.). This is not surprising, because ants are colonial and locally abundant. Based on this data, and the fact that most of the more commonly distributed and “major/minor” alkaloid classes reported here are known from some ant source, the majority of common “minor/major” alkaloids in Table 1 are suspected to be sequestered from dietary ants. The cooccurrence in most frogs of related pumiliotoxin alkaloids **309A** and **325A** (see Fig. 1, Supplementary Information, and Table 1) suggests a possible common alkaloid-source-ant/symbiotic mite for these two alkaloids. Alternatively, *Mantella* could possibly have a hydroxylase to convert **309A** into **325A**, as evidence for frog hydroxylases has been suggested by alkaloid-feeding experiments with captive-born *Dendrobates* spp. (Daly et al., 2003) and *Pseudophryne* spp. (Smith et al., 2002). In one *Mantella* individual, SpiroP **236'** was considerably more abundant than **236** (see Supplementary Information chromatogram for frog #U4), similar to the relative abundance of these spiropyrolizidines reported for the putative source millipede of Clark et al. (2005).

Frog Size and Prey Consumption

M. baroni in this study had an average SVL of 25.5 ± 2.2 mm ($N = 15$), with a range of 22–30 mm identical to a report by Vences et al. (1999) (Table 1). *M. bernhardi* SVLs ranged from 19 to 22 mm in Vences et al. (1999); however, the seven specimens in this study ranged from 17 to 22 mm with an average SVL of 19.9 ± 2.0 mm. *M. baroni* are significantly larger than *M. bernhardi* (t -test, $P < 0.001$), and based on the number of prey per preserved dissected stomach, the larger *Mantella* species consumes more prey (34.7 ± 14.8 prey/frog for 12 *M. baroni* vs. 16.6 ± 11.4 prey/frog for 7 *M. bernhardi*; t -test, $P = 0.009$, from Table 1 data). Furthermore, when performing a regression on one species within a locality, we find a linear relationship for *M. baroni* from Vohi, indicating that larger frogs consumed more prey (slope = 4.949, $P = 0.031$, $R^2 = 0.832$). A partial correlation for the 22 *Mantella*, controlled for by locality, also revealed that frog SVL is positively correlated with both the number of prey (correlation = 0.642, $P = 0.004$) and the total number of alkaloids per frog (correlation = 0.525, $P = 0.025$).

All dietary data represent a “snapshot” of what each specimen ate in the morning of its collection—frogs were preserved within 30 min of capture. In addition to consuming more, and thus probably a greater diversity of prey, it is also likely that some larger prey (that might contain unique alkaloids) are only consumed by larger frogs. Of the 609 *Mantella* stomach arthropods identified by Clark et al. (2005), the two largest prey items were recovered from *M. baroni* (data not shown).

Frog Alkaloid Diversity and Habitat Disturbance

The lower alkaloid diversity (average of 12.0 alkaloids/frog) at Ampa as compared to any other locality could merely be a reflection of the reduced arthropod consumption observed in this smaller species (Table 1, see above). An alternative explanation is suggested by the

notable link between frog alkaloid diversity and observed forest disturbance. The pristine primary forest of Vato is located within Ranomafana Park boundaries and contained *Mantella* with an average of 28.7 known alkaloids per frog (Table 1). Secondary roadside forest localities at the edges of Park boundaries yielded frogs with an intermediate diversity of skin alkaloids (average of 14.5 and 20.2 alkaloids/frog at Saha and at Vohi, respectively). The mean number of alkaloids per frog (mean frog alkaloid diversity) differed: (1) among the three localities of 1100-m elevation (ANOVA, $F = 6.304$, $P = 0.012$, $Eta^2 = 0.492$); (2) more significantly among all four localities (ANOVA, $F = 10.133$, $P < 0.001$, $Eta^2 = 0.628$); and (3) between the two elevation groups, whether (ANOVA, $F = 8.388$, $P = 0.009$, $Eta^2 = 0.295$, $n = 22$) or not (ANOVA, $F = 7.547$, $P = 0.013$, $Eta^2 = 0.284$, $n = 21$) the *M. madagascariensis* data point was included in the analysis. The Eta^2 value suggests that locality differences account for 62.8% of the observed variation in mean frog alkaloid diversity among the four localities.

Fragmented Ampa is clearly the most disturbed forest, with fragments surrounded by rice fields and other agriculture (VCC personal observation; also Rabemananjara et al., 2005). Only 20 different known alkaloids were detected in the six frogs from Ampa, as compared to more than 28 different alkaloids at each of the other localities; this is particularly striking since fewer frogs were sampled at most other localities (Table 1). Fragmentation could lower diversity of alkaloid-source arthropods by eliminating critical microhabitats. Invasive ants can also lower insect diversity in fragmented lowland forests (Fisher et al., 1998; Fisher, 2005). Although confounded by distance, variation in elevation, and frog size/species, this pattern suggests that future conservation research should aim to understand the impact of forest fragmentation on ant assemblages, as invasive ants might decrease the variety of alkaloid-source arthropods available to *Mantella* in Madagascar. Such a scenario could also be imagined for unrelated poison frogs of convergent ecosystems on other continents.

Temporal Variation in Alkaloid Content at Two Localities

Of the 30 alkaloids we detected in the January 1993 Saha 17-skin sample, 15 were absent from the 2003 Saha frogs and four of these were absent from all of the 2003 frog samples (Table 1). Similarly, on average 40% of alkaloids detected in each of the other three localities had been detected in the 1993 Saha sample; 1993 Saha shows little more resemblance to 2003 Saha (15/30 alkaloids) than to Vato (16/33), Vohi (22/59), or Ampa (7/20) frogs in 2003, and 1993 Saha even grouped with the Vohi frogs in the *K*-means cluster analysis (Table 1). In contrast, of the 27 alkaloids we detected in the December 1989 Vato 10-skin sample, only five were absent from Vato and three of these were absent from samples representing the entire region 14 yr later; 11 alkaloids were detected in 2003 Vato but not in 1989 Vato frogs. A comparison of 1989 Vato to different 2003 localities revealed that 1989 Vato shares more alkaloids (66%) with 2003 Vato than with any other locality—only about 33% of 1989—Vato alkaloids were detected in frogs from each of the other localities (17/59 at Vohi, 10/28 at Saha, and 7/20 at Ampa; see Table 1).

Alkaloids not reported in Daly et al. (1996) that are detected here include **151B**, **197E**, **217A**, **223H**, **233A**, **251N**, **251O**, **291E**, **293D**, **307G**, and **323E** in the 1993 Saha sample, and **151B**, **197B**, **251O**, **255B**, **257D**, **271B**, **275C**, **291E**, **293D**, **307A**, **309C**, **323E**, and **325A** in the 1989 Vato sample; these differences are likely a result of the greater sensitivity of the GC-TOF-MS used in this study as compared to the quadrupole GC-MS used in 1996.

Frogs retain their alkaloids for several years in captivity (Daly et al., 1997, 2003 and references therein), so it seems unlikely that the observed temporal variation was influenced

by seasonal variation in time of collection (December/January, the start of the rainy season, vs. March/April, the end of the rainy season). Rather, these results might indicate that the arthropod assemblage was relatively constant in the undisturbed primary forest of Vato, whereas disturbed roadside Saha had undergone a shift in the arthropod community. Disturbance and fragmentation of habitat could decrease the availability of alkaloid-containing prey and pose a possible threat to *Mantella* survivorship. This point is further supported by the observation that the two frog individuals with highest skin-alkaloid diversity both came from pristine Vato, and the six frog individuals with the lowest skin-alkaloid diversity were from disturbed roadside Saha and disturbed Ampa forest fragments (Table 1). A potential threshold diversity for alkaloid-containing arthropods required to maintain wild populations of poison frogs has not yet been determined.

Nonlethal Collection of Frog Skin Alkaloids with the TAS

There is no significant difference in the mean number of frog skin alkaloids detected using a nonlethal TAS vs. the traditional method (Daly et al., 1994b) of lethal full-skin extraction (*t*-test, $P > 0.05$, CI contains zero, for eight *M. baroni* of Vohi). The single TAS frog sample acquired at Saha contained more detectable known alkaloids than two skin samples and fewer alkaloids than one skin sample from Saha. However, quantitative detection by a flame-ionization GC-MS revealed that only 25% of the amount of each alkaloid is recovered with the TAS as compared to full-skin extraction (John W. Daly, personal communication). Based on our results, future investigations on frog alkaloid profiles should rely on the nonlethal TAS, especially when sampling endangered amphibians (listed on CITES appendices). The TAS is particularly useful for obtaining defensive compounds from frogs as it elicits a predator response in the frogs. The resulting TAS extract requires no further chemical workup, yet yields clean gas chromatograms consisting primarily of alkaloids.

Conclusions

Our data suggest that frog alkaloid profile variation is attributable to differences in arthropod assemblages among localities, and as such may give an indication of local ecosystem health. Specifically, variation in frog skin alkaloids appeared: (1) greatest among frog individuals of more geographically distant localities that varied in elevation and size of resident species; (2) lower among individuals from geographically close localities at the same elevation; and (3) lowest among *Mantella* individuals within a locality. In a comparison of frog alkaloid samples over a 10- or 14-yr period, alkaloid turnover, and thus presumably alkaloid-source arthropod turnover, was high in a disturbed locality and low in the pristine primary forest locality.

As all alkaloids in Malagasy poison frogs appear to be obtained from their diet, the local distribution of alkaloid-containing arthropods is likely key to variations in frog skin-alkaloid content among and within localities. Also, larger frog species consume more and larger prey and this increased arthropod sampling likely contributes to greater alkaloid diversity observed in frog skin. Further ecological studies on variation in poison frog alkaloids should use the TAS to evaluate in greater detail factors that might affect alkaloid diversity, such as habitat disturbance and elevation, frog gender, and especially frog age/size. Greater numbers of sympatric *Mantella* species should also be analyzed for variation in their defensive skin chemistry; only one *M. madagascariensis* was compared to several sympatric *M. baroni* (Clark et al., 2005; current study).

It appears that the alkaloid content of Malagasy poison frogs is most affected by locality, and that poison frogs continue to accumulate alkaloids as they age. This poison collection may reach some maximum diversity dependent on the assemblage of alkaloid-source species in the frogs' local habitat and/or individual territories. Because the alkaloids sequestered by frogs vary considerably in toxicity, it is to the frogs' advantage to acquire as many different defensive chemicals as possible. Accordingly, frogs living in particularly arthropod-rich habitats could obtain more dietary alkaloids.

Habitat quality and local arthropod diversity appear to affect how well poison frogs are chemically defended. Therefore, habitat disturbance and fragmentation might decrease *Mantella* survivorship or even exclude the poison frogs altogether *via* selective pressures. Future studies need to address this relationship and determine the threshold of alkaloid-containing arthropods required in habitat suitable for *Mantella* poison frogs.

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Introduction

Tannins are commonly considered dominant defensive compounds in the leaves of many tree species. Temperate deciduous trees, for example, produce tannins ranging from 0.5% to 20% dry weight (Swain, 1979; Ossipov et al., 2001; Salminen et al., 2004). Terrestrial plant tannins are classified as condensed tannins (polymers of flavan-3-ols) or hydrolyzable tannins. Within the hydrolyzable tannins are galloyl glucoses and gallotannins (gallic acid esters of glucose or other polyols) and ellagitannins. The ellagitannins, which are distinguished by the presence of oxidatively coupled galloyl groups, were recently found to have unusually high oxidative activities (Barbehenn et al., 2006, this issue). Tannins in this relatively unexplored group formed higher concentrations of semiquinone radicals and browned at higher rates compared with other hydrolyzable and condensed tannins.

The leaves of many trees contain mixtures of hydrolyzable and condensed tannins (e.g., Baldwin et al., 1987; Kinney et al., 1997; Salminen et al., 2004), but the potential impact of tannin composition on herbivores is poorly understood. Research on the roles of phenolic compounds in protecting leaves from insect herbivores has focused on their oxidative activities (Appel, 1993; Summers and Felton, 1994; Barbehenn et al., 2005). Phenolic oxidation produces reactive oxygen species, which have the potential to damage essential nutrients and/or cause oxidative stress in herbivores (Summers and Felton, 1994; Bi and Felton, 1995, 1997; Barbehenn et al., 2005). The oxidation of ingested phenolics is minimized in caterpillars by co-ingested ascorbate, suggesting that the oxidative activities of leaves depend on the ratio of phenolics to ascorbate (Felton and Duffey, 1992; Barbehenn et al., 2001, 2003a, 2005). However, if the oxidative activities of different phenolics vary greatly, then the oxidative activities of leaves would also depend on phenolic composition and not merely on total phenolic and ascorbate levels.

A major goal of this study was to examine the importance of phenolic composition on the oxidative activities of tree leaves. Previous work showed that sugar maple leaves have greater oxidative activity when ingested by caterpillars than do red oak leaves (Barbehenn et al., 2005). Here, we examined the association between tannin composition and oxidative activities in maple leaf phenolic fractions, which contain a wide range of tannin mixtures. Our initial expectation was that the oxidative activities of leaves could be predicted from the sum of the activities of their component phenolics. However, it is also possible that redox reactions among phenolics could change the overall rate of oxidation of their mixtures (e.g., Hoover et al., 1998). The potential for chemical interactions among tannins was examined with two hydrolyzable tannins in mixtures containing one of four types of condensed tannins: monomeric to trimeric procyanidins, oligomeric and polymeric procyanidins, or polymeric prodelphinidin-rich condensed tannins.

Oxidative activity was measured at pH 10 (representative of many caterpillar midguts), and was defined by three parameters: semiquinone radical concentration, the rate of decay of these semiquinones, and the rate of browning of phenolics. Electron paramagnetic resonance (EPR) spectrometry was used to measure semiquinone radicals (one-electron oxidation products of phenolics). A UV-visible spectrophotometric assay was used to measure the rate of formation of brown pigments (quinones and quinoidal polymers) that are produced by oxidized phenolics (Richard-Forget and Gauillard, 1997). Although the EPR assay (anoxic) is more relevant to the chemistry of the midgut fluids of many insect herbivores (Johnson and Barbehenn, 2000), the browning assay (ambient oxygen) was developed as a relatively simple assay that would provide a second, independent measure of oxidative activity.

Phenolic compounds from ingested leaf tissues are present in herbivore gut fluids in much more complex chemical mixtures than are phenolics in crude or semipurified solvent

extracts from plants. It would be most relevant to measure the oxidative activities of phenolics in the gut fluids of animals that have ingested them. However, a useful approximation of the oxidative activities of ingested phenolics can be obtained by examining whole-leaf extracts. Therefore, we used EPR spectrometry to develop a time-course assay for quantifying the relative oxidative activities in ethanolic extracts of leaves.

This study examined three questions aimed at explaining the large differences between the oxidative activities of red oak and sugar maple leaves: (1) Does tannin composition affect the oxidative activities of tree leaves? (2) Do chemical interactions between tannins affect the oxidative activities of mixtures of tannins? (3) Are the oxidative activities of whole-leaf extracts useful for comparing tree leaf quality?

Methods and Materials

Extraction and Analysis of Leaf Phenolics

Leaves used were from three tagged red oak and sugar maple trees on the University of Michigan campus in Ann Arbor, MI, USA. The same trees have been used in a series of experiments (Barbehenn et al., 2003a, b, 2005). Branch tips were cut from the sunny side of the trees (June 22, 2004) and immediately placed in flasks of water. Within 1 hr, the midribs were removed, and the leaves were freeze-dried and air-mailed to Turku, Finland. Leaves were combined within species (19.2 g red oak and 17.3 g sugar maple) and ground into a homogeneous powder with an IKA Labortechnik A10 mill (Janke & Kunkel GmbH, Staufen, Germany). To identify and quantify the complete phenolic compositions, leaf powder (20 mg) from each species was extracted with 800 μ l of 70% acetone (containing 0.1% ascorbic acid) for 1 hr. The extraction was repeated $\times 4$, and the extracts were pooled. Acetone was removed with an Eppendorf concentrator 5301 (Eppendorf AG, Hamburg, Germany), and the remaining aqueous extract was freeze-dried. The freeze-dried sample was dissolved in 600 μ l of water and filtered (0.45 μ m). Using high-performance liquid chromatography and a diode array detector (HPLC-DAD), hydrolyzable tannins were quantified in pentagalloyl glucose equivalents (280 nm), flavonoids in quercetin equivalents (349 nm), chlorogenic acids in chlorogenic acid equivalents (315 nm), coumaroylquinic acids in coumaric acid equivalents (315 nm), and the remaining phenolics (other than condensed tannin) as gallic acid equivalents (280 nm; Salminen et al., 1999). Monomeric to trimeric condensed tannins were quantified by reverse-phase HPLC, and total condensed tannins were estimated with the acid-butanol assay (Ossipova et al., 2001), with purified birch leaf condensed tannins as a standard.

Foliar Phenolic Fractions

The bulk of the powdered sugar maple leaves was extracted with 70% acetone (5 \times 500 ml). Extracts were combined, and the acetone was rotary evaporated. After centrifugation (10 min, 2500 \times g, room temperature), the supernatant solution was freeze-dried. The crude phenolic extract from sugar maple leaves was separated into nine fractions using a Sephadex LH-20 column (Salminen et al., 1999). The following series of solvents was used sequentially to elute each fraction: water (150 ml), water (350 ml), 30% aqueous methanol (500 ml), 50% methanol (500 ml), 10% acetone (200 ml), 30% acetone (500 ml), 50% acetone (500 ml), 70% acetone (500 ml), and 85% acetone (500 ml). Each fraction was analyzed by HPLC-

DAD, rotary evaporated, and freeze dried. The 50–85% acetone fractions had similar, tannin-rich compositions. To examine these fractions further, they were combined, dissolved in 40 ml of water, and applied to a Sephadex LH-20 column. Twelve fractions were collected with a graded series of aqueous acetone solvents, ranging from 10% acetone to 85% acetone (500 ml each). Phenolics in each fraction and subfraction were identified and quantified as described.

EPR Assay for Oxidative Activity

Pedunculagin, pentagalloyl glucose, and condensed tannins were isolated as described previously (Barbehenn et al., 2006, this issue). Crude phenolics, phenolic-containing fractions, and purified tannins were weighed to the nearest microgram on a Cahn 25 Electobalance (Cahn Instruments, Inc., Cerritos, CA, USA). Immediately before analysis, each sample was solubilized in 70% aqueous ethanol, which was prepared with nitrogen-purged, double-distilled water and ethanol (each bubbled for 1 min/ml). Solubilized samples were further diluted to produce a series of three concentrations per sample. The range of concentrations varied from sample to sample to produce semiquinone levels that were in a linear range (e.g., sample final concentrations of 0.2–0.7 mg/ml). EPR assays were carried out as described previously (Barbehenn et al., 2006, this issue), with the exception that one scan per sample was run. Samples were scanned for an additional period of time (up to 45 min) to determine the linear rate of semiquinone decay. One replicate was run for each sample. Radicals were identified by comparison of the line shapes and center field values with EPR spectra of semiquinone radicals formed by phenolic and ascorbic acid standards (Barbehenn et al., 2003a, 2006). Standard solutions of the stable free radical, 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO, Aldrich Chemical Co.) were run to confirm that the method was consistent through time, and to provide a free radical standard for converting double-integrals of spectra to a concentration basis (in nM; Barbehenn et al., 2006, this issue). Plots were made of semiquinone concentration vs. sample concentration for each series of concentrations. The slope of the regression line represents the semiquinone concentration (nM) normalized for sample concentration (mg/ml). The rate of decay of semiquinone radicals was normalized by using the semiquinone radical concentration present at the start of the decay period.

Phenolic fractions from sugar maple that contained elevated levels of gallic acid ($N = 5$) or that did not produce a useable time-course ($N = 2$) were not included in correlation analyses. Semiquinone decay rates were positively correlated with gallic acid content ($r = 0.77$), and inclusion of these fractions obscured correlations between tannin content and oxidative activity. In two sugar maple fractions, no EPR data were obtained because the semiquinone radical concentration continued to increase for over 45 min, i.e., no decay rate or maximum semiquinone radical concentration could be obtained. Correlations between the tannin composition of leaf fractions and semiquinone radical concentrations were only examined in sugar maple because of the lack of hydrolyzable tannins in red oak.

Phenolic Browning Assay

The browning of phenolics in an alkaline solution was used to compare the rate of oxidation of purified tannins and crude extracts (Barbehenn et al., 2006, this issue). Final sample concentrations typically ranged from 0.01 to 0.1 mg/ml of 70% aqueous ethanol (nitrogen purged). At least three replicate reactions were run from separately weighed samples, as well as a pentagalloyl glucose standard to confirm that measurements were consistent

through time. Sample concentration was plotted against browning rate for each sample, and the slope of each regression represents the browning rate normalized for sample concentration.

The unusually high rate of browning of gallic acid (Barbehenn et al., 2006, this issue) could potentially dominate the overall oxidative activity of sugar maple fractions containing gallic acid ($N = 5$). As expected, there was a strong correlation between gallic acid content and the browning rates of sugar maple fractions ($r = 0.97$). Therefore, correlations between tannin content and oxidative activity excluded samples containing gallic acid to examine the effects of tannins more clearly. Other low-molecular-weight phenolics could potentially have a similar strong effect on the oxidative activities of phenolic fractions. However, chlorogenic acid, one of the major low-molecular-weight phenolics, had only 1% of the rate of oxidation of gallic acid (on a mg/ml basis; Barbehenn et al., 2006, this issue).

Effects of Condensed Tannins on Hydrolyzable Tannin Oxidation Rates

The EPR assay was used to measure semiquinone concentrations from pedunculagin (0.046 mg/ml, final concentration) and pentagalloyl glucose (0.091 mg/ml) in the presence or absence of condensed tannins (0.046 and 0.091 mg/ml). Pedunculagin was chosen as an example of an oxidatively active ellagitannin, and pentagalloyl glucose as a less-active gallotannin precursor (Barbehenn et al., 2006, this issue). A lower concentration of pedunculagin than pentagalloyl glucose was used to produce semiquinone concentrations in the linear range for pedunculagin, whereas a higher concentration of pentagalloyl glucose helped to measure changes in its smaller semiquinone concentrations with greater accuracy. Four types of condensed tannins were used: monomeric to trimeric, oligomeric and polymeric procyanidins, and polymeric prodelphinidin-rich condensed tannins. Condensed tannins (2.0 mg/ml) were solubilized in oxygen-free 70% ethanol immediately before use. A 1.0 mg/ml condensed tannin solution was prepared by diluting the 2.0 mg/ml solution 1:1 with 70% ethanol. A 20- μ l aliquot of the hydrolyzable tannin solution was mixed with a 20- μ l aliquot of the condensed tannin solution, or 70% ethanol (control). An aliquot (20- μ l) of the tannin mixture was then mixed with 200 μ l of the pH 10 buffer, and the EPR spectrum was obtained (Barbehenn et al., 2006, this issue). Each condensed tannin and hydrolyzable tannin was examined alone to determine mean concentrations of semiquinone radicals. Each treatment was examined using 4–6 measurements (replicates) with independently weighed tannin samples. The ratios of condensed tannin/ellagitannin and condensed tannin/hydrolyzable tannin were chosen to encompass those found in trees such as sugar maple. Due to the length of each experiment, each of the four condensed tannins were examined with both hydrolyzable tannins on separate days. TEMPO standards ($N = 2$) were run on each day, and used to confirm that day-to-day variation in EPR measurements had a negligible effect on the results. In the absence of condensed tannins, semiquinone concentrations from replicate hydrolyzable tannin measurements were divided by the mean semiquinone concentration, defining the expected value as 100 percent (Table 3). Expected values for semiquinone concentrations in tannin mixtures were calculated from the sum of the semiquinone concentrations from the tannins that were examined separately in each experiment.

The browning assay was used to measure the effect of each of the four condensed tannins on the rate of browning of pedunculagin and pentagalloyl glucose. The experimental design was the same as described for the EPR assay, with the following exceptions: A total of 80 μ l of each tannin mixture was prepared, from which three

replicate 20- μ l aliquots were placed in a 96-well microplate. pH 10 buffer (180 μ l, ambient oxygen level) was added to start these reactions. Thus, tannin final concentrations were slightly higher in the browning assay than in the EPR assay. Either four or five replicate measurements were made for each treatment (tannin concentration and mixture). Pentagalloyl glucose browning rates were measured in each run to ensure that day-to-day variation was negligible.

EPR Time-Course Assay on Whole-Leaf Extracts

Fresh red oak and sugar maple leaves ($N = 3$ trees/species) were ground under liquid nitrogen and extracted in nitrogen-purged ethanol (6 ml) in June and July 2003, as described previously (Barbehenn et al., 2005). Phenolics from the same trees were analyzed in this study, but were sampled in 2004. Extracts were stored at -80°C under a nitrogen atmosphere in plug-seal screw-cap centrifuge tubes. Leaf extracts stored in this manner appear to remain stable for a period of at least 4–7 months (Barbehenn et al., 2003b), but neither the stability of the phenolics over longer time periods nor the potential effects of ethanolysis on phenolic composition were examined (Mueller-Harvey, 2001). Aliquots (20 μ l) were taken from each extract, mixed with the pH 10 buffer (200 μ l), placed in a flat cell, and observed with EPR, as described above. EPR was used to identify the radicals formed (ascorbyl or semiquinone) in each sample and to follow their concentrations over a 45-min period. Samples were rescanned at five-min intervals. Spectra were identified and quantified, as described above. As ascorbyl radical levels in sugar maple samples decayed and semiquinone radicals formed, a spectrum in which small amounts of both radicals were present was formed. These intermediate spectra were regarded as 0 nM for each radical type due the difficulty of quantifying small amounts of these radicals in mixtures. TEMPO radical standards were used to calculate ascorbyl and semiquinone radical concentrations (Barbehenn et al., 2006, this issue). The fresh weight of each leaf sample was used to standardize radical concentrations, i.e., radical concentration/mg. Ascorbyl radical decay rates were normalized for ascorbyl radical concentrations by dividing them by the ascorbyl radical concentration at the start of the time course.

Statistical Analyses

The relationships between the percentage of three groups of tannins in sugar maple phenolic fractions and the oxidative activities of these fractions were examined with linear regressions (Wilkinson, 2000). The effects of condensed tannins on hydrolyzable tannin semiquinone radical concentrations and browning rates were examined by two-way ANOVA (SAS; PROC MIXED procedure), using hydrolyzable tannin type, condensed tannin type, and condensed tannin concentration and their two-way and three-way interactions in the model (SAS, 2000). The percent of the expected oxidation rate was calculated as the actual rate of oxidation (semiquinone concentration or browning rate) divided by the sum of the browning rates for the condensed tannin and hydrolyzable tannin when measured alone. The percent of expected values for pedunculagin and pentagalloyl glucose controls (i.e., without added condensed tannin) were calculated as the individual variate/mean, with the mean expected rates of pure hydrolyzable tannins defined as 100%. Data from both EPR and browning assays were arcsin square root transformed to fit a normal distribution. Pairwise multiple comparisons were made using the differences of least-square means (PROC MIXED) to test *a priori* hypotheses for concentration effects (i.e., control oxidation > 0.05 mg/ml > 0.10 mg/ml). In each ANOVA analysis, the main

effects and interactions were significant ($P < 0.001$), and specific comparisons of interest are reported in Results. All summary statistics are presented as the mean \pm SEM.

Results

Red oak and sugar maple leaves contained distinctly different classes and concentrations of phenolics (Table 1). Red oak contained approximately one fourth the amount of total phenolics found in sugar maple leaves. The bulk of the tannins in red oak were condensed tannins, with little ellagitannin and no galloyl glucoses found. Sugar maple contained substantial amounts of ellagitannins and condensed tannins, as well as a wide range of galloyl glucoses. Neither species contained high-molecular-weight gallotannins (i.e., galloyl glucoses larger than pentagalloyl glucose). Further characterization of the low-molecular-weight condensed tannins showed that red oak and sugar maple contained 0.5 and 2.5 mg/g monomeric–trimeric condensed tannins, respectively. The crude phenolics from red oak produced broad, weak semiquinone radical spectra, similar to those of oligomeric and polymeric condensed tannins (Barbehenn et al., 2006 this issue). The crude phenolics and

Table 1 Phenolic compounds in red oak (*Quercus rubra*) and sugar maple (*Acer saccharum*) leaves

Phenolic compound	Red oak		Sugar maple	
	mg/g DW	Percent total phenolics	mg/g DW	Percent total phenolics
Gallic acid and galloyl glucoses				
Gallic acid	–	–	0.7	0.5%
Monogalloyl glucose	–	–	1.5	1.1%
Digalloyl glucose	–	–	0.7	0.5%
Trigalloyl glucose	–	–	0.1	0.1%
Tetragalloyl glucose	–	–	9.5	7.3%
Pentagalloyl glucose	–	–	4.3	3.3%
Ellagitannins				
Oenothien B	0.3	1.0%	–	–
Galloyl-HHDP-glucose	–	–	2.6	2.0%
Trigalloyl-HHDP-glucose	–	–	11.6	8.9%
Ellagitannin (950 Da)	–	–	2.0	1.6%
Ellagitannin (952 Da)	–	–	8.9	6.8%
Ellagitannin (1110 Da)	–	–	8.3	6.4%
Ellagitannin (1120 Da)	–	–	1.3	1.0%
Other ellagitannins	–	–	10.0	7.6%
Condensed tannins				
Total condensed tannins	23.7	70.7%	51.0	39.1%
Other phenolics				
Chlorogenic acid or its derivatives	4.1	12.1%	6.7	5.1%
Coumaroyl quinic acid or its derivatives	0.1	0.3%	0.2	0.2%
Flavonoid glycosides	3.6	10.7%	5.8	4.4%
Noncharacterized phenolics	1.7	5.2%	5.3	4.1%
Sum of phenolic compounds	33.5	100.0%	114.8	100.0%

phenolic fractions from sugar maple produced semiquinone radical spectra with line shapes that were similar to spectra from hydrolyzable tannins (Barbehenn et al., 2003a, 2006).

The overall oxidative activities of sugar maple and red oak phenolics were compared with three measures: semiquinone concentration, semiquinone decay rate, and browning rate. Each of these measures was significantly higher in sugar maple phenolics than in red oak phenolics ($P < 0.05$; Table 2).

Significant regressions relating tannin composition and the rate of browning were observed in phenolic fractions from sugar maple leaves (Fig. 1a,b,c). Browning rates increased as the percentage of ellagitannins in the fractions increased ($R^2 = 0.76$, $P < 0.001$). By contrast, the rate of browning decreased as the percentage of condensed tannins in these fractions increased ($R^2 = 0.74$, $P = 0.001$), and browning rates also decreased as the percentage of galloyl glucoses increased ($R^2 = 0.41$, $P = 0.034$). Similarly, the relationship between each of the three types of tannins and semiquinone decay rates showed the same direction of association as did browning rates, i.e., increasing with higher ellagitannin content ($R^2 = 0.29$, $P = 0.14$), decreasing with higher condensed tannin content ($R^2 = 0.62$, $P = 0.012$), and decreasing with higher galloyl glucose content ($R^2 = 0.23$, $P = 0.19$). Although not statistically significant, the association between each of the three types of tannins and semiquinone concentrations also showed the same direction of association as did browning rates and semiquinone decay rates ($R^2 = 0.10$ – 0.23).

Ellagitannin and condensed tannin levels were themselves negatively correlated in the maple fractions ($r = -0.73$), suggesting that lower oxidation rates in condensed tannin-rich fractions could either be caused by a direct effect of condensed tannins on oxidation rates or the result of decreased concentrations of ellagitannins in fractions that were rich in condensed tannins. Therefore, the direct effects of condensed tannins on hydrolyzable tannin oxidation rates were tested to understand the basis for the negative relationship between condensed tannins and oxidative activities in phenolic fractions from maple leaves. Semiquinone radical concentrations from pedunculagin (ellagitannin) and pentagalloyl glucose were significantly decreased in the presence of all four types of condensed tannins tested (Table 3). The expected concentrations of semiquinones from pedunculagin were decreased between 17% and 90% by an equal concentration of condensed tannins, and decreased by 29–92% by twice the concentration of condensed tannins, a small, but significant decrease from the higher condensed tannin concentration ($P = 0.015$). Similarly, pentagalloyl glucose formed lower concentrations of semiquinone radicals in the presence of equal or greater concentrations of condensed tannins ($P < 0.001$; Table 3). Condensed tannins decreased semiquinone concentrations from pentagalloyl glucose by 42–85% and 74–91% by an equal concentration or twice the condensed tannin concentration, respectively. Thus, condensed tannins had a greater antioxidant effect on pentagalloyl glucose than on pedunculagin ($P < 0.001$). Prodelphinidin-rich condensed tannins were the

Table 2 Oxidative activities of red oak (*Quercus rubra*) and sugar maple (*Acer saccharum*) foliar phenolics (70% acetone extracts) measured with EPR spectrometry and spectrophotometry (415 nm)

Tree species	Semiquinone concentration [nM radical (mg/ml) ⁻¹]	Semiquinone decay rate [nM radical (mg/ml) ⁻¹ sec ⁻¹]	Browning rate [mAbs/min (mg/ml) ⁻¹ min ⁻¹]	<i>N</i>
Red oak	133 ± 24 ^a	64 ± 17 ^a	0.23 ± 0.14 ^a	4
Sugar maple	424 ± 120 ^b	310 ± 9 ^b	3.45 ± 0.70 ^b	4

Data are presented as mean ± SE. Different letters comparing tree species denote significant differences ($P < 0.05$).

Fig. 1 Relationship between tannin composition of phenolic fractions from sugar maple leaves and the rate of browning (415 nm) at pH 10. (a) Ellagitannins ($R^2 = 0.76$, $P < 0.001$). (b) Condensed tannins ($R^2 = 0.74$, $P = 0.001$). (c) Galloyl glucoses ($R^2 = 0.41$, $P = 0.034$). Browning rates were normalized for phenolic concentration, producing units of mAbs/min per phenolic concentration (mg/ml)

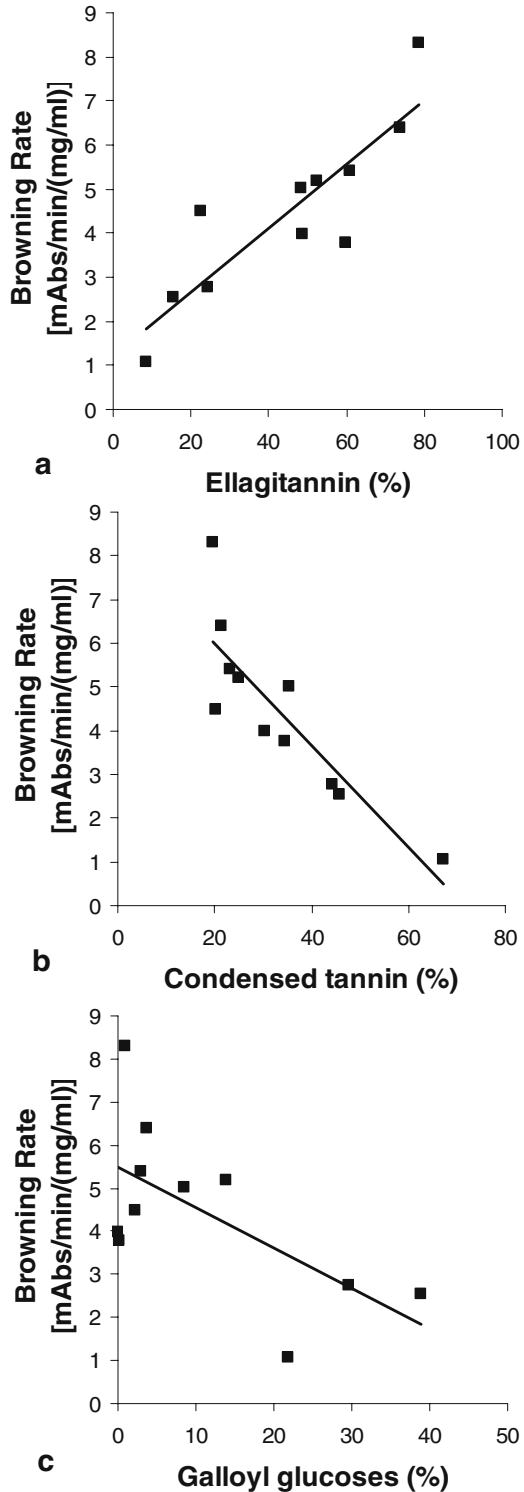


Table 3 Effects of condensed tannins on semiquinone radical concentrations formed by pedunculagin and pentagalloyl glucose at pH 10

Hydrolyzable tannin	Condensed tannin concentration (mg/ml)	Condensed tannin tested							
		PC monomers-trimers		PC oligomers		PC polymers		PD-rich polymers	
		Semiquinone concentration [nM (mg/ml) ⁻¹]	Percent expected value	Semiquinone concentration [nM (mg/ml) ⁻¹]	Percent expected value	Semiquinone concentration [nM (mg/ml) ⁻¹]	Percent expected value	Semiquinone concentration [nM (mg/ml) ⁻¹]	Percent expected value
Pedunculagin (0.046 mg/ml)	0.000	796 ± 52 ^a	100.0 ± 6.5 ^a	785 ± 45 ^a	100.0 ± 5.8 ^a	774 ± 50 ^a	100.0 ± 6.5 ^a	870 ± 86 ^a	100.0 ± 9.9 ^a
	0.046	708 ± 35 ^a	78.9 ± 3.9 ^b	553 ± 15 ^b	61.6 ± 1.7 ^b	690 ± 40 ^{ab}	81.9 ± 4.8 ^b	89 ± 13 ^b	9.5 ± 1.4 ^b
	0.091	657 ± 44 ^a	71.3 ± 4.7 ^b	525 ± 49 ^b	56.6 ± 5.2 ^c	548 ± 37 ^b	63.3 ± 4.2 ^c	70 ± 6 ^b	7.5 ± 0.6 ^b
Pentagalloyl glucose (0.091 mg/ml)	0.000	372 ± 26 ^a	100.0 ± 6.9 ^a	343 ± 31 ^a	100.0 ± 8.9 ^a	330 ± 14 ^a	100.0 ± 4.1 ^a	429 ± 34 ^a	100.0 ± 7.8 ^a
	0.046	179 ± 28 ^b	37.8 ± 5.9 ^b	121 ± 13 ^b	26.5 ± 2.9 ^b	152 ± 20 ^b	38.2 ± 5.0 ^b	75 ± 10 ^b	15.2 ± 2.0 ^b
	0.091	130 ± 7 ^c	26.0 ± 1.3 ^c	111 ± 3 ^b	22.9 ± 0.6 ^b	104 ± 12 ^c	24.7 ± 2.9 ^c	53 ± 10 ^c	10.7 ± 1.9 ^b

Concentrations of semiquinone radicals are presented as mean ± SE from four independent measurements. Different letters indicate significant differences between means within each tannin combination. Semiquinone concentrations for procyanidin (PC) monomers-trimers, oligomers, procyanidin polymers, and prodelphinidin (PD)-rich (55%) polymers averaged 102, 112, 69, 66 μM, respectively, at 0.046 mg/ml, and averaged 126, 142, 91, 69 μM at 0.091 mg/ml.

Table 4 Effects of condensed tannins on the rate of browning of pedunculagin and pentagalloyl glucose at pH 10

Hydrolyzable tannin	Condensed tannin concentration (mg/ml)	Condensed tannin tested							
		PC monomers-trimers		PC oligomers		PC polymers		PD-rich polymers	
		Browning rate [mAbs/min (mg/ml) ⁻¹]	Percent expected value	Browning rate [mAbs/min (mg/ml) ⁻¹]	Percent expected value	Browning rate [mAbs/min (mg/ml) ⁻¹]	Percent expected value	Browning rate [mAbs/min (mg/ml) ⁻¹]	Percent expected value
Pedunculagin (0.05 mg/ml)	0.000	17.6 ± 0.5 ^a	100.0 ± 2.9 ^a	19.7 ± 1.0 ^a	100.0 ± 5.0 ^a	17.1 ± 0.9 ^a	100.0 ± 1.6 ^a	18.3 ± 0.9 ^a	100.0 ± 2.5 ^a
	0.050	24.2 ± 1.0 ^b	135.5 ± 5.4 ^b	19.2 ± 0.8 ^a	96.4 ± 4.1 ^b	17.5 ± 0.8 ^a	102.3 ± 4.7 ^a	10.8 ± 0.9 ^b	46.4 ± 4.0 ^b
	0.100	33.0 ± 1.4 ^c	185.5 ± 7.9 ^c	17.5 ± 1.7 ^a	86.0 ± 8.3 ^b	18.0 ± 1.2 ^a	98.9 ± 6.5 ^b	11.4 ± 0.7 ^b	43.9 ± 2.9 ^b
Pentagalloyl glucose (0.10 mg/ml)	0.000	2.7 ± 0.2 ^a	100.0 ± 7.1 ^a	2.8 ± 0.2 ^a	100.0 ± 8.0 ^a	3.0 ± 0.1 ^a	100.0 ± 1.6 ^a	2.8 ± 0.5 ^a	100.0 ± 17.0 ^a
	0.050	9.9 ± 0.8 ^b	328.7 ± 25.5 ^b	2.3 ± 0.4 ^a	75.8 ± 12.4 ^b	2.6 ± 0.2 ^a	85.9 ± 6.1 ^b	1.9 ± 0.3 ^a	23.9 ± 3.5 ^b
	0.100	11.1 ± 1.5 ^b	369.3 ± 49.0 ^b	2.8 ± 0.1 ^a	82.8 ± 3.4 ^b	2.4 ± 0.2 ^a	80.3 ± 5.4 ^b	3.6 ± 0.6 ^b	34.4 ± 5.4 ^b

Data are presented as mean ± SE from four or five independent measurements. Different letters indicate significant differences between means within each tannin combination. Browning rates for procyanidin (PC) monomers-trimers, oligomers, procyanidin polymers, and prodelphinidin (PD)-rich (55%) polymers averaged 0.3, 0.2, 0.0, 5.1 mAbs/min, respectively, at 0.05 mg/ml, and averaged 0.3, 0.6, 0.1, 7.7 mAbs/min at 0.10 mg/ml.

most effective antioxidants for both types of hydrolyzable tannins ($P < 0.001$ for each condensed tannin comparison). The condensed tannins themselves formed relatively low levels of semiquinones (Table 3).

Condensed tannins had a wide range of effects on the rates of browning of the hydrolyzable tannins (Table 4). The expected rates of browning of pedunculagin and pentagalloyl glucose were decreased by 54–76% by prodelphinidin-rich condensed tannins

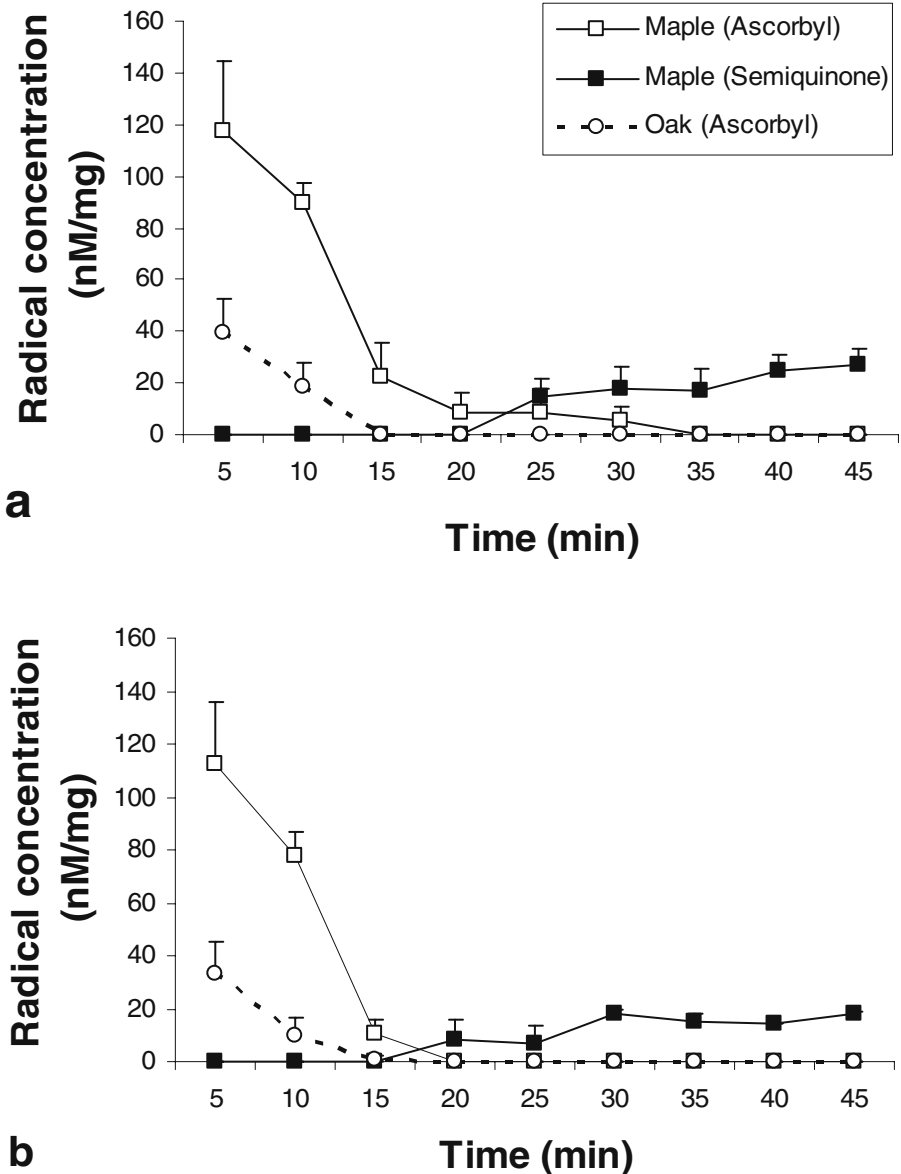


Fig. 2 EPR time-course analyses of ethanolic extracts of red oak and sugar maple leaves in June (a) and July (b) 2003. The same trees were used in 2003 as were used for chemical analyses and measurement of oxidative activities in 2004 in this study. Radical concentrations are presented on a leaf fresh weight basis

($P < 0.001$). Oligomeric and polymeric procyanidin condensed tannins had significant, but weak, antioxidant effects on both hydrolyzable tannins. Low-molecular-weight condensed tannins had a striking prooxidant effect on the hydrolyzable tannins, increasing the browning rate of pedunculagin by 36–86% ($P < 0.005$) and increasing the browning rate of pentagalloyl glucose by 229–269% ($P < 0.001$). Condensed tannins themselves generally had low browning rates, adding little directly to the overall expected rates (Table 4). Prodelphinidin-rich condensed tannins were an exception, having both a markedly higher browning rate than the other condensed tannins, as well as the greatest antioxidant activity.

When red oak leaf extracts from June were examined at pH 10 with EPR, ascorbyl radicals were observed (Fig. 2a). Concentrations of ascorbyl radicals decreased over a 15-min period, but semiquinone radicals were not detected during the following half hour. Similar results were observed from extracts of leaves from July (Fig. 2b). Extracts of sugar maple leaves initially produced ascorbyl radicals at concentrations that were 3.0- to 3.3-fold higher than those from red oak (Fig. 2a,b). When normalized for the initial radical concentration, the rates of decay of ascorbyl radicals during the first 15 min of the assay were similar in the extracts of maple and oak in June (0.45 and 0.48 nM/mg/min, respectively) and July (0.41 and 0.50 nM/mg/min, respectively). However, after ascorbyl radical concentrations fell below detectable levels, only sugar maple extracts produced increasing concentrations of semiquinone radicals.

Discussion

The results of this study on the phenolic fractions, total phenolics, and ethanolic extracts of red oak and sugar maple leaves suggest that the oxidative activities of these leaves can be affected by their tannin composition. The oxidative activities of phenolic fractions from sugar maple were positively associated with their ellagitannin content, but negatively associated with condensed tannins and galloyl glucoses. Although these relationships were statistically significant mainly from the browning assay, regressions for the three measures of oxidative activity for each of three tannin types point toward the same overall conclusions. A positive relationship between ellagitannin concentration and phenolic oxidation rate was expected based on the unusually high rates of oxidation of purified ellagitannins at pH 10 (Barbehenn et al., 2006, this issue). However, the finding that condensed tannins can decrease the oxidation of hydrolyzable tannins was unexpected, and such interactions suggest that tannin composition in plants can have either positive or negative effects on their overall oxidative activities. This study provides a more complete explanation for the greater oxidative activity of sugar maple than red oak for caterpillars than was previously proposed (Barbehenn et al., 2005); in addition to the relatively low ratio of ascorbate/total phenolics in sugar maple, 34% of the phenolics produced by the maple trees were highly active ellagitannins. In contrast, red oak had a higher ratio of ascorbate/total phenolics (Barbehenn et al., 2005), and 71% of its phenolics were condensed tannins with low oxidative, or potentially antioxidant, activity. Only 1% of the phenolics in the red oak leaves were ellagitannins.

An important conclusion of this study is that the oxidative activities of tree leaves cannot be predicted from common measures of phenolics, such as total phenolics or total hydrolyzable tannins. Not only do the oxidative activities of the component phenolics in these measures vary tremendously, but redox reactions between phenolics (e.g., hydrolyzable and condensed tannins) can also change the oxidative activity of the mixture greatly. Dynamic methods for measuring the total oxidative activities of leaves, such as the

EPR time-course assay in this study, provide a useful overview of the complex chemical reactions that occur in the mixtures of phenolics and numerous other compounds from tree leaves. Our results suggest that more oxidatively active leaves, such as those from sugar maple, are marked by the production of semiquinone radicals, once ascorbyl radical (and hence ascorbate) levels have decreased. This pattern has been observed previously (Yamasaki and Grace, 1998; Grace et al., 1999) and reflects the instability of ascorbate at high pH, as well as the loss of ascorbate and ascorbyl radicals upon their reaction with semiquinone radicals and quinones. The higher concentrations of ascorbyl radicals in sugar maple than red oak extracts was the opposite of our expectation based on the higher ascorbate concentrations in oak leaves (Barbehenn et al., 2003b). It is possible that ascorbate oxidizes more rapidly to ascorbyl radicals in sugar maple because of a higher reaction rate between ascorbate and oxidized phenolics in extracts. The lack of observable semiquinone radicals from red oak extracts is consistent with the presence of moderate levels of condensed tannins in this species and the low levels of semiquinone radicals formed by these tannins (Barbehenn et al., 2006, this issue).

An assumption of this work is that ethanolic extracts of leaves contain the bulk of the redox-active compounds that are present in the midgut fluids of caterpillars, e.g., phenolic compounds, ascorbate, and α -tocopherol (Barbehenn et al., 2003b), and that the comparison of the oxidative activities of the two species in this study would not be changed by the inclusion of the insoluble leaf components, e.g., cell wall polymers, starch, and proteins. Some support for this assumption is provided by the results of a previous study showing that the consumption of sugar maple produces higher levels of phenolic oxidation in the midgut fluids of caterpillars than does the consumption of red oak (Barbehenn et al., 2005). Experiments with tannin-treated leaves is needed to confirm that variation in tannin composition can produce the observed differences in levels of phenolic oxidation in caterpillar midgut fluids.

The oak/maple dichotomy observed in this study is not a general pattern found in these plant taxa, because ellagitannins are found in abundance in many oak species (e.g., Haddock et al., 1982; Haslam, 1989). The individual red oak and sugar maple trees examined are unlikely to represent the full range of phenolics (both types and concentrations) found in each of these species over their wide geographical ranges and through time. Indeed, some leaf extracts from sugar maple trees in other study sites produced EPR time-course results that showed either the immediate formation of high levels of semiquinone radicals or ascorbyl radicals without the subsequent formation of semiquinone radicals (unpublished data). It is unclear whether phenolic composition and/or ascorbate concentration might explain this between-tree variation.

Low-molecular-weight phenolics interfered with the determination of the oxidative activities of tannins in some phenolic fractions in two ways: (1) highly active phenolics, such as gallic acid, can dominate the overall activities observed, and (2) less active phenolics, such as chlorogenic acid, can produce steadily increasing concentrations of secondary radicals over time (Grace et al., 1999). In some phenolic fractions, such a continuous increase in semiquinone radicals precluded our ability to measure their concentration maxima. However, the concentrations of low-molecular-weight phenolics in the crude phenolics or whole-leaf extracts from the trees in this study were at sufficiently low levels that they did not dominate the overall oxidative activities of the leaves. For example, gallic acid (0.7 mg/g in sugar maple leaves) can be calculated to contribute only 3–4% of the semiquinone concentration and approximately 11% of the browning rate of the total phenolics (Barbehenn et al., 2006, this study).

Condensed tannins can be found at high levels in many tree leaves and can deter feeding by herbivores, supporting the idea that they may have evolved, in part, as a generalized chemical defense. However, recent studies have found little support for this hypothesis in tree-feeding caterpillars (Ayres et al., 1997; Osier et al., 2000; Kopper et al., 2002). Alternatively, because there are high concentrations of condensed tannins in some leaves, they may act as antioxidants in the midgut contents of caterpillars. Thus, we raise the possibility that positive correlations between condensed tannin levels and caterpillar performance (e.g., Rossiter et al., 1988; Hemming and Lindroth, 1995) could have a substantive biochemical basis. A positive impact might be most pronounced when condensed tannins are ingested in ellagitannin-rich leaves and when the condensed tannins are themselves prodelphinidin-rich.

A prodelphinidin-rich condensed tannin had the greatest ability to decrease the oxidation of hydrolyzable tannins in this study. Other hydrolyzable tannins [cocciferin D₂, vescaligin, and gallotannins (mean MW=1396 Da)] also had substantially lower rates of browning in mixtures with the prodelphinidin-rich condensed tannin (unpublished data). Prodelphinidin subunits are distinguished by a pyrogallol (trihydroxy) substitution on the B-ring, whereas procyanidin subunits have a catechol-type B-ring. A variety of phenolics containing galloyl (trihydroxy) groups have higher redox activities than their structural counterparts containing catechol groups, e.g., flavonoids, anthocyanins, catechins, and condensed tannins (Cao et al., 1997; Guo et al., 1999; Okuda, 1999a,b; Williamson et al., 1999; Kähkönen and Heinonen, 2003). The results of this work suggest that it may be important to measure the prodelphinidin/procyanidin ratio or total prodelphinidins, and not merely total condensed tannins, to understand their impact on caterpillars.

We are unaware of studies that have compared the biological activities of purified ellagitannins, condensed tannins, and their mixtures in herbivorous insects. For example, do condensed tannins decrease the amount of oxidative damage to essential nutrients in the midgut or decrease oxidative stress in midgut tissues when co-ingested with ellagitannins? To the extent that foliar oxidative activity provides a defense against herbivores such as caterpillars, the results of this study suggest that there may be a trade-off between producing ellagitannin- or condensed tannin-rich leaves as effective defenses against different ecological challenges. Further work in these areas would provide a stronger basis for understanding the role of insect herbivory in the evolution of foliar tannin composition.

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Introduction

Tannins in higher plants are broadly categorized as hydrolyzable or condensed (proanthocyanidins; Fig. 1). Hydrolyzable tannins are synthesized from galloyl glucoses, with pentagalloyl glucose serving as the precursor for both higher-molecular-weight gallotannins and ellagitannins. Whereas high-molecular-weight gallotannins contain additional galloyl groups on pentagalloyl glucose, ellagitannins contain two or more neighboring galloyl groups that are oxidatively coupled to form rigid groups such as hexahydroxydiphenoyl (HHDP) units (Okuda et al., 2000; Fig. 1). Condensed tannins are polymers of flavan-3-ols, including procyanidins and prodelphinidins (Fig. 1).

Tannins are both structurally diverse and have diverse ecological functions. They commonly comprise from 0.5% to 10% dry weight of tree leaves, but levels on the order of 20% dry weight are found in some species (Swain, 1979; Salminen et al., 2004). Because tannins are commonly present in much larger amounts than other phenolics, they generally are believed to be a major component of the chemical defenses in leaves. They can act not only as feeding deterrents but also as antimicrobial agents, ultraviolet light protectants, and possibly toxins (Swain, 1979; Hagerman and Butler, 1991; McAllister et al., 2005).

In ecological studies, separate measurements of total gallotannins and condensed tannins are occasionally made, but little attention has been paid to ellagitannins. Despite decades of research, the relative importance of different classes of tannins as antiherbivore defenses remains poorly understood. At root is a lack of understanding of the physiological mechanisms of activity in herbivores and the influence of structure on these mechanisms.

Tannins are defined, in part, by their ability to bind and precipitate proteins, and the ability of tannins to precipitate proteins remains a common assay for their potential biological activity against herbivores (e.g., Ossipov et al., 2001; Riipi et al., 2002). Although tannins do bind proteins in the physiological conditions found in vertebrate herbivores (Hagerman, 1989), this mode of action has not been supported in insect herbivores. First, surfactants in insect gut fluids prevent the formation of insoluble protein–tannin complexes (Martin et al., 1987). Second, when phenolics are present in pH conditions that exceed their pK_a values (ca. pH 9), they are ionized and lose much of their hydrogen-bonding (and protein-binding) ability (Hagerman and Butler, 1978). However, polyphenols are susceptible to oxidation at high pH (Simic and Jovanovich, 1994). Thus, in herbivores with high gut pH, such as caterpillars (Appel and Martin, 1990), one alternative mode of action for phenolic compounds is their prooxidant activity. Although many phenolics are excellent antioxidants at acidic to neutral pH (e.g., Rice-Evans et al., 1996; Hagerman et al., 1998), at high pH phenolics and their oxidation products may act as prooxidants, promoting damage to nutrients in the gut lumen and causing oxidative stress in the surrounding gut tissues (Appel, 1993; Summers and Felton, 1994; Barbehenn et al., 2005). In general, quinones are reactive and often toxic compounds in biological systems (Gant et al., 1988; Thiboldeaux et al., 1998).

Recent work comparing the oxidative activities of sugar maple (*Acer saccharum*) and red oak (*Quercus rubra*) leaves in caterpillars found elevated levels of semiquinone radicals, protein carbonyls, and total peroxides in the gut fluids of caterpillars that ingested sugar maple leaves (Barbehenn et al., 2005). This study concluded that sugar maple might have greater prooxidant activity than red oak, in part as a result of its tannin composition, especially ellagitannins. Sugar maple contained relatively large amounts of ellagitannins, along with galloyl glucoses and condensed tannins, whereas red oak contained smaller amounts of condensed tannins, but little or no hydrolyzable tannins.

To test the hypothesis that ellagitannins have greater oxidative activities than galloyl glucoses or condensed tannins, we compared the oxidative activities of a structurally diverse set of polyphenols (Fig. 4). The hydrolyzable tannins chosen for comparison represent six of the different branches of the biosynthetic pathway of hydrolyzable tannins found in higher plants (cf. Gross, 1999; Salminen et al., 2001, 2004). The galloyl glucoses examined (monogalloyl to pentagalloyl glucose) are biosynthetic precursors to gallotannins and ellagitannins. For this study, we have grouped galloyl glucoses and high-molecular-weight gallotannins together because these phenolics lack the distinctive oxidatively coupled galloyl groups present in ellagitannins. The ellagitannins examined represent a range of types, including molecules with cyclic or acyclic glucose cores, and dimers in which both types of subunits are combined. The condensed tannins examined represent molecules composed entirely of procyanidin units or largely of prodelphinidin units, and a wide range of molecular weights (Fig. 1).

The main goal of this study was to assess the extent to which different tannins might act as prooxidants in biological systems at high pH. The oxidative activities of tannins were characterized primarily by two measures: the concentration of semiquinone radicals formed and the rate of browning. A third characteristic, semiquinone radical decay rate, was also measured, but was less useful as a means of distinguishing between different groups. Measurements of semiquinone radicals and brown pigments (formed by quinones) provide two different estimates of the propensity of phenolics to form reactive oxygen species. Electron paramagnetic resonance (EPR) spectrometry measured semiquinone radicals, which are formed by the one-electron oxidation of phenolics. A browning assay was developed to provide an independent measure of the rate of phenolic oxidation to quinones following a loss of two electrons. The oxidative activities of tannins in the pH and oxygen conditions relevant to many caterpillar midgut fluids (pH 10 and low oxygen concentration; Johnson and Barbehenn, 2000) were approximated in the EPR assay, whereas the browning assay was carried out in solutions at ambient oxygen concentration. By examining the oxidative activities of three different classes of tannins, this study addressed their relative potential as plant oxidative defenses against herbivores, such as caterpillars.

Methods and Materials

Tannin Purification

1-*O*-galloylglucose (purity 91.2%), 1,6-di-*O*-galloylglucose (>99.9%), 1,2,6-tri-*O*-galloylglucose (>99.9%), and 1,2,3,6-tetra-*O*-galloylglucose (>99.9%) were isolated from leaves of *Betula pubescens* as described in Salminen et al. (2001). 1,2,3,4,6-Penta-*O*-galloylglucose (>99.9%) was purified from tannic acid (Baker) as described by Salminen and Lempa (2002). A mixture of high-molecular-weight gallotannins, including hexa- to tridecagalloylglucoses (>99.9%, mean MW 1396 Da), was isolated from tannic acid (Baker) by column chromatography with Sephadex LH-20 using the elution profile described in Salminen et al. (1999). Pedunculagin (95.8%) was isolated from leaves of *Betula nana* as described by Salminen et al. (1999, 2001). Oenothien B (>99.9%) was isolated and purified from the inflorescences of *Epilobium angustifolium* with a combination of Sephadex LH-20 and preparative high-performance liquid chromatography (HPLC) as described previously (Salminen et al., 1999, 2001). Cocciferin D₂ (94.3%) was isolated from the leaves of

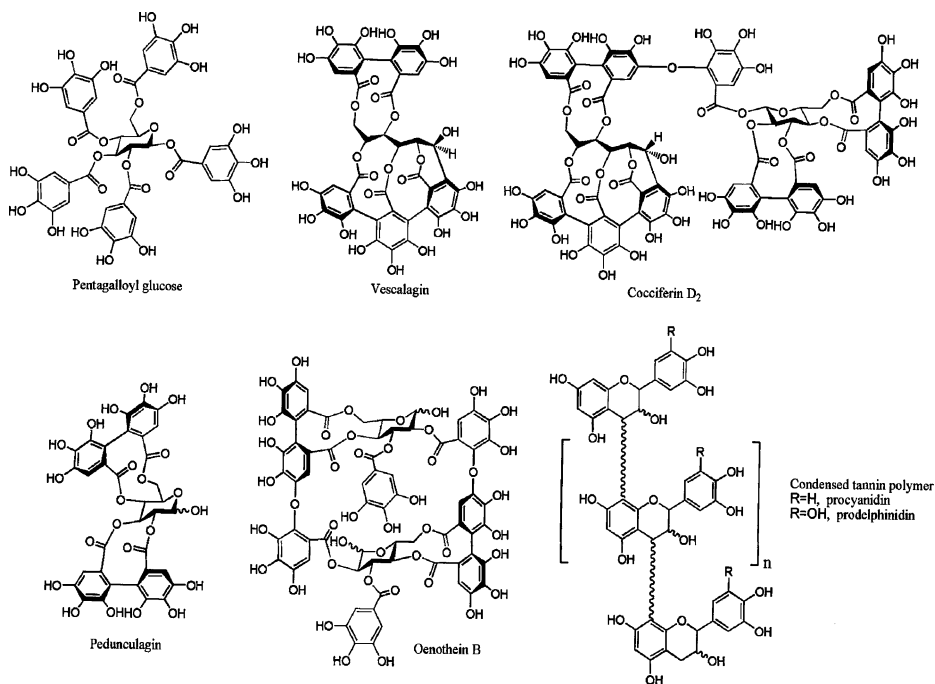


Fig. 1 Structures of some examined ellagitannins, a galloyl glucose, and condensed tannins. Pentagalloyl glucose is the core metabolite of the hydrolyzable tannins, and can be further esterified with galloyl groups to yield gallotannins. Oxidative coupling of galloyl groups in galloyl glucoses and gallotannins yield ellagitannins, including pedunculagin, cocciferin D₂, vescalagin, and oenothin B. Condensed tannins are typically formed by 4→8- or 4→6-coupled flavan-3-ols such as catechin or gallicocatechin

Quercus robur, as described in Salminen et al. (2004). Vescalagin was isolated from the leaves of *Q. robur*, and included castavalonic acid as an impurity (ratio 3:1, ellagitannin purity >99.9%, approximately MW 990 Da). Castavalonic acid is a galloylated form of castalagin, an isomer of vescalagin. Compound purities were measured with HPLC coupled with a diode array detector (280 nm). Identities were confirmed by UV and mass spectra, and by nuclear magnetic resonance spectroscopy (Salminen et al., 2001, 2004, unpublished data).

Condensed tannins, i.e., procyanidin monomers to trimers (average MW=578 Da), procyanidin oligomers (average MW=1442 Da), and procyanidin polymers (average MW=2882 Da) were isolated from bark of *Pinus sylvestris* by column chromatography on Sephadex LH-20 as described in Karonen et al. (2004). Procyanidins were identified on the basis of retention times and UV and mass spectra (well-defined molecular ions and fragment ions) characteristic for procyanidins. High-molecular-weight prodelphinidin-rich condensed tannin polymers (prodelphinidin/procyanidin=55:45, average MW=9026 Da) were obtained from mountain birch leaves (*B. pubescens* ssp. *czerepanovii*). These tannins were purified by using a Sephadex LH-20 column with an elution profile as described in Ossipova et al. (2001). Prodelphinidin/procyanidin ratios and approximate molecular weights were characterized with ¹³C-NMR spectroscopy as described in Karonen

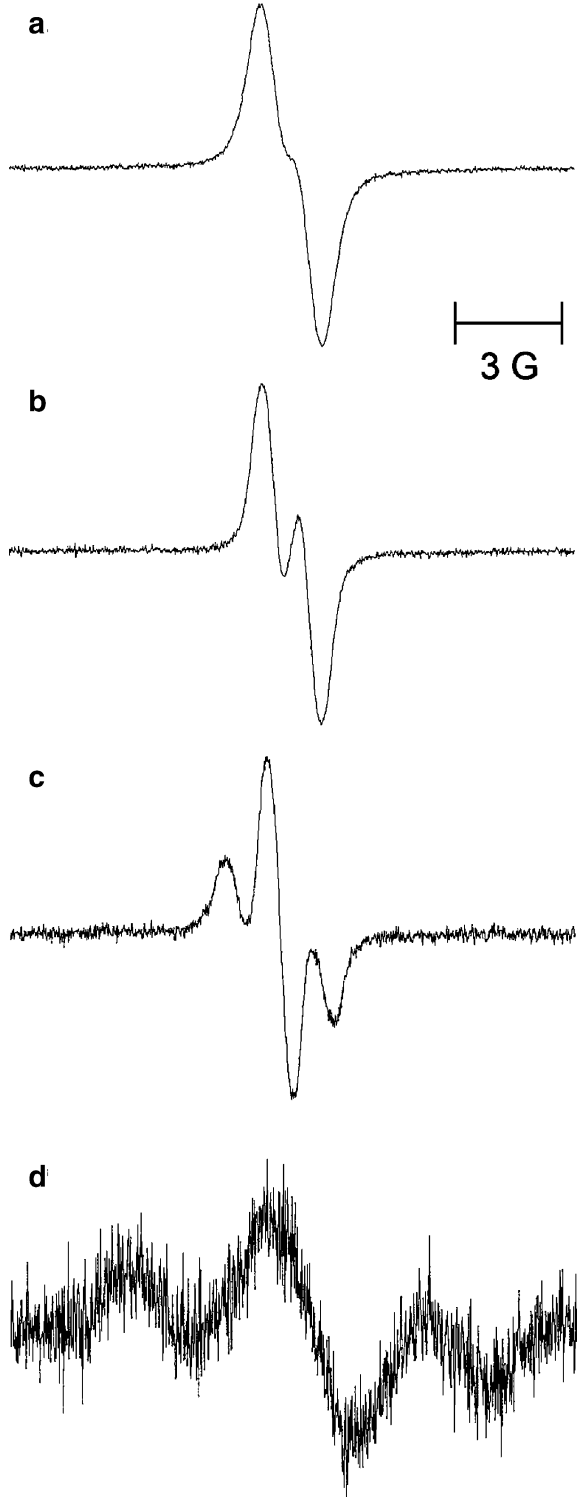
et al. (2006). Condensed tannin samples were highly pure (>99.9%), as measured by normal-phase HPLC (Karonen et al., 2006).

EPR Spectrometry

Tannin samples were weighed to the nearest microgram on a Cahn 25 Electobalance (Cahn Instruments, Inc., Cerritos, CA, USA). Solvents for EPR experiments were purged with nitrogen by bubbling them for 1 min/ml, and kept under a nitrogen atmosphere. Immediately before analysis, samples were dissolved in ethanol/double-distilled water (70:30, v/v) and kept under a nitrogen atmosphere. The stock tannin solution was serially diluted with 70% ethanol to produce final concentrations ranging from 0.5 to 3.0 mg/ml. To achieve reaction mixtures containing 20–300 μM tannin, a 20- μl aliquot of each tannin solution was added to a 2-ml centrifuge tube containing 200 μl of pH 10 carbonate buffer (70 mM), containing 10% (v/v) dimethylsulfoxide to help stabilize semiquinone radicals. A 200- μl aliquot of this mixture was placed in a quartz flat cell (Wilmad Glass Co., Buena, NJ, USA) that was pretuned in a cylindrical TM₁₁₀ cavity. EPR spectra (Fig. 2) were obtained within 30 sec of sample mixing, and recorded at room temperature with a Bruker EMX spectrometer (X-band frequency). Instrument parameters were as follows: center field, 3483 G; sweep width, 15 G; point resolution, 1024; frequency, 9.76 GHz; microwave power, 20.17 mW; gain, 5.02e^5 ; modulation frequency, 100 kHz; modulation amplitude, 1.00 G; time constant, 10.24 msec; scan rate, 0.24 G/sec. A single scan per sample was used for hydrolyzable tannins. Because of the low signal produced by condensed tannins, these were scanned five times. Hydrolyzable tannin samples were rescanned at 84-sec intervals for up to 35 min to determine the rate of radical decay, and the condensed tannins were rescanned at 7-min intervals. Because the kinetics of radical production differed among compounds, we measured the maximum semiquinone concentration produced for each compound, rather than the concentration at a fixed time after starting the reaction. Likewise, the time at which the maximum signal was produced served as the starting point for determining the rate of radical decay. The time to produce the maximum semiquinone radical concentration varied from less than 30 sec for tannins such as pedunculagin to as much as 11 min for tannins such as oenothlein. Radicals produced by purified tannins were identified as semiquinones based on comparisons of the line shapes and center field positions with EPR spectra from previous studies using similar conditions (e.g., Bors et al., 2000; Barbehenn et al., 2003). The area of each first derivative spectrum was quantified by double integration using WinEPR software (Bruker Instruments, Billerica, MA, USA). The calculated areas of condensed tannin spectra were divided by five, which accurately corrected for the higher number of scans of these samples (unpublished data). Between two and four replicate samples of separately weighed samples were run for each tannin.

Standard solutions of the stable free radical, 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO, Aldrich Chemical Co.) were made in 70% ethanol (0.58 μM final concentration) and analyzed with EPR in pH 10 buffer using identical EPR conditions as described above. Daily measurement of TEMPO standards ($N = 2$ independent solutions/d) confirmed that the method was consistent through time, and provided a free radical standard for converting double integrals of spectra to molar concentrations. The area obtained by double integration of the spectra from TEMPO standards averaged 0.224 ± 0.006 area units per nanomolar TEMPO from 22 runs over a 4-mo period.

Fig. 2 Representative EPR spectra of ellagitannins, galloyl glucoses, and condensed tannins. (a) Oenothein B (ellagitannin). (b) Pedunculagin (ellagitannin). (c) Pentagalloyl glucose. (d) Condensed tannin oligomers. Hydrolyzable tannins were scanned three times for presentation, whereas the condensed tannin oligomer sample was scanned 30 times. Center field and field width of all spectra were 3483 and 15 G, respectively



Regressions relating semiquinone concentration and tannin concentration were plotted for each phenolic compound (Fig. 3a). The slope of each regression represents the semiquinone concentration normalized for sample concentration, and this value was used for calculating the semiquinone concentration produced by each phenolic (i.e., nM radical/ μ M phenolic). These plots also confirmed that the range of concentrations examined were within the linear range for measuring semiquinone concentrations. The rate of decay of semiquinone radicals was normalized for the effect of semiquinone concentration by using the concentration of radicals at the start of the decay period.

Phenolic Browning Assay

A spectrophotometric measure of the rate of browning of a low-molecular-weight phenolic (Misra and Fridovich, 1972) was adapted to measure the browning of tannins with a microplate reader. Samples were weighed as described above, but were dissolved in 400 μ l of 70% ethanol (nitrogen-purged), and kept under a nitrogen atmosphere until used. Purified tannins were serially diluted 1:1 with 70% ethanol to produce a series of four tannin concentrations that were kept under nitrogen. The same pH 10 buffer used for EPR was used in this assay, but it was not bubbled with nitrogen, because oxygen was necessary for the formation of brown pigments within a 3-min period. Aliquots (20- μ l, 3 replicates) of each sample solution were placed in a 96-well microplate (3 columns per run). Phenolic concentrations in the reaction mixture typically ranged from 0.01 to 0.1 mM, and oxygen concentration was estimated to be about 0.25 mM, based on saturation levels at typical room temperature and pressure. To start the reaction, 180 μ l of pH 10 buffer were added to each well with an 8-channel multiple pipetter. Absorbance was measured at 415 nm, beginning 15 sec after the addition of the buffer. Subsequent measurements were made with a kinetic protocol every 8 sec over a period of 3 min by using a Bio-Rad Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). The initial, linear rate of browning

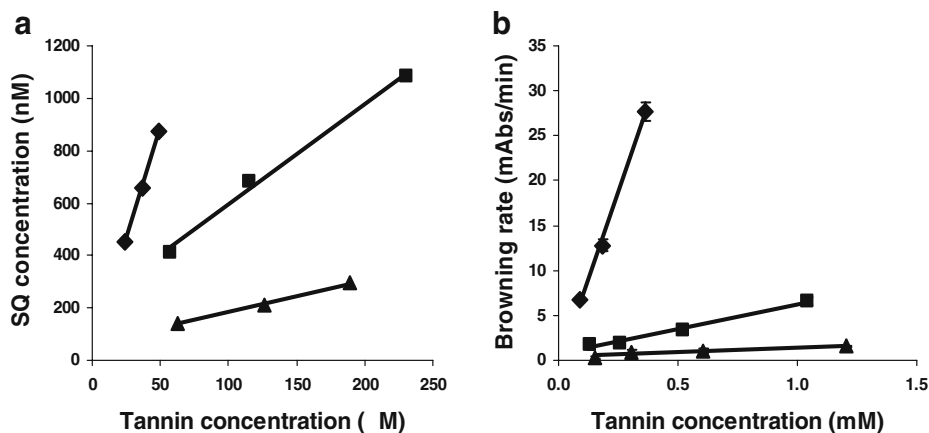


Fig. 3 Representative plots used to normalize semiquinone concentration (a) and browning rate (b) for the effect of tannin concentration. Tannins plotted include coccoiferin D₂ (ellagitannin; ♦), tetragalloyl glucose (■), and condensed tannin oligomers (▲). Slopes of these regressions represent the normalized values for each assay. Semiquinone concentrations are plotted for the single replicate analyzed per tannin concentration from one assay. Between two and three separate assays were run for each tannin. Mean browning rates are plotted from three replicate measurements per tannin concentration (\pm SEM) from one assay. Between two and seven assays were run for each tannin. Where not visible, the SEM is smaller than the symbol in (b)

(mAbs/min) was measured within the first 60 sec of the reaction. Between 2–6 replicate reactions were run for separately weighed samples. A pentagalloyl glucose standard was run on each day to confirm that measurements were consistent through time. Plots of browning rate vs. sample concentration (mM) were made for each sample to confirm that rate was a linear function of concentration for each compound (Fig. 3b). The slopes of these plots were used as the browning rates (normalized for sample concentration as mAbs/min/mM phenolic).

Statistical Analysis

The relationships between semiquinone concentration, semiquinone decay rate, and browning rate vs. the number of galloyl groups in galloyl glucoses were determined with Model II regressions (Wilkinson, 2000). The associations between semiquinone decay rate and semiquinone concentration, and between semiquinone decay and browning rates for each of the tannin groups were examined with correlations (Wilkinson, 2000). Because of non-normal data and small sample sizes, comparisons of mean semiquinone radical concentrations, semiquinone decay rates, and browning rates between tannin groups were made by using Kruskal–Wallis nonparametric tests (Wilkinson, 2000). All summary statistics are presented as the mean \pm SEM.

Results

Ellagitannins, galloyl glucoses, and gallotannins produced EPR spectra similar to those reported for other phenolics (Bors et al., 2000), with little hyperfine structure (Fig. 2). Condensed tannins produced spectra with broad line shapes and much smaller amplitudes than those of hydrolyzable tannins. The maximum semiquinone concentration produced and the rate of browning differentiated the three tannin groups (Figs. 4a,c and 6a,b). In general, ellagitannins formed the highest concentrations of semiquinone radicals, whereas galloyl glucoses and condensed tannins formed lower levels of semiquinones. Semiquinone decay rates did not differ significantly between the tannin groups, although the high decay rate of one ellagitannin, vescalagin, suggested that there may be substantial variation within this tannin group (Fig. 4b). Higher semiquinone radical concentrations were formed by more highly esterified galloyl glucoses ($R^2 = 0.88$, $P < 0.05$; Fig. 5a), the opposite of the relationship between the rate of decay of galloyl glucose semiquinones and the number of galloyl groups ($R^2 = 0.96$, $P < 0.05$; Fig. 5b).

The browning rates of ellagitannins were generally higher than those of galloyl glucoses and high-molecular-weight gallotannins, which generally browned at higher rates than did condensed tannins (Figs. 4c and 6b). Among the galloyl glucoses, browning rates decreased as the number of galloyl groups increased ($R^2 = 0.83$, $P < 0.05$; Fig. 5c). Oenothin B, the only ellagitannin tested that had both HHDP and free galloyl groups, browned more slowly than did the other ellagitannins. Among the condensed tannins, the prodelphinidin-rich polymeric condensed tannins had an unusually high browning rate. In general, tannins that formed higher concentrations of semiquinones also browned more rapidly, but there were contrary examples within the hydrolyzable tannins, such as vescalagin and oenothin B. Consistent relationships were observed for each of the three types of tannins between the measures of oxidative activity: A negative association between semiquinone radical decay rate and semiquinone concentration was observed for ellagitannins ($r = -0.88$), galloyl glucoses ($r = -0.89$), and condensed tannins ($r = -0.48$), whereas semiquinone radical

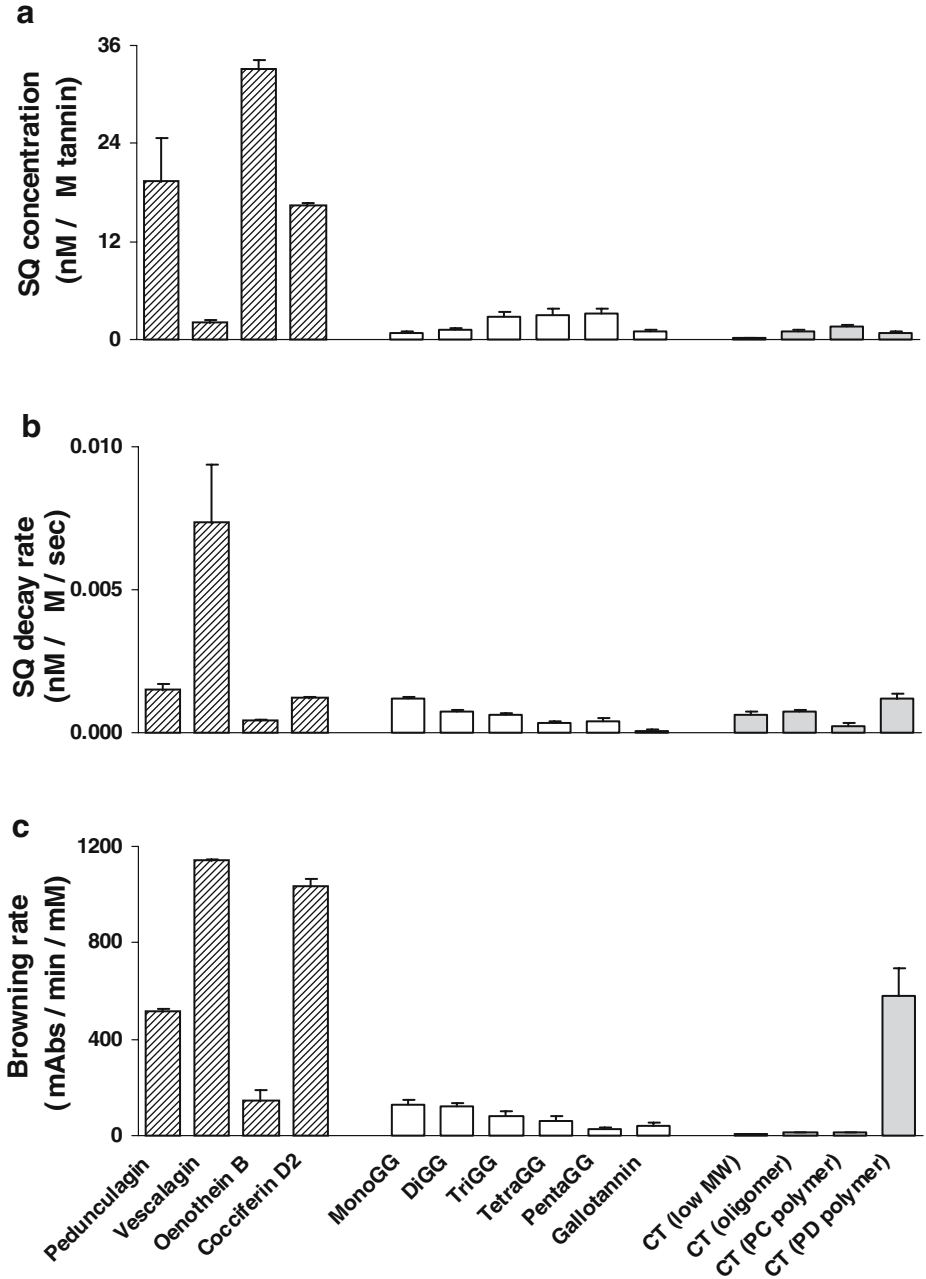


Fig. 4 (a) Semiquinone radical concentrations formed by ellagitannins, galloyl glucoses (and high-molecular-weight gallotannins), and condensed tannins at pH 10 (anoxic conditions). (b) Tannin semiquinone radical decay rates. (c) Browning rates of tannins at pH 10 (ambient oxygen level). ▨, ellagitannins; □, galloyl glucoses; ▤, condensed tannins. Data are presented as mean ± SEM (*N* = 2–7 assays per tannin). SQ, semiquinone radical; GG, galloyl glucose; CT, condensed tannin; PC, procyanidin; PD, prodelphinidin

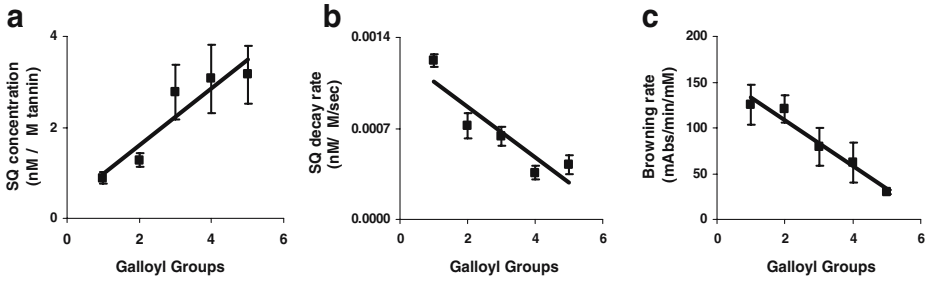


Fig. 5 (a) Relationship between the number of galloyl groups on galloyl glucoses (not including high-molecular-weight gallotannins) and semiquinone concentrations ($R^2 = 0.88$). (b) Relationship between the number of galloyl glucoses and semiquinone decay rates ($R^2 = 0.96$). (c) Relationship between the number of galloyl groups on galloyl glucoses and browning rates ($R^2 = 0.83$). Galloyl glucose semiquinone concentrations and browning rates were normalized for the initial molar concentrations of the galloyl glucoses. Semiquinone decay rates were normalized for the initial semiquinone radical concentrations. Data are presented as mean \pm SEM ($N = 2-7$). SQ, semiquinone radical

decay rate and browning rate were positively associated among ellagitannins ($r = 0.69$), galloyl glucoses ($r = 0.86$), and condensed tannins ($r = 0.82$).

To relate our results both to chemical mechanisms and ecological interactions, mean semiquinone concentrations and browning rates were compared on a molar and mass concentration basis (Fig. 6a,b). Semiquinone decay rates cannot be normalized on a mass basis. Similar values were obtained when the data were normalized based on mass ($\mu\text{g/ml}$) or molarity (μM) for most of the tannins tested, as expected for compounds with an average molecular weight of 1130 Da. The prodelphinidin-rich condensed tannin (average MW= 9026 Da) was a notable exception, with a browning rate that was nine-fold lower when

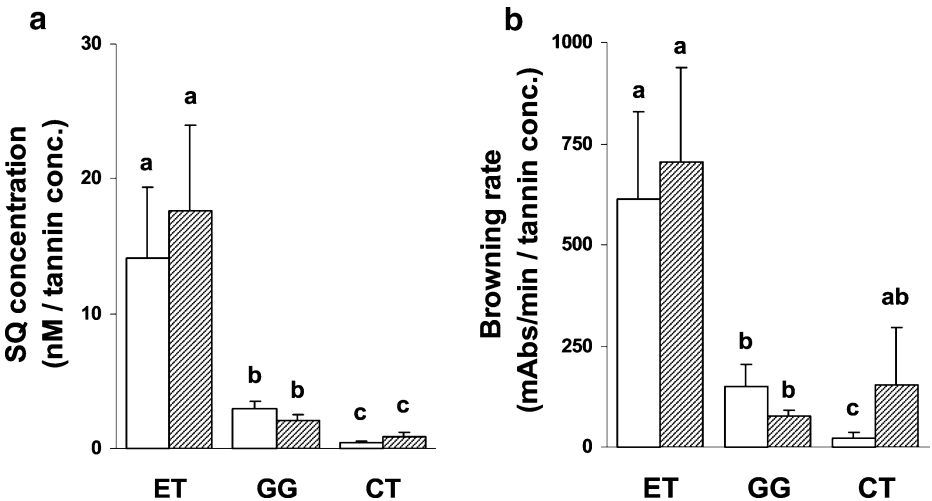


Fig. 6 Semiquinone radical concentrations (a) and browning rates (b) of ellagitannins, galloyl glucoses (including gallotannins), and condensed tannins when normalized for tannin concentration on a per mass basis ($\mu\text{g tannin/ml}$) or a molar basis. Data are presented as mean \pm SEM ($N = 4-6$). Different letters within each method of comparison indicate significant differences between tannin types. ET, ellagitannin; GG, galloyl glucose; CT, condensed tannin. \square , $\mu\text{g/ml}$ basis; ▨ , μM basis

normalized by mass concentration than when normalized by molarity. Thus, each method generally produced a similar pattern of oxidative activity among the tannin groups: ellagitannins > galloyl glucoses > condensed tannins.

Discussion

This study provides the first comparison of the oxidative activities of a wide variety of tannins at pH 10. Under these conditions, ellagitannins typically form high levels of semiquinone radicals and brown at high rates compared with galloyl glucoses, gallotannins, and condensed tannins. Because the oxidation of phenolics produces reactive oxygen species that can damage biological molecules, the results suggest that ellagitannin-rich plants have active oxidative defenses against herbivores with high gut pH. This expectation is consistent with the production of higher levels of peroxides and oxidized proteins in caterpillars that fed on sugar maple (ellagitannin-rich) compared with those that fed on red oak (condensed tannin-rich; Barbehenn et al., 2005).

An important conclusion of this study is that structural features of tannins affect their oxidative activities. Although the number of tannins examined was small, the results suggest some structure–activity mechanisms. Ellagitannins have oxidatively linked galloyl groups, which limit their ability to form resonance-stabilized radicals and potentially increase their oxidative activities (Wright et al., 1997; Bors et al., 2001b; Ito et al., 2002; Fukuhara et al., 2003; Quideau et al., 2004). Second, condensed tannins with a trihydroxyl substitution pattern on the B-ring (prodelphinidins) have greater browning rates than those with an *ortho*-dihydroxyl substitution pattern (procyanidins). This is consistent with previous findings of greater redox activity in pyrogallol- or galloyl-containing phenolics (Yoshioka et al., 1991; Cao et al., 1997; Guo et al., 1999; Okuda, 1999a,b; Fukumoto and Mazza, 2000; Mukai et al., 2005). The browning rate of gallic acid (a trihydroxyl phenol) was also 50-fold higher than that of chlorogenic acid (an *ortho*-dihydroxyl phenol; unpublished data).

In general, the EPR and browning assays produce similar rankings of oxidative activities among tannins (Fig. 6a,b). However, there are also differences between the two assays for certain compounds, e.g., oenothin B forms high concentrations of semiquinone radicals but has a low browning rate. In contrast, vescalagin forms low concentrations of semiquinones but has a high browning rate. These differences may be the result of using assays that are run under different oxygen concentrations and measure different oxidation products. EPR spectrometry measured the primary and secondary semiquinone radicals formed by phenolics under anoxic conditions, whereas the browning assay measured quinones and polymeric pigments under ambient oxygen conditions (Waite, 1976; Cilliers and Singleton, 1989; Grace et al., 1999). However, it is possible that the results of the three measures of oxidative activity are consistent with an oxidation pathway leading from phenolic to semiquinone radical to quinone. The negative correlation between semiquinone radical concentration and decay rate that was observed for each of the groups of tannins would be expected if rapid radical decay rates prevented the accumulation of high radical concentrations. Similarly, the positive correlation between semiquinone radical decay rate and browning rate that was observed for each of the groups of tannins would be expected if semiquinone radicals decayed to form brown quinones and other oxidation products. Although our data do not provide explicit support for this pathway, because radical and browning reactions were studied in different oxygen conditions, radical intermediates previously have been proposed for browning pathways for phenolics (Bors et al., 2000).

Further work is needed to explore the above relationships between measures of phenolic oxidative activity and the extent of oxidative damage to biologically important molecules. The formation of high concentrations of semiquinone radicals in a pH 10 buffer does not necessarily mean that a tannin would act as a prooxidant in a biological system. For example, although oenothin B forms high semiquinone concentrations, it might act as an antioxidant at high pH because its radicals are relatively stable (Bors and Michel, 1999). If this was the case, it would be reflected by the formation of low levels of oxidized markers, much like the formation of relatively low levels of brown oxidation products by oenothin B. In general, one would expect to find that (1) tannins with higher oxidative activities produce higher concentrations of biomarkers of oxidative damage, and (2) measures of oxidative activity that are most relevant to the mechanisms of oxidative damage are more strongly correlated with oxidative damage than are less relevant measures.

Polyphenolic compounds are often regarded as excellent antioxidants, in part because they can act as effective radical scavengers. The unusually long half-lives of the semiquinone radicals produced by tannins in the high pH, anoxic conditions that were employed in this study (1–24 min) are one indication of this potential (Pryor, 1986; Bors et al., 2001a). However, there is little in common between previous comparisons of radical scavenging by tannins and our measurements of their oxidative activities. For example, galloyl glucoses scavenged DPPH radicals in methanolic solutions at least as effectively as ellagitannins (Vuorela et al., 2005). Similarly, galloyl glucoses and monomeric ellagitannins had equivalent superoxide anion and DPPH radical-scavenging abilities (Hatano et al., 1989). In contrast, ellagitannins in our study were generally more susceptible to oxidation to semiquinone radicals and quinones than were gallotannins. The different conclusions produced by these different comparisons emphasize the need to examine phenolic activities in physiologically relevant conditions.

The high antioxidant or prooxidant activities of high-molecular-weight tannins is in part a result of the increased number of phenolic hydroxyl groups present per molecule as molecular weight increases (Hatano et al., 1989; Bors and Michel, 1999). For the lower-molecular-weight condensed tannins, radical production and browning rate are largely a function of the number of catechol B rings (Fig. 4a). However, the high-molecular-weight, prodelphinin-rich condensed tannins formed lower concentrations of semiquinone radicals and browned more rapidly than expected based solely on their degree of polymerization. Structural factors such as intramolecular resonance stabilization and coupling reactions may affect the oxidative activity of high-molecular-weight phenolics. Other features of tannins that potentially affect their oxidative activities include their redox potential and steric factors (Musso, 1967; Hodnick et al., 1988; Jovanovic et al., 1995; Chan et al., 1999; Guo et al., 1999; Mukai et al., 2005). For example, the fact that browning rate decreases linearly as the number of galloyl groups increases for the galloyl glucoses (Fig. 5c) suggests that intramolecular reactions, such as coupling, may take place rather than browning when several properly oriented galloyl groups are found on a single glucose. Further work is needed to examine the structural basis for the widely varying oxidative activities of tannins, with an emphasis on testing a larger number of examples of the different types of ellagitannins and condensed tannins.

Because both the number of phenolic hydroxyl groups and unknown structural features of tannins affect their prooxidant activities, it is not surprising that activities calculated on a mass basis yield somewhat different relationships than activities calculated on a molar basis. This was most apparent in the case of condensed tannins with high masses (e.g., 9026 Da), and would be particularly relevant in many ecological studies, in which tannin levels are

reported as a percentage of total mass. To understand the potential ecological and evolutionary significance of tannin composition, it is important to consider the impact of different tannin structural types on herbivores on a mass basis. For a given mass of tannins produced by a plant, and ingested by a caterpillar, the results suggest that ellagitannins would be most effective as oxidative defenses. By contrast, galloyl glucoses and condensed tannins would be less effective (Fig. 6a,b).

If we have correctly identified the relative reactivities of the major groups of tannins in high pH conditions, then it follows that the synthesis of substantial amounts of such different types of tannins by plants is the result of diverse selective pressures (e.g., Hagerman and Robbins, 1993; Northrup et al., 1998; Aerts et al., 1999). Our results not only point to the need to differentiate among several major groups of tannins in studies of plant–herbivore interactions, but also show that there can be a large amount of variation within the groups. Thus, some caution would be necessary when generalizing about the oxidative activities of leaves in which different tannin groups are quantified, but the composition of the tannins within each group are unknown. A better understanding of the structure–activity relationships of tannins should lead to new models for plant chemical defenses and herbivore adaptations to these defenses.

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Keywords Community · Condensed tannins · Developmental trajectory · Ontogeny · Phenolic glycosides · Phytochemistry · *Populus*

Introduction

Plant chemistry affects the behavior, distribution, performance, and population dynamics of herbivores (Frankel, 1959; Schultz, 1988; Bernays and Chapman, 1994; Seldal et al., 1994; Karban and Baldwin, 1997; Larsson et al., 2000; Shelton, 2000). Variation in chemistry among plants within a species results from genetic and environmental causes. Concentrations of many plant antiherbivore compounds, such as phenolic glycosides and condensed tannins, are under genetic control (Orians et al., 1993; Hwang and Lindroth, 1997; Lindroth et al., 2002), but also respond to environmental factors such as resource availability (Kinney et al., 1997; Lindroth et al., 1997; Orians et al., 2003).

Plant development can drive the distribution and/or performance of both pathogens and herbivores (Zagory and Libby, 1985; Kearsley and Whitham, 1989; Kearsley and Whitham, 1998; Karban and Thaler, 1999; Brennan and Weinbaum, 2001; Swihart and Bryant, 2001; Lawrence et al., 2003; Barrett and Agrawal, 2004) and can have community-level consequences (Waltz and Whitham, 1997). Well-characterized major cellular, physiological, and structural changes occur as plants develop (Cronk et al., 2002; Srivastava, 2002), but much less is known about the effects of development on plant chemistry (Edwards, 1982; Baldwin, 1999; Ohnmeiss and Baldwin, 2000). Some work has demonstrated higher levels of chemical defenses in juvenile shoots or ramets of the Salicaceae (Tahvanainen et al., 1985; Reichardt et al., 1990; Martinsen et al., 1998), but each of these studies was limited to a single tree species. Most other studies of the interaction of plant development and chemistry in trees have measured phytochemical concentrations in leaves of different ages within small saplings (e.g., Bingaman and Hart, 1993; Kleiner et al., 2003), as opposed to the variation that exists at any given time in mature, canopy-sized trees.

Here, we focus on plant development as a major source of phytochemical variation within individual trees. Developmental processes in plants can be generalized into two broad categories, ontogenetic and physiological or environmental (e.g., Lawson and Poethig, 1995). Ontogenetic variation arises from changes in gene expression in plant meristems (Poethig, 1990; Wiltshire et al., 1994, 1998; Jordan et al., 1999, 2000). It is widespread and results in changes in vegetative structures across whole-plant gradients (Jones, 1999). In contrast, environmental changes result from alteration of the local meristem environment by factors such as shading, water, and nutrient relations. This distinction is important, because only ontogenetically derived traits are heritable and subject to natural selection. Few studies have actually quantified the ontogenetic component of developmental traits that affects herbivores (but see Lawrence et al., 2003).

To assess developmentally based variation in plant chemistry, we studied the phytochemical variation in a hybrid swarm composed of Fremont and narrowleaf cottonwood (*Populus fremontii* and *P. angustifolia*, respectively), their F₁ hybrids, and complex backcrosses to narrowleaf cottonwood. Collectively, these are referred to as “cross types” (Wimp et al., 2004). Fremont cottonwood occurs at lower elevations, narrowleaf at higher elevations. The two species hybridize naturally in zones where their distributions overlap throughout the western United States (Eckenwalder, 1984), and they dominate many riparian zones in the intermountain west. The results of previous community studies performed in this hybrid system motivated several of the hypotheses of this study.

First, based on the distribution and survival of common herbivores (Kearsley and Whitham, 1989, 1998; Waltz and Whitham, 1997), we hypothesized that plant phytochemistry varies predictably within a tree across three developmental zones (juvenile ramets, juvenile zone, and mature zone). Because Waltz and Whitham (1997) showed that the juvenile zone was intermediate in its arthropod community to the mature zone and juvenile ramets, we hypothesized that it would also have intermediate phytochemistry. Second, we hypothesized that this predictable within-tree variation, which we term “developmental trajectory in phytochemistry,” has a genetic basis and would differ among cross types. Third, because different cross types support different arthropod communities in the wild and in a common garden (Wimp et al., 2005), we hypothesized that phytochemistry differs predictably among cross types (Fremont, narrowleaf, and F₁ and backcross hybrids). Last, because the leaf feeding beetle, *Chrysomela confluenta*, sequesters toxic phenolic glycosides and feeds preferentially on juvenile ramets, we predicted that juvenile ramets would be highest in phenolic glycosides.

This work is unique in that we studied phytochemical variation on multiple scales simultaneously: seasonal, within and among species, as well as within individuals, by using mature, canopy-sized trees. We document developmentally based phytochemical differences within trees that exist at any given time, as opposed to only the differences that occur as a result of aging of leaves at a given position in the tree through a growing season. Ultimately, because plant development affects whole arthropod communities (Waltz and Whitham, 1997), these findings could provide additional genetic and phytochemical mechanisms for understanding biodiversity, community structure, and ecosystem processes.

Methods and Materials

Common Garden Survey

To test the genetic basis of differences in phytochemistry among cross types, we sampled trees in a common garden established in 1991 at the Ogden Nature Center, Ogden, UT, USA. This study included 11 Fremont, 11 F₁ hybrid, 34 backcross hybrid, and 13 narrowleaf trees. The particular genotypes included were chosen as a representative sample of each cross type. Only leaves from the juvenile zone were sampled. Collection dates as well as sampling, chemical, and statistical methods were the same as for the field study, described below.

Field Survey

We studied cottonwoods growing along the Weber River in the vicinity of Ogden, UT, USA, where many population, community, and ecosystem-level studies have been conducted. We sampled leaves from trees at seven sites in the 13-km-long hybrid zone as well as from the pure species zones (one Fremont site, two narrowleaf sites) on three occasions: 21–24 May, 20–23 June, and 14–18 August 2001. At the seven sites in the hybrid/overlap zone, 2–4 of the cross types co-occurred at each site. Based on restriction fragment length polymorphism marker studies that genetically characterized the species or hybrid status of individual trees (Keim et al., 1989; Martinsen et al., 2001), our collection consisted of 10 Fremont, 14 F₁ hybrid, 13 backcross hybrid, and 13 narrowleaf trees. These were chosen based on their genetic characterization and presence of all desired developmental zones. All trees were reproductively mature in the upper crown, but juvenile at the base. Leaves were collected

from the mature and juvenile zones of all trees. All cross types, except Fremont, reproduce vegetatively by root suckering (Schweitzer et al., 2002), and leaves were also sampled from these small (<1.5 m tall) juvenile saplings that we refer to as “juvenile ramets,” reflecting their asexual origins. Thus, for the four cross types and three possible developmental zones, eleven combinations exist. Sampling from the different developmental zones of the same trees was carried out because one of our major aims was to test for and document phytochemical differences among zones of the same trees in the field.

Leaves from the mature zone were sampled with a 6-m-long pole pruner, and the reproductive status was verified by the presence of catkins and/or their scars. Each sample consisted of 15–25 leaves taken from each zone of the tree, and these leaves were collected from around the entire circumference of the canopy within that zone. Each leaf was removed by cutting at the lamina–petiole junction, and was the fifth or middle leaf from the current year’s growth to standardize leaf age. All leaf samples were frozen between blocks of dry ice and kept under dry ice until storage at -20°C . Leaves were lyophilized and ground to pass a 40-mesh screen on a Wiley Mill.

Nitrogen is often of critical importance to herbivores (Mattson, 1980; White, 1984), whereas condensed tannins and phenolic glycosides are the major secondary metabolites in the genus *Populus* (Palo, 1984; Lindroth et al., 1987). Nitrogen was measured by using an elemental analyzer (LECO, St. Joseph, MI, USA). After an exhaustive extraction of leaf tissue in 70% acetone with 1 mM ascorbate at 4°C , condensed tannins were determined with the acid butanol assay (Porter et al., 1986) by using tannins prepared from narrowleaf cottonwood as the standard (Hagerman and Butler, 1980; Waterman and Mole, 1994). Phenolic glycoside concentrations were determined by HPLC as described by Lindroth et al. (1993). The compounds salicortin and HCH-salicortin (hydroxy-cyclohexen-on-oyl-salicortin) are the major phenolic glycosides in this system (Rehill et al., 2005), but only Fremont cottonwood and F_1 hybrids contain detectable amounts of HCH-salicortin. Given this distribution, all analyses and figures for HCH-salicortin are based on data from Fremont and F_1 hybrids only. Both compounds were purified from cottonwood leaves by liquid–liquid extraction (Lindroth et al., 1986), followed by medium-pressure, “flash” chromatography (Still et al., 1978) and were used as standards. Salicortin has known antiherbivore properties (Lindroth and Hwang, 1996), and based on its structure the same is likely to be true of HCH-salicortin. Total phenolic glycosides were calculated as the sum of salicortin and HCH-salicortin. This pooled value has frequently been shown to affect herbivore preference and performance (e.g., Osier and Lindroth, 2001). All chemical measures are reported on the basis of percentage dry weight (% DW).

Data were analyzed by using repeated-measures analysis of variance [PROC MIXED, version 8, SAS (SAS Institute)] with an unstructured variance–covariance matrix and individual trees treated as subjects. Despite our explicit hypothesis for phenolic glycosides, we had no *a priori* assumptions about the trajectories of developmentally based variation for all the measured phytochemicals among all cross types. Thus, developmental zones were nested within cross type to prevent bias. Pairwise comparisons of least squares means were made with a Tukey adjustment and were considered significant when $\alpha < 0.05$.

Although some minor heteroscedasticity may have been present (e.g., compare Fremont vs. narrowleaf tannin concentrations and associated standard errors), we did not use any data transformations for several reasons. Valavanis (1959) indicates that for maximum likelihood methods (as used by SAS PROC MIXED), heteroscedasticity will not bias parameter estimates. Neter et al. (1996) and Montgomery (1997) both point out that as sample size increases, F tests approach permutation tests, which are distribution-free, and if total sample

size is not small, F tests can be considered in this light. Our total sample size was 420 observations for each chemical measured, with 50 test subjects (trees). With a sample size this large, we can invoke the Central Limit Theorem (Sokal and Rohlf, 1995), that the means of samples drawn from a population of any distribution will approach a normal distribution as sample size increases. Given the potential for somewhat ambiguous interpretation of transformed vs. untransformed data, we erred on the side of clarity of interpretation.

We used regression to make comparisons between cross types of the phytochemical shifts among developmental zones within trees. Because Fremont does not produce juvenile ramets, we used only juvenile and mature zone measures of condensed tannins, salicortin, and total phenolic glycosides in comparison among cross types. Each of these variables was regressed against the two developmental zones to determine the slope of the change (“developmental trajectory”), and slopes were compared. Thus, the slope indicates the direction and magnitude of the average change in concentration between the juvenile and mature zones of each cross type.

Results

Common Garden Survey of Phytochemical Variation

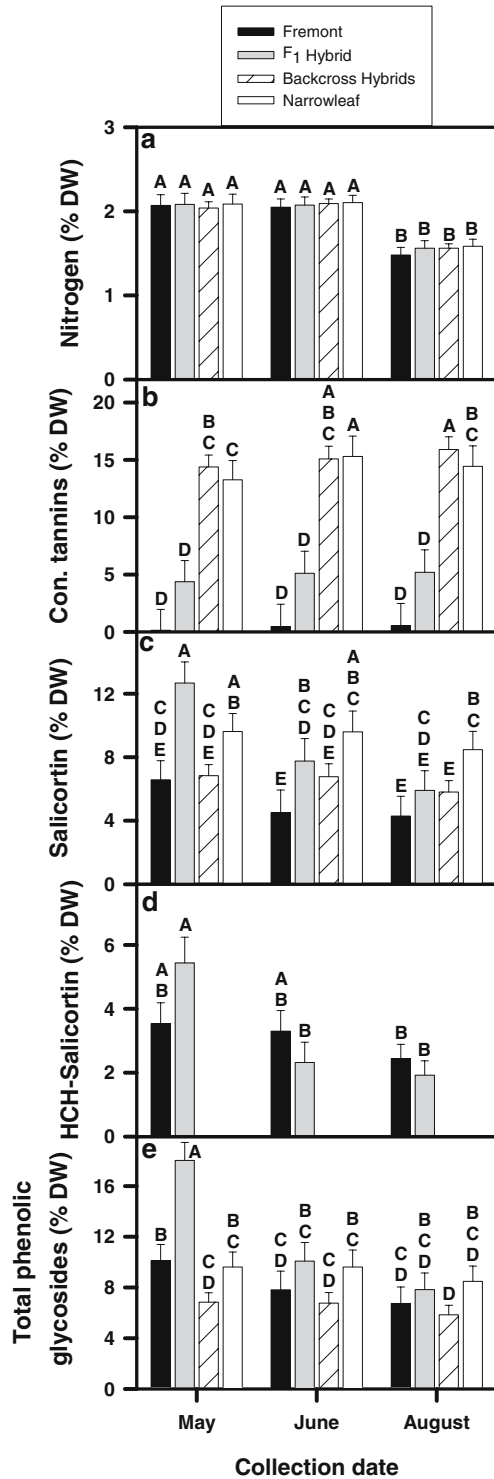
Condensed tannins, salicortin, and total phenolic glycosides in juvenile zone foliage differed significantly among cross types. Levels of seasonal variation differed widely among chemical measures and cross types.

Nitrogen concentrations were the same among cross types but did show significant seasonal variation (Table 1; Fig. 1a). Condensed tannin concentrations increased between the May and June collection dates and differed greatly among cross types (backcross = narrowleaf > F_1 = Fremont). In contrast, concentrations of salicortin decreased throughout the survey (Table 1; Fig. 1c), but also differed among cross types. These differences among cross types changed during the survey, likely as a result of different seasonal changes in each type (Fig. 1c). Concentrations of HCH-salicortin decreased during the survey (Table 1; Fig. 1d), but no consistent differences existed throughout the growing season between F_1 hybrids and Fremont cottonwood. Total phenolic glycoside concentrations, driven mostly by the concentrations of salicortin, decreased over the growing season (Table 1; Fig. 1e) and differed among cross types. Also, cross types differed in the amount of seasonal variation (see Fig. 1e).

Table 1 Type III tests of effects for the repeated measures anova of concentrations of nitrogen, condensed tannins, salicortin, HCH-salicortin, and total phenolic glycosides in the common garden survey

Effect	Nitrogen		Condensed tannins		Salicortin		HCH-salicortin		Total phenolic glycosides	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Collection date	126.45 _{2,65}	<0.001	8.73 _{2,65}	<0.001	29.99 _{2,65}	<0.001	8.35 _{2,20}	0.002	43.43 _{2,65}	<0.001
Cross type	0.02 _{3,65}	0.997	20.92 _{3,65}	<0.001	2.98 _{3,65}	0.037	0.04 _{1,20}	0.837	5.08 _{3,65}	0.003
Date × cross type	0.37 _{6,65}	0.894	1.14 _{6,65}	0.351	5.36 _{6,65}	<0.001	3.27 _{2,20}	0.059	9.58 _{6,65}	0.001

Fig. 1 Nitrogen, condensed tannin, salicortin, HCH-salicortin, and total phenolic glycoside concentrations in the common garden survey showing levels among cross types among the collection dates. HCH-salicortin was not measured in narrowleaf and backcross foliage. Each bar represents the least squares mean plus one standard error of the mean. Bars with different letters represent least squares means that are significantly different at $\alpha = 0.05$ using a Tukey adjustment



Field Survey: Chemistry

Statistical analysis of the effects of cross type, developmental zone, and collection date on foliar chemistry showed major response patterns, and revealed numerous higher-order interactions (e.g., cross type \times zone for a particular date; see Table 2) that occasionally varied from the dominant patterns. To facilitate clear communication of these results, we emphasize the major patterns for each chemical variable.

Nitrogen levels decreased slightly over the course of the growing season (Table 2; Fig. 2a). Concentrations of nitrogen did not differ consistently among cross types or developmental zones within each cross type (Fig. 2b).

Concentrations of condensed tannins did not differ among collection dates, but did differ among cross types and developmental zones, consistent with our hypotheses. Tannin levels varied greatly (ca. 40-fold) among cross types (Table 2; Fig. 2c). Backcross hybrid foliage had the highest concentrations, narrowleaf had intermediate concentrations, and F₁ and Fremont foliage both had very low concentrations (backcross > narrowleaf > F₁ \cong Fremont; 16.7%, 10.5%, 2.3%, and 0.4% DW, respectively).

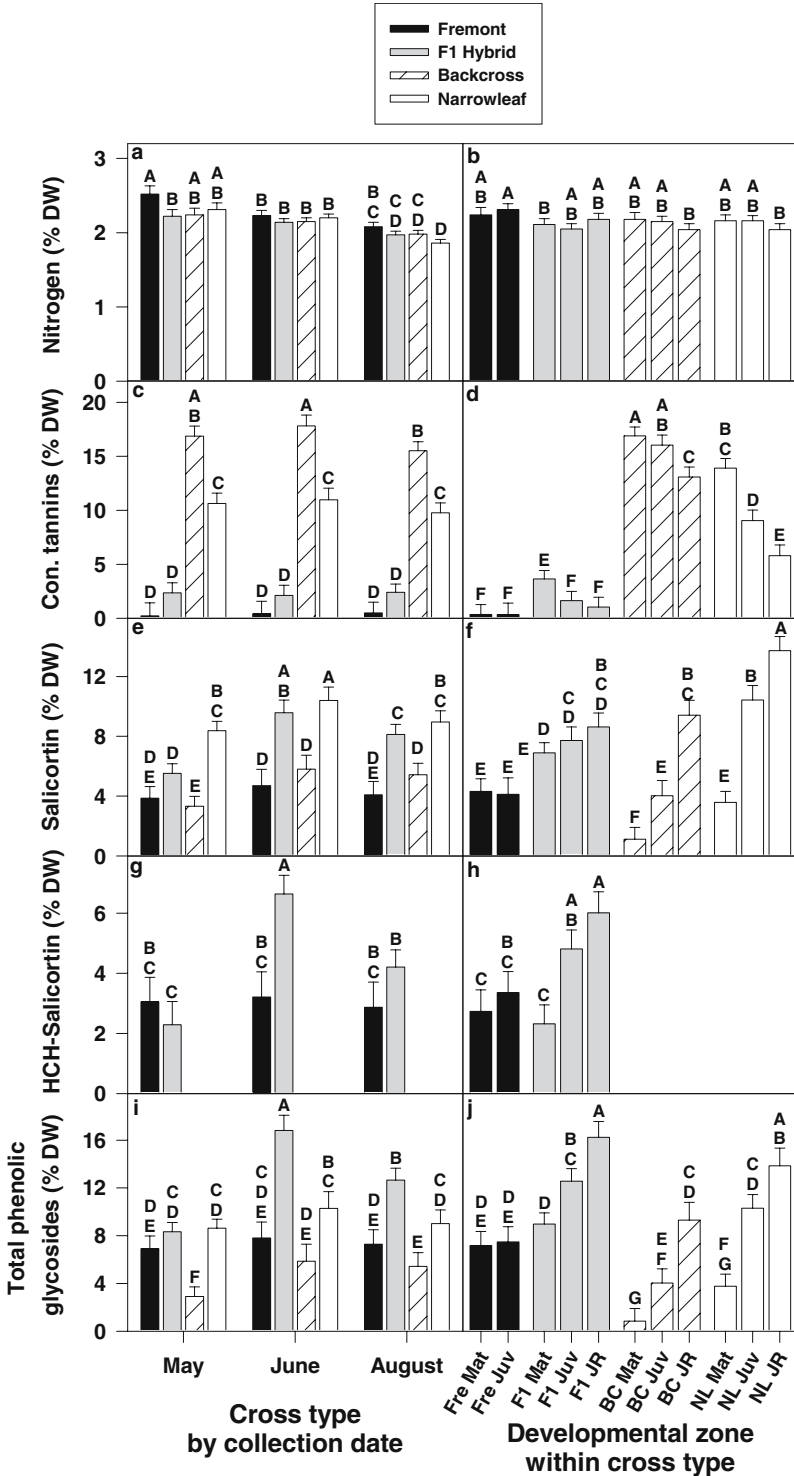
Tannin levels differed significantly among developmental zones for backcross hybrids, narrowleaf, and F₁ hybrids (Fig. 2d). These patterns among developmental zones within cross types changed seasonally (Fig. 3). Thus, relative to our initial prediction, the juvenile zone was frequently, but not always, intermediate in condensed tannin concentrations relative to the mature zone and juvenile ramets.

Salicortin concentrations showed strong seasonal variation, but as hypothesized, the levels also differed among cross types and within individual trees. Concentrations were lowest in May, peaked in June, and decreased to an intermediate level in August (Table 2; Figs. 2e and 4). Cross types separated into two groups: salicortin levels in both narrowleaf and F₁ hybrids were greater than those in backcross hybrids and Fremont (Fig. 2e).

Within all cross types except Fremont, salicortin concentrations differed among developmental zones, and the patterns were generally the opposite of those for condensed tannins (Fig. 2f). For both narrowleaf and backcross hybrids, the ranks of salicortin levels among developmental zones were the same (juvenile ramets > juvenile > mature). These results correspond well with our predictions of juvenile zone intermediacy and higher

Table 2 Type III tests of effects for the repeated measures anova of concentrations of nitrogen, condensed tannins, salicortin, HCH-salicortin, and total phenolic glycosides in the field survey

Effect	Nitrogen		Condensed tannins		Salicortin		HCH-salicortin		Total phenolic glycosides	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Collection date	77.03 _{2,48}	<0.001	2.33 _{2,47}	0.109	21.21 _{2,48}	<0.001	5.74 _{2,38}	0.007	15.39 _{2,48}	<0.001
Cross type	1.36 _{3,48}	0.266	83.31 _{3,47}	<0.001	11.08 _{3,48}	<0.001	3.71 _{1,24}	0.066	13.41 _{3,48}	<0.001
Date \times cross type	3.08 _{6,48}	0.013	1.46 _{6,47}	0.212	2.47 _{6,48}	0.037	4.25 _{2,38}	0.022	4.33 _{6,48}	0.001
Developmental zone (cross type)	0.97 _{7,48}	0.464	14.09 _{7,47}	<0.001	24.28 _{7,48}	<0.001	6.63 _{3,37}	0.001	20.34 _{7,48}	<0.001
Date \times developmental zone (cross type)	2.81 _{14,48}	0.004	7.46 _{14,47}	<0.001	4.22 _{14,48}	<0.001	1.29 _{6,49}	0.278	2.85 _{14,48}	0.004



◀**Fig. 2** Nitrogen, condensed tannin, salicortin, HCH-salicortin, and total phenolic glycoside concentrations of four cross types in the field survey. HCH-salicortin was not measured in narrowleaf and backcross foliage. The left set of panels (a, c, e, g, i) highlight differences among cross types among the collection dates for all developmental zones combined. The right set of panels (b, d, f, h, j) highlight differences among developmental zones within cross types for all collection dates combined. Cross types are abbreviated as follows: NL, narrowleaf; BC, backcross; F₁, F₁ hybrid; and Fre, Fremont. Developmental zones are abbreviated as follows: Mat, mature zone; Juv, juvenile zone; and JR, juvenile ramet. Each bar represents the least squares mean plus one standard error of the mean. Bars with different letters represent least squares means that are significantly different at $\alpha = 0.05$ using a Tukey adjustment *within the same panel only*

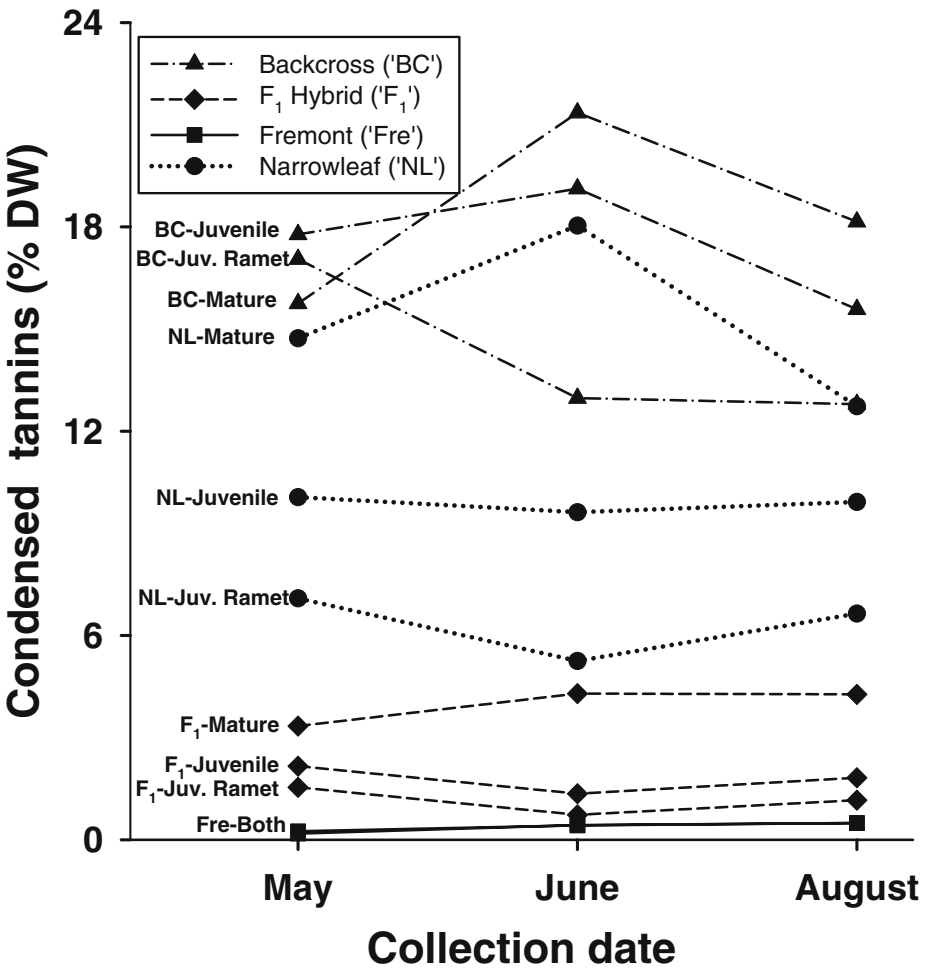


Fig. 3 Least squares means of condensed tannin concentrations for all developmental zones within cross types at the three collection dates. Cross types are abbreviated as follows: NL, narrowleaf; BC, backcross; F₁, F₁ hybrid; and Fre, Fremont. Data for both juvenile and mature zones of Fremont cottonwood are nearly identical, hence the designation “Fre-Both” for the superimposed lines. Differences among developmental zones for narrowleaf and both hybrid cross types account for much of the variation in the system

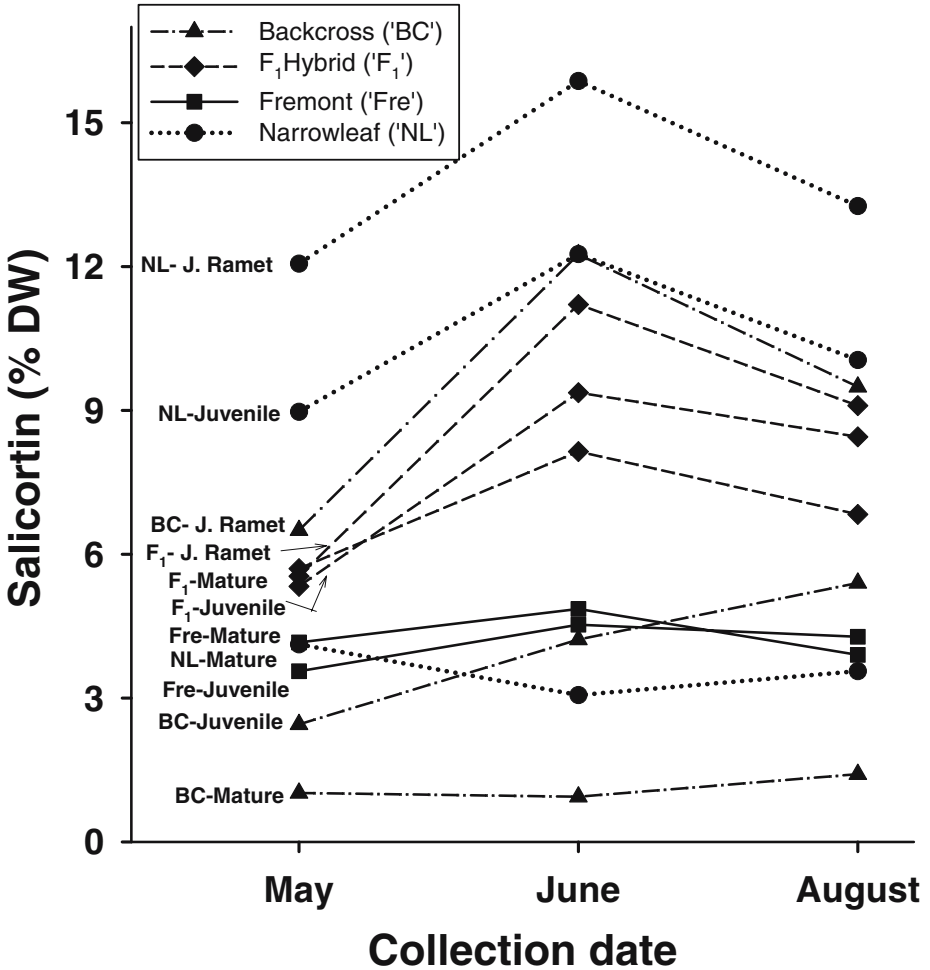


Fig. 4 Least squares means of salicortin concentrations for all developmental zones within cross types at the three collection dates. Cross types are abbreviated as in Fig. 3. As with condensed tannins, differences among developmental zones for narrowleaf and both hybrid cross types account for much of the variation in the system

phenolic glycoside concentrations in juvenile ramets. A similar but subtler pattern appeared in F₁ hybrids (juvenile ramets = juvenile, juvenile = mature, but juvenile ramets > mature). As with tannins, developmentally based differences varied among collection dates (see Fig. 4).

For salicortin concentrations, intraplant variation eclipsed interplant variation (Fig. 4). Whereas the greatest interplant variation encompassed a two-fold range (Fremont vs. narrowleaf, 4.2% vs. 9.3% DW, respectively), within narrowleaf salicortin concentrations varied over a four-fold range (3.6% vs. 13.7% DW, mature vs. juvenile ramet, respectively) and in backcross over an eight-fold range (1.1% vs. 9.4%, mature vs. juvenile ramet, respectively).

HCH-salicortin showed significant seasonal variation, which confounded differences in concentration among cross types. HCH-salicortin levels were higher in F₁ hybrids than Fremont in June only (Fig. 2g). Concentrations were the same between juvenile and mature foliage of Fremont, but highest in the foliage of juvenile zone and juvenile ramets of F₁

hybrids, both of which differed from F_1 mature foliage (Fig. 2h). Whereas HCH-salicortin concentrations among plants varied over a 1.5-fold range, and the difference was not statistically significant (F_1 vs. Fremont, 4.5 vs. 3.1%, respectively), within F_1 hybrids concentrations ranged over a 2.5-fold range, and the difference was statistically significant (6.2% vs. 2.5%, juvenile ramet vs. mature zone, respectively). Overall, the patterns in HCH-salicortin concentrations differed from those of condensed tannins and salicortin.

Composite concentrations of phenolic glycosides were lowest in May, peaked in June, and decreased to an intermediate level in August (Table 2; Fig. 2i), and differed significantly among cross types (Fig. 2j). Salicortin was the dominant or only phenolic glycoside contained in most foliage (ca. 66% in Fremont, 60% in F_1 hybrids, and 100% in narrowleaf and backcross hybrids). Thus, the patterns of differences among developmental zones within cross types were similar to those of salicortin, as were the shifts in the patterns among months. Narrowleaf and F_1 juvenile ramets had the greatest concentrations, whereas backcross and narrowleaf mature foliage both had the lowest. Also, as with salicortin and HCH-salicortin, intraplant variation driven by developmental trajectories exceeded interplant variation (contrast Fig. 2i with Fig. 2j).

Field Survey: Developmental Trajectories

Consistent with our prediction, developmental trajectories differed among cross types for condensed tannins and phenolic glycosides. Narrowleaf showed the greatest change in condensed tannin concentrations between juvenile and mature zones, F_1 was intermediate, and backcross and Fremont showed no change (respectively, $N = 63, 65, 74,$ and 59 ; $P < 0.05$ for all pairwise differences between slopes). A slightly different pattern was found for salicortin: narrowleaf showed the greatest change, backcross was intermediate, and F_1 and Fremont showed no change. Changes in total phenolic glycoside concentrations between juvenile and mature zones were greatest in narrowleaf, intermediate in hybrids, and least in Fremont. Overall, as predicted, the phytochemical trajectories of both the F_1 and backcross hybrids were intermediate to those of narrowleaf and Fremont cottonwood, supporting our hypothesis of an ontogenetic basis for these patterns.

Discussion

Genetic and Ontogenetic Control of Phytochemistry

The data from the common garden survey argue that both condensed tannins and salicortin (and so total phenolic glycosides) are under genetic control. Because HCH-salicortin is present only in the foliage of Fremont and F_1 hybrids (Rehill et al., 2005), but not narrowleaf or backcross hybrids, it, too, is under genetic control, at least at the level of presence/absence (likelihood ratio test: $G = 71.1, P < 0.001$). Differences among cross types in the common garden experiment (where only juvenile zone foliage was sampled) were identical (HCH-salicortin and total phenolic glycosides) or similar (condensed tannins and salicortin) to those of the juvenile zone in the field survey. It seems unlikely that field conditions, along an altitudinal gradient of the Weber River, duplicate the conditions of the common garden. The resemblance of common garden chemistry to the juvenile zone chemistry in the field would be consistent with genetic control of these secondary plant metabolites. Thus, in aggregate, the data indicate that the major defensive chemicals in this hybrid system—condensed tannins and phenolic glycosides—vary predictably among cross

types, establishing a genetic component to the phytochemical variation. The phenotypic variation in phytochemistry found among cross types in the field survey is likely to be genetically based.

The data do not constitute direct experimental evidence for genetic control of phytochemistry in the mature zone of the canopy, because the common garden study was carried out only with juvenile zone foliage. However, any uncertainty that this might introduce to our conclusions should be considered in the following light. First, if chemistry in the mature zone is under less genetic control than chemistry in the juvenile zone for all cross types, we would have expected a less consistent pattern of changes between developmental zones within cross types, given environmental heterogeneity in the field survey. Second, most plant secondary metabolites appear to be under at least some level of genetic control (Hamilton et al., 2001). Last, even if chemistry in the mature zone is more phenotypically plastic than that in the juvenile zone, and the observed differences between juvenile and mature zones occur as a result of microenvironmental variation, the increase in phenotypic plasticity would still constitute an ontogenetic shift in the control of chemistry, because phenotypic plasticity itself can be considered a trait under genetic control (Callahan et al., 1997).

In the field survey, although the specific patterns differed among phytochemicals, the overall pattern is of predictable changes both within an individual tree and among different cross types. In total, these changes constitute evidence for ontogenetically based developmental trajectories of phytochemistry in this *Populus* system. Several lines of evidence support this conclusion. First, plant development affected phytochemistry as much or more than other variables normally emphasized in phytochemical studies, such as seasonal variation. Second, although some or all of the cross types co-occurred at most of the field sites, different cross types exhibited major differences in both their phytochemistry and the developmental trajectories of specific phytochemicals. These findings argue for a genetic basis to both phytochemistry and developmental trajectory. Third, the magnitude of change both within an individual tree and among cross types was great, e.g., up to 40-fold differences in concentration. Fourth, condensed tannins, salicortin, and HCH-salicortin show drastically different patterns of change among cross types, indicating that the changes do not occur purely because of resource availability for secondary plant metabolites. Finally, in both studies, nitrogen concentrations showed little variation, whereas the other phytochemicals varied greatly. This argues against any changes in condensed tannins or phenolic glycosides as a result of differences in available nitrogen, leading to potential protein competition with phenolic metabolites (Jones and Hartley, 1999). Because these phytochemicals are ecologically important, the developmental trajectories have the potential to affect populations, communities, and ecosystems.

Plant Developmental Trajectory

We refer to predictable, developmentally based changes in plant traits (such as the phytochemistry we measured) as a plant's "developmental trajectory." For the sake of analysis, we concentrated on differences between the lower (juvenile) vs. upper (mature) portions of the canopy, and used the slope of the difference between these zones of the tree as a metric. The use of such a linear measure does not imply that all developmentally based changes occur in a gradual, linear fashion, or that all the traits of a given plant species change in the same manner or extent with development. Rather, this slope is intended only as a readily comprehensible means of referring to the change between developmental zones of the same cross type (if they occur), and of comparing the extent of change among cross

types. Further, we acknowledge that many developmentally based changes occur outside the scope of our measure (between juvenile and mature zones of a canopy sized tree). However, we concentrated on these developmental zones as a result of the potential major community and ecosystem consequences (most of a tree's biomass is in these zones) and for uniformity of comparison, because Fremont cottonwoods have no juvenile ramets.

We found striking differences in trajectories among cross types. Fremont cottonwood exhibits no significant trajectory, whereas narrowleaf cottonwood exhibits the steepest trajectory, and the hybrids are generally intermediate. The intermediate hybrid trajectories suggest an additive genetic mechanism to the phytochemical variation and argue that it has a genetic (i.e., ontogenetic) rather than environmental basis.

Developmental Trajectories and Community Structure

The findings suggest a potential phytochemical basis for the distribution of two herbivores in this system with known community effects, *Pemphigus betae*, a galling aphid, and *C. confluens*, a leaf-feeding beetle. For example, for *C. confluens*, which prefers to feed on juvenile ramets (Kearsley and Whitham, 1989), tannin levels explain ca. 50% of the variation in larval weight gain (Rehill et al., unpublished data), consistent with our finding of lower tannin concentrations in juvenile ramets. Concurrently, the foliage of juvenile ramets is also highest in phenolic glycosides, which larvae sequester and convert to their own defenses (Martinsen et al., 1998).

Developmental variation in phytochemistry may be one mechanism that could cause pronounced differences in arthropod communities associated with different developmental zones of the same tree, and this leads to two predictions: First, the greater the chemical differences among developmental zones, the greater the difference in the respective communities among those zones; Second, because phytochemistry differs among developmental zones, arthropod diversity in a stand of trees is a combination of the interplant genetic diversity (Wimp et al., 2004) as well as the intraplant ontogenetic diversity (see also Kearsley and Whitham, 1989, 1998; Waltz and Whitham, 1997; Lawrence et al., 2003). At present, we know of no studies on the relationship between developmental trajectories and herbivore communities. However, for both *Quercus velutina* and *Q. alba*, condensed tannin concentrations are higher in leaves of the upper canopy vs. those of the understory, and they explain ca. 25% of the variation in leaf-chewing insect community composition (Forkner et al., 2004).

Developmental Impacts on Ecosystem Processes

The marked increase in condensed tannin concentrations in mature foliage of narrowleaf and backcross trees, as well as the modest increase in F₁ hybrids, may have ecosystem consequences. Condensed tannins influence decomposition in this (Driebe and Whitham, 2000) and many other systems (Hattenschwiler and Vitousek, 2000; Hattenschwiler et al., 2003; Schweitzer et al., 2004). Increased tannin levels in the mature zone, which usually represents the majority of foliar mass in mature trees, may represent a means of controlling nitrogen cycling in the immediate environment of the tree (Northup et al., 1998; Van Breemen and Finzi, 1998; Kraus et al., 2003). Such control may be particularly advantageous to clonally propagating cross types, such as narrowleaf and backcrosses, as well as other clonal tree species (i.e., those that reproduce vegetatively), such as aspen (*Populus tremuloides*). Because leaves from ramets within a clone are more likely to fall and decompose under another member of the same clone (genotype) than nonclonal species

(i.e., an area effect), we predict that clonal species will undergo more pronounced developmental shifts in phytochemistry than will nonclonal species. Aspen has been found to have similar developmentally based chemical shifts to narrowleaf (Donaldson et al., 2006).

Evolutionary Pressures Leading to Genetic and Ontogenetic Differentiation

The reciprocal trajectories of phenolic glycosides (decreasing with maturity) and condensed tannins (increasing with maturity) in narrowleaf, backcross, and F_1 hybrids could represent a selective regime shaped by competing pressures (similar to aspen, Donaldson et al., 2006). Intense herbivory in the early life and lower portion of a tree's canopy may be balanced by the need to control nutrient cycling. This ensures adequate resources once sexual reproduction has begun. Such a pattern of competing selection pressures would be consistent with the shift from higher to lower phenolic glycosides from juvenile to mature foliage (better defended leaves in the lower canopy against herbivory) and the opposite trend for condensed tannins (thus, altering rates of decomposition and nitrogen mineralization).

The difference in the developmental trajectories of two congeneric trees (narrowleaf and Fremont cottonwoods) that have been in close proximity and/or had overlapping distributions for at least 12 million years (Eckenwalder, 1984) emphasizes that patterns of ontogenetic shifts in plant chemistry may be widespread. Phylogenetic constraints, however, seem unlikely to explain the flat trajectory of Fremont cottonwood. Selection pressures differ among environments for multiple reasons, such as the extent of herbivory, the costs of producing different defenses, and the availability of required resources such as minerals. Therefore, it seems likely that different selective regimes have led to different developmental trajectories. Developmental trajectories can be under genetic control (Wiltshire et al., 1998; Jordan et al., 1999), and variation in developmental trajectories within species may be an adaptation to environmental heterogeneity (Jordan et al., 2000).

The findings prompt a methodological caveat. Some studies of heritability compare traits of seedlings with those of mature trees. However, we have demonstrated major differences in ecologically important traits among the developmental zones of mature trees. Based on these data, if developmental trajectories of phytochemistry apply throughout the life of a tree, then the foliar chemistry of seedlings, and, therefore, resistance to natural enemies, may differ substantially from the mature zone of large trees. This may give an inaccurate representation of the genetic component of the studied traits, as others have suggested (Cornelissen et al., 2003).

Finally, given the established role of plant quality along with the potential for within-plant variation to affect herbivores (Stockhoff, 1993; Suomela and Ayres, 1994; Shelton, 2000), it is clear that developmental trajectories of phytochemistry could be important in regulating herbivore populations, structuring communities, and influencing ecosystems. More mechanistic studies are required to evaluate the community- and ecosystem-level importance of ontogenetically based chemical variation by linking the performance and distribution of keystone herbivores to plant chemistry. Such studies are a focus of continuing research with this hybrid system.

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Plants defend themselves against herbivores by using various mechanisms, including chemical ones, and defense has frequently been found to be most intense during the juvenile stage (Kozłowski, 1971; Bryant and Kuropat, 1980; Bryant et al., 1983; Reichardt et al., 1984; Tahvanainen et al., 1985; Mauricio, 2000). Boreal tree species, for example, have chemical defenses against feeding by moose, hares, and voles (e.g., Tahvanainen et al., 1985, 1991a), and the phenolic compounds found in birch (Hegnauer, 1989) are used in defense against herbivores (Palo, 1985; Palo et al., 1985; Tahvanainen et al., 1991a, b). They have been reported to affect food preferences of bank voles *Clethrionomys glareolus* (Hjältén et al., 1996), and phenolic substances on the base of birch bark may affect their palatability to voles (Rousi et al., 1993). However, there have been no studies of the role of phenolic compounds in birch seedlings as a defense against voles.

Voles of the genus *Microtus* are common in boreal forests at the early successional stage in Northern Fennoscandia (Hansson, 1977), but their population densities undergo cyclical fluctuations (Hansson and Henttonen, 1988). Their diet consists primarily of grasses, sedges, and forbs (Myllymäki, 1977; Ostfeld, 1985), but they also gnaw tree seedlings and saplings, and are an important cause of tree seedling mortality, especially during high-density years (Byers, 1984; Rousi et al., 1990; Hansson, 1977). For voles, tree seedlings are a poor-quality food source (Stenseth et al., 1977; Harju and Tahvanainen, 1997; Iason and Van Wieren, 1999) so they browse on them mainly in winter, when their preferred food sources are scarce (Rousi et al., 1993).

Voles seem to be selective when they feed on tree seedlings. Pusenius et al. (2002) found that field voles, *Microtus agrestis*, discriminated between different genotypes of silver birch, *Betula pendula*, seedlings, preferring to attack genotypes that had achieved competitive dominance in dense populations, i.e., those with the highest growth rate. Vole preference also correlated negatively with the genotypic mean of resin droplet density, suggesting that the chemistry of seedling bark is a factor in their choice. However, resin droplets are mostly found at the top of the *B. pendula* seedlings (Tahvanainen et al., 1991b) with fewer droplets in the lower part of the stems (Rousi et al., 1990; Bryant and Julkunen-Tiitto, 1995), where voles usually feed (Tahvanainen et al., 1991b; Rousi et al., 1997). On the other hand, both the upper and lower parts of the seedlings have similar concentrations of phenolics (Taipale et al., 1994) and we wanted to see whether these are responsible for variation in birch resistance to vole attack.

Variation in the size of seedlings on a spatial scale relevant to foraging voles (Hansson, 1968; Pusenius and Viitala, 1993) can be produced by intraspecific competition through at least two different mechanisms. First, in the process of becoming established, seedlings may be exposed to different existing local densities, yielding different mean seedling sizes among local patches, according to the self-thinning rule (Westoby, 1984; Crawley, 1997). Second, the differential success of individual seedlings within patches of competing seedlings may give rise to size differences, especially in high-density patches with asymmetrical competition (Weiner et al., 2001).

Production of secondary compounds is thought to be costly to a plant's energy and nutrient budget (Bryant and Julkunen-Tiitto, 1995), possibly even limiting its growth potential, and there is thus a potential trade-off between defense and growth (Rhoades, 1979). The costs of resistance are hypothesized to increase under resource-limiting conditions (Weis and Hochberg, 2000), so concentrations of defense compounds may vary with the density of competitors and the ability to acquire resources in competitive conditions, largely determined by seedling size (e.g., Tiffin, 2002).

The effect of intraspecific interactions on plant susceptibility to herbivores has been little studied (Hjältén et al., 1994). There have been several studies of the secondary chemistry of

silver birch (e.g., Laitinen et al., 2000, 2002), but nothing is known about the effects of intraspecific competition on the secondary chemistry of birch stems. This study focuses on intraspecific competition between silver birch seedlings and its effects on phenolic compounds, growth, and palatability to field voles. We studied the effects of the intensity of competition, as indicated by seedling density, and the effects of competitive success within populations, as indicated by seedling size, on quality and susceptibility to vole attacks. We asked the following questions: (1) How does intraspecific competition affect the growth of silver birch seedlings? (2) Do intraspecific interactions affect secondary chemistry and nitrogen content of the stems of the seedlings? (3) Do potential quality changes caused by intraspecific interactions affect the food selection of voles?

Methods and Materials

Birch Seedling Experiments

The study site was located in Punkaharju, southeastern Finland (61°41'N, 29°20'). Silver birch (*B. pendula*) seedlings were grown from a mixture of seeds collected from 27 randomly chosen, open-pollinated trees growing in a naturally regenerated birch forest. Seeds were sown in early May 2001 in peat pot trays, which disintegrate 3–4 wk after being planted, allowing plant roots to grow freely into the soil. Each tray was 0.4 × 0.6 m and consisted of 130 hexagonal pots, or cells, 5.1 cm wide and 7.5 cm deep in rows of 10. Homogenized peat (Kekkilä M6) was pressed into the cells and watered. Two days later, a mixture of seeds was sown randomly onto the trays. Sowing was carried out in a greenhouse to exclude external seeds. There was no artificial lightning or heating in the greenhouse, and the trays were watered as necessary. Five wk after sowing, trays were placed, still intact, onto a strip of bare soil in an abandoned field, and the seedlings were left to grow in natural conditions for one growing season. They were not watered, but weeds were removed from around them.

The seedlings established in each tray, or plot, constituted an experimental population. These populations were subsequently randomized for different treatments. Seedling populations were divided into two groups of 24, which were located close to each other. One group was subjected to a density manipulation (DM) experiment: Seedlings were thinned to obtain populations with different predominant heights. Densities chosen were based on prior experience (e.g., Pritinen et al., 2003) such that no significant size–hierarchy or seedling mortality would occur during the experiment. The other group was subjected to a competitive success (CS) experiment: A seedling density was allowed that was high enough for a clear size–hierarchy to be formed. In the DM experiment, thinning started about 2 wk after sowing, when the seedlings had established themselves. Seedlings were thinned to three different densities: low (one seedling in every other cell, 65 seedlings per plot, 270/m²), moderate (one seedling per cell, 128 seedling per plot, 533/m²), and high (two seedlings per cell, 260 seedlings per plot, 1083/m²). In thinning the populations to their final densities, we selected seedlings located at the centers of the cells. Where cells contained no seedlings or only weak ones, seedlings were transplanted from nearby cells. Each treatment was applied to eight randomly selected plots.

In the CS experiment, seedlings were not thinned at all. At the end of the growing season, the mean density was 429 per plot (1787/m²), which was 1.7 times greater than the highest density in the DM experiment. The processes of producing these densities were

different, and there was almost no mortality in the DM experiment, whereas mortality was high in the CS experiment.

The Vole Feeding Experiments

The voles used in the feeding experiments were trapped at Konnevesi, central Finland, in August 2000 (see Puseenius et al., 2002). They were housed in a 6×12 m outdoor enclosure containing nesting boxes and hay bales to provide shelter. Vegetation within the enclosure included natural food plants (Teivainen et al., 1986).

Our aim was to study whether voles choose among different-sized seedlings when the size difference is due to (1) density differences among stands, or (2) variable competitive success in a high-density population. Thus, we conducted two feeding experiments in the fall, when the seedlings were in winter dormancy. In the first, we allowed voles to choose among seedlings grown at the three different densities of the DM experiment. We constructed 15 replicated 0.4×0.6 m stands, each containing 10 randomly chosen seedlings from each of the three treatments, transplanted with their entire root ball. Each stand was enclosed within a 0.8×0.8 m sheet metal pen. One vole was introduced into each pen and allowed to feed. We monitored the feeding closely and recorded the order in which the three different classes of seedlings were attacked. The experiment was halted when at least two thirds of the seedlings had been attacked.

In the second experiment, we allowed voles to choose among different-sized seedlings from the CS experiment. Sixteen 0.4×0.6 m experimental stands were constructed, each containing 10 dominant, 10 medium, and 10 subordinate seedlings (see below). The other experimental conditions were identical to those of the first experiment.

Measurements

Seedlings from the DM experiment were harvested after the growing season, and their biomass, height, and root biomass were measured. Seedlings from the CS experiment were harvested in November, after the vole feeding experiment, when they were in winter dormancy. Because the ground was already frozen, it was not possible to recover roots. We randomly sampled seedlings from three distinct categories identified by their size in relation to the prevailing canopy within each experimental stand. The size–hierarchy categories were as follows: subordinate (well below the prevailing canopy), intermediate (prevailing canopy), and dominant (well above the prevailing canopy). We measured the height of the seedlings. The mean heights (\pm standard deviation) of dominant, intermediate, and subordinate seedlings were 15.2 ± 3.6 , 12.6 ± 2.3 , and 8.8 ± 0.9 cm, respectively.

Chemical Analysis

Sampling and Measurement of the Seedlings We randomly sampled two individual seedlings from eight different plots for each of the studied densities (DM experiment) and size–hierarchy categories (CS experiment). The total number of sampled seedlings in each case was 48: 3 densities \times 8 plots per density \times 2 seedlings per plot (DM experiment) and 3 size–hierarchy categories \times 2 seedlings per category per plot \times 8 plots (CS experiment).

Seedlings were collected for stem analysis in November, when the leaves had fallen off and they were in winter dormancy. All seedlings were stored in paper bags and left to dry at room temperature for 2–3 wk until the stems were dry and then stored in a freezer (-20°C).

Air drying was used on the basis of previous studies (e.g., Julkunen-Tiitto and Tahvanainen, 1989; Keinänen and Julkunen-Tiitto, 1996; Keinänen et al., 1999; Mutikainen et al., 2000; Julkunen-Tiitto and Sorsa, 2001). Forty-eight seedlings were taken randomly from the density experiment for measurements of their physical properties. They were taken from the ground with their roots intact and air-dried as described above. The dry weight of the roots and stems and the height of the stems were measured.

Stem Extraction Each stem sample was taken from two seedlings from each plot with the same density (DM experiment) or size category (CS experiment). A piece of stem (5 cm long) was cut 4 cm from the base of both seedlings and combined. Any buds were removed without breaking the stem. Stems were cut into 5 mm pieces, weighed, put into a 50 ml centrifuge tube, and stored in a freezer (-20°C). For extraction, 10 ml of methanol were added, the sample was homogenized for 2 min with an Ultra-Thorax homogenizer, and it was incubated on ice for 15 min. The residue was extracted three more times. Methanol was evaporated with a vacuum evaporator, and the dry sample was redissolved in 8 ml of methanol. Aliquots of 2 and 1 ml were taken for HPLC analyses and condensed tannins, respectively. The extraction residue was air-dried overnight and stored in a freezer. The internal standard was recorsin, which was added at the beginning of the extraction procedure (480 μg , ca. 99%, Ega-Cheme). The internal standard showed 74.9% recovery in the DM experiment and 74.8% in the CS experiment, respectively.

Colorimetric Test for Condensed Tannins The amounts of condensed tannins were determined with the colorimetric test (e.g., Porter et al., 1986., Julkunen-Tiitto and Sorsa, 2001). Condensed tannins were estimated as proanthocyanidins. The stem dry sample was redissolved in 1 ml of methanol. Six ml of butanol-HCl and 200 μl of iron reagent were then added to 100 μl of the stem sample. Samples were hydrolyzed in boiling water for 50 min and cooled. Absorbance at 550 nm was measured. Condensed tannins purified from *Betula nana* leaves were used as a standard.

HPLC Stem phenolic compounds were analyzed by HPLC. The HPLC system was a Hewlett-Packard HP 1050 instrument (Avondale, PA, USA) with a quaternary pump, a vacuum degasser, an autosampler, a thermostatic column oven, and a photodiode array detector (HP 1040A) combined with an HP Chemstation. A 3- μm HP Hypersil ODS column (60 \times 4.6 mm i.d.) was used. The solvents were aqueous 1.5% tetrahydrofuran + 0.25% orthophosphoric acid (A) and methanol (B). The solvent gradient was as follows: 0–5 min 100% A; 5–10 min 85% A, 15% B; 10–20 min 70% A, 30% B; 20–30 min 65% A, 35% B; 30–50 min 50% A, 50% B; 50–55 min 100% B; 55–60 min 100% A (Julkunen-Tiitto and Sorsa, 2001). The flow rate was 2 ml/min, and the injection volume 20 μl . The column oven was set at 30°C . The analysis was monitored at 220, 270, 280, 320, and 360 nm.

The phenolic compounds identified were quantified with commercial standards: myricetin-3-*O*-rhamnoside (Apin Chemicals, Oxon, UK) for myricetin derivatives, quercetin-3-*O*-glucoside (Extrasynthèse, Genay, France) for all quercetin and rhamnetin derivatives, kaempferol-3-*O*-rhamnoside (Extrasynthèse, Genay, France) for all kaempferol derivatives, chlorogenic acid (Aldrich, Steinheim, Germany) for cinnamic acid derivatives, salidroside (a gift from H. Prof. Thieme, Germany) for rhododendrine derivatives, and platyphylloside.

Eighteen individual compounds were found in stems (Fig. 1; Table 1). Identification of the compounds was based on comparisons of retention times and spectral characteristics (Keinänen and Julkunen-Tiitto, 1998). The compounds were grouped on the basis of their

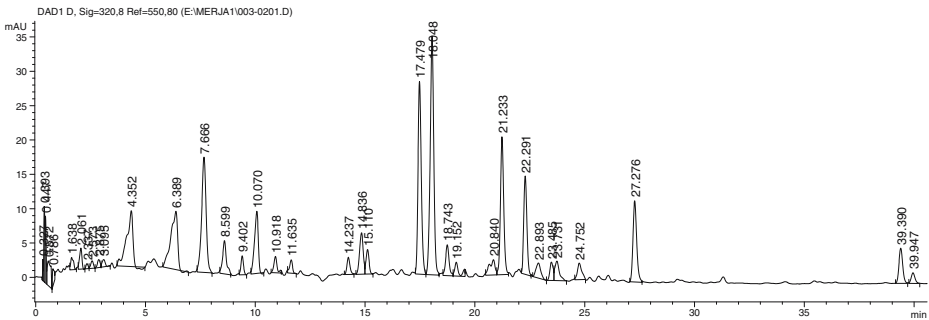


Fig. 1 HPLC chromatogram of stem phenolic compounds monitored at 320 nm

chemical derivatives. Stem phenolics were grouped as flavonol glycosides, chlorogenic acid derivatives, catechine derivatives, rhododendrin derivatives, platyphylloside, cinnamic acid derivative, and monocoumarylstragalol (Table 1). The total concentration of the phenolics was calculated by adding the concentrations of condensed tannins and individual compounds together.

Nitrogen Analysis The total nitrogen content of dried stems was analyzed. Stem samples of 40–190 mg were taken from directly above the part that had been taken for phenolic extraction and cut into smaller pieces (1–2 mm). Samples were analyzed with a nitrogen analyser (Leco FP-528). The Leco Corn Flour calibration sample (1.7% N \pm 0.03) was used as a standard.

Statistical Analyses

The effects of density on seedling height and stem and root biomass were analyzed by multivariate analysis of variance (MANOVA). Effects of density and competitive success (seedling size category) on the concentrations of individual chemical compounds and groups of compounds in the stems were analyzed by MANOVA. These groups included phenolics from the HPLC analysis that were categorized on the basis of their chemical derivatives, and condensed tannins. Where MANOVA revealed a significant effect of treatment, we made corresponding univariate ANOVAs. In addition, we analyzed the effects of density and competitive success on the total concentration of phenolics, summed across individual compounds, by univariate ANOVA. The normality of residuals for each compound and compound group was tested with the Kolmogorov–Smirnov test, and compounds with normally distributed concentrations were included in the MANOVA. The *F*-values of the MANOVAs were converted from Pillai's trace statistics. Certain compounds were detected in only one or two samples, or not at all, resulting in non-normal distributions for these compounds (Table 1). The effect of treatment on the concentrations of these was tested with Kruskal–Wallis nonparametric ANOVA, using Bonferroni-adjusted significance level to compensate for the large number of tests. Pairwise differences in height, biomass, and chemistry between different densities or height classes were assessed using a *post hoc* test (Bonferroni). The vole feeding preferences were tested with Friedman's nonparametric ANOVA. All analyses were conducted using the SPSS statistical package for Windows 11.0 (SPSS, Inc.).

Table 1 The detected phenolic compounds, their retention times and significances *B. pandula* obtained from univariate tests

Compound	Retention time (min)	DM		CS	
		<i>P</i>	Bon	<i>P</i>	Bon
Flavonol glycosides				<0.0005	
Myricetin-diglycoside	14.8	0.047	NS	0.011	NS
Myricetin derivate	15.1	0.366	NS	0.305	NS
Hyperin	17.4			0.017	
Quercetin-3-glucoside and glucuronide	18.0			0.037	
Quercetin-3-arabinoside	18.7			0.023	NS
Quercitrin	21.2			0.301	
Kaempferol derivate	23.7			0.013	NS
Chlorogenic acid derivatives				0.003	
Chlorogenic acid	7.9			0.005	
Chlorogenic acid derivate 1	22.3	0.629	NS	0.007	NS
Chlorogenic acid derivate 2	23.4	0.038	NS	0.124	NS
Chlorogenic acid derivate 3	24.7	0.038	NS	0.368	NS
Chlorogenic acid derivate 4	27.2	0.124	NS	0.751	NS
Catechine derivatives				<0.0005	
Catechin-glycoside	6.5			<0.0005	
(+)-Catechin	7.5			<0.0005	
Rhododendrin derivatives				<0.0005	
Rhododendrin 1	9.6	0.922	NS	0.742	NS
Rhododendrin 2	9.8			<0.0005	
Rhododendrin aglycone	11.1			<0.0005	
Cinnamic acid derivative	24,8			0.200	NS
Platyphylloside	20.2			0.003	
Monocoumarylastragalol	39.3			0.090	
Condensed tannins				0.115	

The analyses of compounds with non-normally distributed concentrations were performed with Kruskal–Wallis test and include a Bonferroni-corrected significance. The significances with no Bonferroni correction are based on univariate ANOVAs performed after a significant MANOVA. Significances are lacking in the case of a nonsignificant MANOVA. Bon: Bonferroni-corrected significance; DM: density manipulation experiment; CS: competitive success experiment.

Results

Density, Biomass, and Height Root and stem biomass, root-to-shoot ratio, and stem height varied among the populations grown at different densities created by thinning (MANOVA, $F_{8,38} = 6.90$, $P < 0.0005$). Univariate *F*-tests indicated a significant ($P < 0.05$) effect of density for all variables. All measures of seedling size decreased with increasing density. Root biomass differed between all studied densities (Bonferroni multiple comparison, $P < 0.05$; Fig. 2a). Stem biomass differed between low and moderate, and low and high densities (Bonferroni multiple comparison, $P < 0.05$; Fig. 2b). The differences in root and stem biomass between high-density and low-density seedlings were 2.5- and 2.0-fold, respectively. Root–shoot ratio and stem height differed between low densities and high densities, and between moderate densities and high densities (Bonferroni multiple comparison, $P < 0.05$; Fig. 2c and 2d). The mean height of seedlings from the CS experiment was less than that of DM seedlings, being 12.2 ± 2.6 cm, which was less than

the mean height of seedlings from the highest density (15.1 ± 3.9 cm) in the DM experiment. ($t = 3.39$, $df = 62$, $P < 0.01$; Fig. 2d).

The Effects of Density on the Secondary Compound and Nitrogen Concentrations of Stems MANOVA did not indicate any effect of density on concentrations of phenolic compounds in stems in the DM experiment ($F_{26,20} = 0.82$, $P = 0.69$), and density had no effect on the concentrations of the individual compounds with non-normal distributions (Kruskall–Wallis ANOVA with Bonferroni-adjusted significance levels, $0.05/7 = 0.0071$; Table 1). Neither was there any effect of density on the concentrations of groups of phenolic compounds (MANOVA, $F_{16,30} = 1.35$, $P = 0.24$). We also compared total phenolic concentrations, summed across individual compounds, in the seedlings grown at different densities. Density had an effect on the concentration of total phenolics in stems (ANOVA, $F_{2,21} = 4.64$, $P = 0.021$; Fig. 3a). Seedlings from low densities had higher concentrations of total phenolics than those from moderate or high densities (Bonferroni multiple comparisons, $P < 0.05$). The effect of density on nitrogen concentrations (% dry weight) in stems was marginally nonsignificant ($F_{2,21} = 2.83$, $P = 0.081$; Fig. 4a).

The Effects of Seedling Size on the Chemical Composition of Stems In the CS experiment, seedling size had a significant effect on the concentrations of total phenolics (ANOVA, $F_{2,21} = 6.56$, $P = 0.006$). The highest concentration of total phenolics occurred in the tallest seedlings and the lowest in the smallest ones (Fig. 3b). Nitrogen concentrations (%) varied among seedlings of different size classes (ANOVA, $F_{2,21} = 4.51$, $P = 0.023$). The highest nitrogen concentrations were found in the smallest seedlings and vice versa (Fig. 4b).

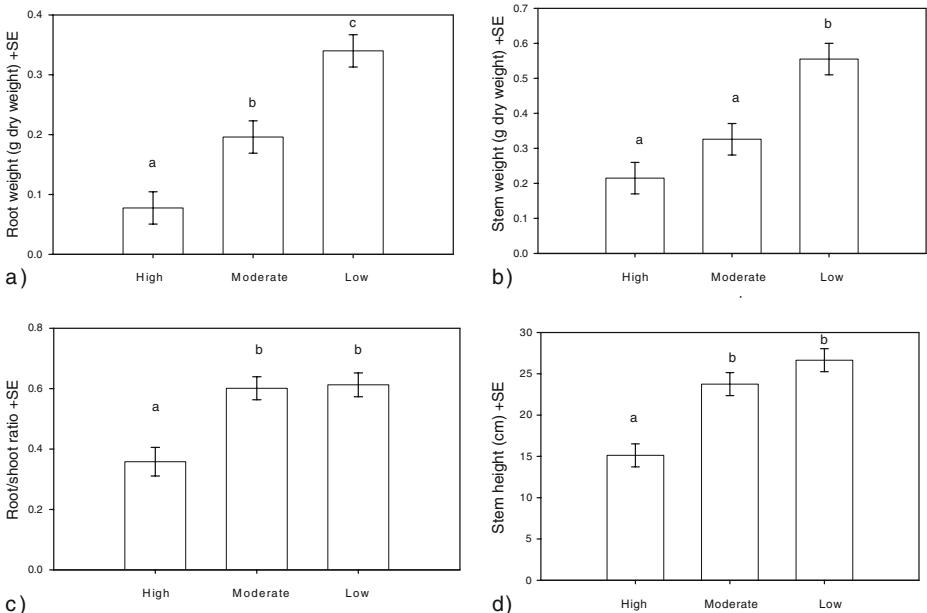


Fig. 2 Root weight (a), stem weight (b), root/shoot ratio (c), and stem height (d) in the three different densities of DM experiment. The means represented by bars with a same letter do not differ from each other (Bonferroni, $P < 0.05$)

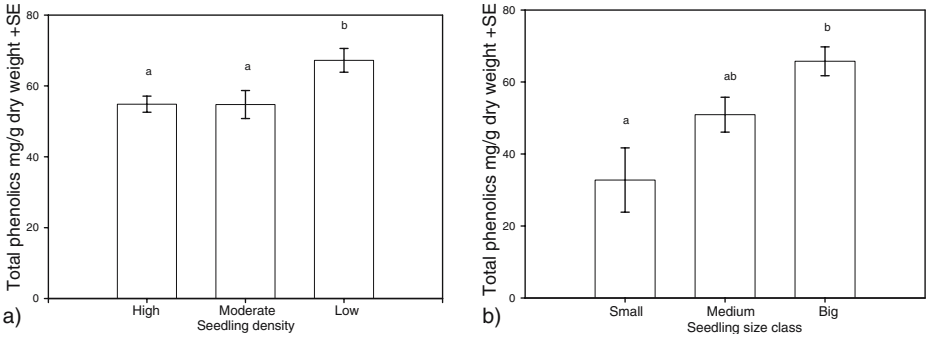


Fig. 3 Total phenolics concentration in stems: (a) DM experiment and (b) CS experiment seedlings

Hence, the pattern of variation in nitrogen in relation to density was the opposite of that observed for phenolic compounds.

Concentrations of phenolic compounds of stems varied among seedlings of different size–hierarchy categories (MANOVA, $F_{22,24} = 3.18$, $P = 0.003$). Univariate F -tests of the individual compounds (Table 1) and pairwise comparisons (Bonferroni, $P < 0.05$) revealed that the concentrations of catechin glycoside increased with seedling size (there were significant differences between all size classes). Concentrations of (+)-catechin, rhododendrin (2), and chlorogenic acid (A, B) were lower among small seedlings than among medium and large ones. The concentrations of rhododendrin aglycone were lower among small and medium seedlings than among large ones and the concentrations of hyperin, quersetin-3-glucoside, and platyphylloside were lower among small seedlings than among large ones. Seedling size had no significant effects on the concentrations of non-normally distributed individual compounds (Kruskal–Wallis ANOVA, Bonferroni-adjusted significance level $0.05/10 = 0.005$; Table 1). The concentrations of different compound groups also differed between the different-sized seedlings (MANOVA, $F_{16,30} = 2.42$, $P = 0.018$). Univariate F -tests indicate that there were differences in chlorogenic acids, flavonol glycosides, rhododendrins, catechins, and platyphylloside, but not condensed tannins, monocoumarylstragalol, and cinnamin acid derivative. In general, tall seedlings had higher concentrations than medium-sized or small ones (Fig. 5a–d).

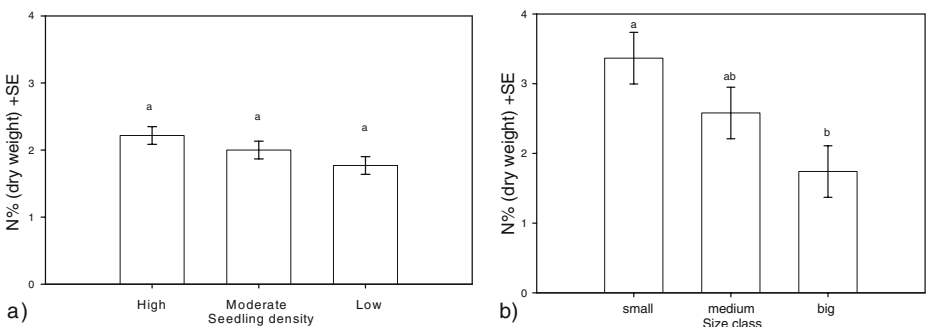


Fig. 4 Nitrogen content (% dry weight) in DM experiment (a) and in CS experiment (b) seedlings

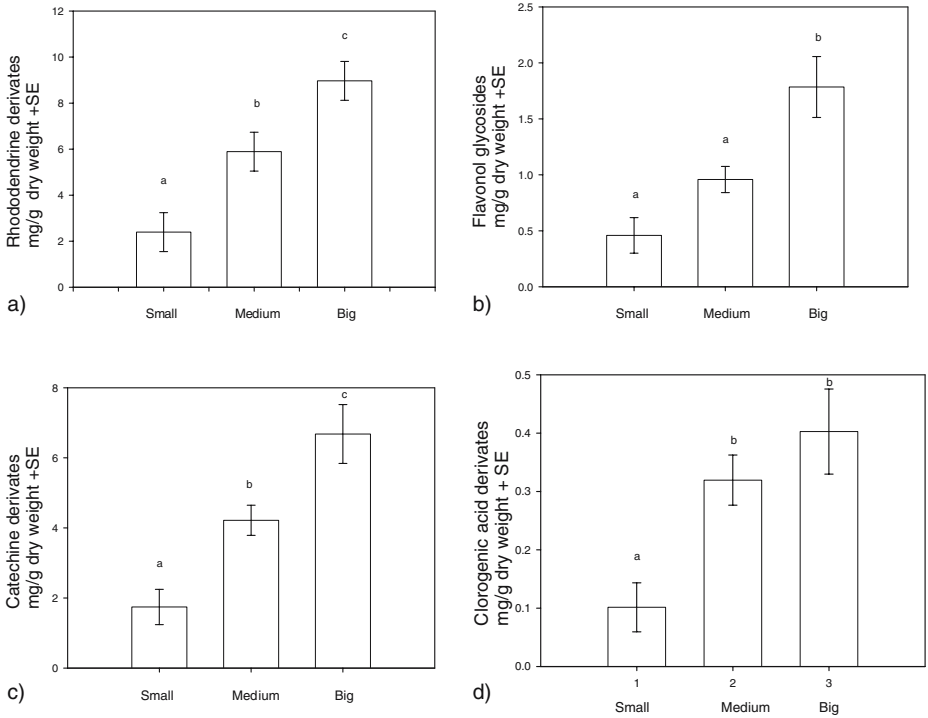


Fig. 5 Concentration (mg/g) of rhododendrin derivatives, catechin derivatives, flavonol glycosides, and chlorogenic acid derivatives in CS experiment seedlings

Vole Feeding Preferences Voles did not select among seedlings grown at different densities (Friedman’s nonparametric ANOVA, $\chi^2 = 0.93$, $N = 15$, $df = 2$, $P = 0.63$; Fig. 6a), but they preferred large seedlings to small seedlings from the CS experiment (Friedman’s nonparametric ANOVA, $\chi^2 = 7.12$, $N = 16$, $df = 2$, $P = 0.029$; Fig. 6b).

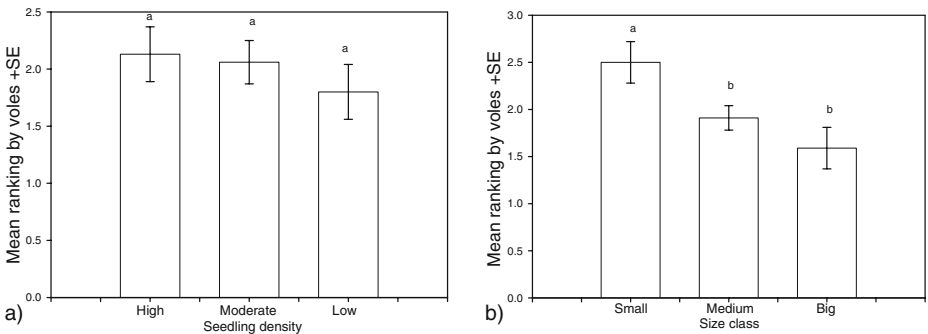


Fig. 6 Vole preference in DM (a) and CS (b) experiments

Discussion

We studied the extent to which size variation generated by variable population density and variable competitive success within high-density populations is related to the chemical quality and herbivore susceptibility of silver birch seedlings. Intraspecific competition reduced the growth of juvenile birches, as indicated by the negative relationship between population density and seedling size. Population density had an effect on stem total phenolics, but no effect on the concentrations of individual phenolic compounds, groups of phenolic compounds, or total nitrogen. Voles also did not show any preference between seedlings grown at different densities.

Large, competitively dominant seedlings generally had higher concentrations of phenolic compounds and lower concentrations of nitrogen than small, competitively inferior seedlings. Voles preferentially attacked large seedlings when these were offered along with small ones. Thus, the relationship between feeding preference and nitrogen and/or secondary compound concentrations was not in accordance with the expected role of these constituents as factors affecting plant susceptibility to herbivores (e.g., Stephens and Krebs, 1986; Marquis and Batzli, 1989; Hjäältén et al., 1994).

The observed decrease in seedling size with increasing density was as predicted on the basis of yield density curves constructed for several plant species (e.g., Silvertown and Charlesworth, 2001). Biomass tended to decrease more rapidly than height with the increase of density from low (270 seedlings/m²) to moderate (533 seedlings/m²), suggesting that the seedlings prioritized allocation for growth in height and thus the ability to intercept light. The intense competition in the populations of the CS experiment resulted in a considerable degree of variation in height and also in mortality, which was not observed in the DM experiment. Patterns of variation observed in the CS experiment are characteristic of asymmetrical competition, which is particularly liable to occur when seedlings compete for light (Weiner et al., 2001). Seedlings from the CS experiment were also smaller at mean height than those from the DM experiment, indicating that competition was more intense in the former.

There were significant variations in the concentrations of individual phenolic compounds among the different-sized seedlings from the CS experiment: Tall seedlings had higher concentrations than smaller ones. Moreover, seedlings from the low-density treatment in the DM experiment, i.e., the largest seedlings, had higher concentrations of total phenolics than those from moderate- and high-density treatments. This is consistent with the notion that resource competition constrains the production of chemical defenses (Cipollini and Bergelson, 2002). Stamp et al. (2004) have shown that the concentration of allelochemicals is inversely related to the intensity of competition. On the other hand, variation in nitrogen concentrations showed the opposite pattern to that found in carbon-based phenolic compounds. Nitrogen concentrations increased with decreasing seedling size in the CS experiment and tended to be highest in seedlings at the highest density, i.e., the smallest seedlings, of the DM experiment. These patterns in covariation of seedling size and concentrations of phenolics and nitrogen suggest that the growth of small seedlings was limited by the availability of light, and the growth of taller seedlings was limited by shortage of nitrogen. Nutrient availability is known to affect growth and resistance traits (Herms and Mattson, 1992; Tiffin, 2002). The CNB hypothesis predicts that if nutrient deficiency limits growth more than photosynthesis, it leads to the accumulation of carbon-based secondary chemicals (Bryant et al., 1988). It is likely that large seedlings had better access to light than smaller ones and were better able to synthesize carbon-based compounds. Several studies have shown that the concentration of phenolic compounds

increase under high light conditions (Larsson et al., 1986; Lavola, 1998; Lavola et al., 2000). Small, probably more shaded seedlings suffered from a shortage of light and a low potential to produce carbon-based compounds via photosynthesis. Consequently, these seedlings were unable to utilize the available nitrogen, which then accumulated (Rousi et al., 1993).

Plant defense is thought to be metabolically costly, and a trade-off between growth and defense has been proposed (Rhoades, 1979; Herms and Mattson, 1992). The positive relationship between phenolic concentrations and plant size observed in the present study seem to contradict this idea and differential access to light among different-sized seedlings is likely to be important here. In competitive conditions, variation in the acquisition of resources is a factor that can mask potential trade-offs. If the acquisition of resources is more variable than variability in allocation to different traits, a positive correlation is likely to be observed between the two traits in spite of the trade-off between them (Van Noordwijk and De Jong, 1986). Thus, we suggest that the differential competitive success associated with differential growth results in differential nutrient balance and differences in the concentrations of secondary metabolites.

In the DM experiment, the size differences between seedlings from the three densities were not associated with such clear differences in chemistry as the size differences resulting from competitive success in the CS experiment. The effect of competition on the chemical quality of the seedlings was not as evident as its effects on their physical characteristics. It is likely that competitive success was affected by seedling genotype: Previous studies of silver birch seedlings have revealed genotypic variation in both growth (e.g., Viherä-Aarnio and Velling, 2001; Pusenius et al., 2002; Pritinen et al., 2003) and chemistry (Keinänen et al., 1999; Laitinen et al., 2000, 2002), so the size differences and associated differences in chemistry observed in the CS experiment may have resulted from genotypic differences. It has been noted that random birch families have different growth rates in competitive conditions (Pusenius et al., 2002; Pritinen et al., 2003) and it is possible that seedlings that grew taller in the CS experiment were of certain genotypes. In the DM experiment, size differences as a result of different densities were probably not related to genotypic variation, and the assortment of genotypes within each of the groups of different size may have been wider than in the CS experiment.

The vole feeding experiments showed no preferences between seedlings grown at different densities. This is consistent with the findings of Hjältén et al. (1994). However, voles did choose between different-sized seedlings from the CS experiment, preferring the tallest ones. A study by Pusenius et al. (2002) showed that field voles preferentially attacked competitively dominant, fast-growing genotypes of silver birch seedlings, and other mammalian browsers, such as moose and deer, prefer large seedlings (for a review see Price, 1991; Shabel and Peart, 1994). Fast-growing plants seem to have relatively fewer fibres than slow-growing ones and this might make them more palatable to voles (e.g., Iason and Van Wieren, 1999), but it does not explain the differential results of the experiment with CS and DM seedlings. Again, this might be because of differences in genotype composition among different-sized seedlings in the CS experiment, but not in the DM experiment. However, the results pertaining to the relationship between chemistry and seedling size, i.e., the highest amounts of phenolics and lowest amounts of nitrogen in the largest seedlings, conflict with previous findings on the relationship between plant chemistry and the food preference of voles (Bergeron and Jodoin, 1987; Marquis and Batzli, 1989).

In summary, we can say that growth and biomass of the silver birch seedlings studied were affected by stand density. The resulting differences in seedling size were associated with differences in concentration of total phenolics, but they did not affect the feeding

preferences of voles. However, size differences resulting from differential competitive success were more clearly associated with variation in nitrogen concentrations and the concentration of various secondary compounds. Voles also selected seedlings that were successful in competition. Differences in the set of characteristics associated with size variation in relation to density variation vs. variation in competitive success were possibly related to genotypic differences in competitive success (Pusenius et al., 2002). Our results suggest that the ecological consequences of size variation may be contingent on the mechanism that created the variation.

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Introduction

In the early 1980s, the horse chestnut leafminer, *Cameraria ohridella* (Lep., Gracillariidae), was observed for the first time in Macedonia, near Ohrid Lake (Deschka and Dimic, 1986). Since that outbreak, *C. ohridella* spread rapidly and is now established throughout central Europe (Skuhrovy, 1999; Hellrigl, 2001; ˇSefrova, 2003). The larval stages of this insect mine in leaves of the horse chestnut *Aesculus hippocastanum* (Sap., Hippocastanaceae). The eggs are deposited singly on veins of the upper leaf surface. The insect develops within leaves through one to five feeding larval stages, two prepupal stages, and one pupal stage (ˇSefrova and Skuhrovy, 2000; Freise, 2001; De Prins et al., 2003; Zunke and Doobe, 2003; John e et al., 2005). Larvae of the first feeding stage (L1) create a 1- to 3-mm-long mine parallel to the leaf vein. The second feeding stage (L2) enlarges the mine to a nonuniform circle with a diameter of approximately 3 mm, which is broadened to 8 mm by the third stage (L3). Older feeding larvae (L4, L5) broaden the mines so much that one mine can cover several square centimeters. In early larval stages (L1–L3), leaf veins limit the expansion of mines. During development of late larval stages (L4, L5), leaf veins are destroyed and, in heavy infestations, mines merge and can totally cover the leaf surface. Larvae in early stages are considered to be of the “sap-sipping” type, whereas those in late stages are of the “tissue-feeding” type (Pschorn-Walcher, 1994).

The origin of this invasive insect species is still unknown (Freise et al., 2002). Moreover, it is not known whether the horse chestnut tree is the original host plant or whether *C. ohridella* performed a host shift (Hellrigl, 1999). The sex pheromone of the moth was identified by Svatoš et al. (1999). In this study, the olfactory basis of oviposition site selection by *C. ohridella* was examined. The insect has three generations (spring, summer, and autumn) in Central Europe (Pschorn-Walcher, 1994; Hellrigl, 1999; Skuhrovy, 1999). At high population densities, the mining larvae are able to completely destroy the leaf surface (Tomiczek and Krehan, 1998; Gilbert and Gregoire, 2003). During the development of the first generation in spring, the host leaves are green and the trees are flowering (John e et al., 2003). The second and third generations encounter increasingly damaged leaf tissue caused by mining conspecifics and decreasing green leaf area suitable for oviposition. Because of these factors, the moths of various generations encounter different olfactory environments. The odor patterns of green and damaged leaves were analyzed by gas chromatography-mass spectrometry (GC-MS) and GC-electroantennographic detection (GC-MS/EAD). Identified compounds were provided to the insect antennae in dilution series to examine quantitative antennal responses of moths. In bioassays, leaves of *A. hippocastanum* infested with different larval stages and with different degrees of browning were tested for their possible effect on adults. Moreover, oviposition choices by female moths were tested in two choice tests, by using green leaves treated with single compounds induced by larval mining in leaves, and that elicited responses from antennae of adult moths.

Methods and Materials

Locations and Trees

Between July 2002 and October 2004, leaves for odor sampling were taken from trees in Goettingen (Lower Saxony, Germany, 8°C mean annual temperature, 600–700 mm rainfall).

Trees were situated in different types of locations: directly in the city (sealed soil), in a suburban area (grassland), and in a meadow surrounded by forest. Microclimatic conditions and removal of foliage were monitored.

Sampling of Volatiles

Odor samples for GC-MS/EAD analysis were obtained by using the closed-loop stripping analysis (CLSA) method (Boland et al., 1984). At each location, leaf volatiles from three trees of *A. hippocastanum* were sampled three times during the appearance of *C. ohridella* adults and once before and after each generation. From 2002 till 2004, there were three generations per year in Goettingen. For collection of volatiles, five leaves per tree were taken from different branches. Volatiles released in response to stress or attack by phytophagous insects can be released locally at the site of damage or systemically from other parts of the plant (Röse et al., 1996; Dicke, 1999). To rule out position effects within one tree, one branch pointing in each direction (north, south, west, and east) was chosen. The fifth leaf was taken from near the trunk of the tree. Five leaves per tree were put into a single 250-ml glass flask with ground glass joints. The degree of browned, mined leaf surface was noted for each leaf (rating: 0%, 1–5%, 6–10%, 11–30%, 31–50%, 51–70%, 71–90%, and 91–100%). Leaves with 0% leaf browning were checked to ensure that no larvae were developing in the tissue. It was not possible to open mines to remove the larvae and their associated products without complete destruction of the leaf. Thus, during collection of volatiles, the larvae remained inside their mines. Stainless steel capillaries (1 mm ID) were fed through the stoppers (PTFE) of the flasks. Miniature pumps (type DC12/16NK; Fürgut, Tannheim, Germany) were used to circulate air from the flasks to adsorbent traps (type “LR”; CLSA-Filter, Daumazan sur Arize, France). Traps consisted of glass tubes (6 cm long and 0.3 mm ID) loaded with 1.5 mg charcoal. The sampling time for leaf odor was 45 min at a flow rate of 1 l/min. Volatiles were eluted from the traps with 75 μ l solvent ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 2:1, Suprasolv-quality; Merck/VWR, Darmstadt, Germany). After elution, the samples were stored at -80°C . In May 2004, in addition to leaf odor, floral volatiles were obtained by the same method, with a few modifications. Thus, flowers were enclosed in a plastic roasting bag (Melitta GmbH, Minden, Germany) directly on the trees and volatiles were sampled for 4 hr (11:30 AM–3:30 PM; flow rate, 1 l/min).

GC-MS/EAD System

Samples were analyzed by coupled GC-MS/EAD (Weissbecker et al., 2004), using a 6890N gas chromatograph (Agilent, Palo Alto, CA, USA) and a 5973N mass spectrometer (Agilent). The GC was equipped with a split/splitless (S/SL) injector and an HP-5MS column (Agilent; 30 m, 0.25 mm I.D., film thickness 0.25 μm). A GRAPHPACK 3D/2 flow splitter (Gerstel, Mülheim, Germany) was used to split the effluent from the column into a capillary (1 m long, 0.1 mm ID) leading to the MS and another (1 m long, 0.15 mm ID) leading to the EAD. The restriction capillaries resulted in an equal split of the gas flow into the two setups.

A modified “olfactory detector port” (ODP-2; Gerstel; Weissbecker et al., 2004) guided the capillary out of the GC oven within a flexible heating sleeve. Volatiles eluted from the column into a flow of helium make-up gas, and then were mixed with humidified air (23°C, 80% RH). The airflow (flow rate, 400 ml/min) was directed through the flow tube (15 cm long, 6 mm ID, PTFE) to the insect antennae preparation that was housed in a PTFE detector cell.

Excised antennae of *C. ohridella* were placed into an antenna holder milled from a perspex disc (Färbert et al., 1997). The origins of the moths used were noted so that possible differences in electrophysiological responses among individuals from different locations could be traced. Within the holder, the ends of the antennae contacted an electrolyte solution that provided electrical contact to a pair of Ag/AgCl electrodes. EAD potentials were amplified by a factor of 100 with a high-impedance amplifier (input impedance 100 M Ω ; Prof. Koch, Kaiserslautern, Germany) containing a built-in low-pass filter set to a cutoff frequency of 1 Hz to suppress the ubiquitous electrical supply frequency of 50 Hz. An additional high pass filter set at a cutoff frequency of 0.01 Hz was used to suppress drift of the EAD signal. The amplified and filtered signal was digitized by using a 35900E A/D converter (Agilent) and recorded by the GC ChemStation software (Agilent).

Analytical Conditions

One- μ l aliquots of the samples were injected into the S/SL injector (temperature, 250°C). The GC oven was programmed from 50°C for 1.5 min, then 6°C/min to 200°C; hold for 5 min. Helium was used as carrier gas (1 ml/min, 24 cm/sec). The GC-MS interface was held at 280°C. The heating sleeve of the ODP was set to 230°C. The MS used electron impact ionization (EI) at 70 eV, in scan mode (35–300 mass units, 2.78 scans/sec). For preliminary peak identification, the NIST mass spectral library (National Institute of Standards and Technology, Gaithersburg, MD USA) and the MassFinder 3.0 software (Hochmuth, 2004) together with the library “Terpenoids and Related Constituents of Essential Oils” (König et al., 2004) were used. Subsequently, the spectra and retention times of compounds were compared with those of synthetic standards, and identifications were confirmed by coinjection. To ensure the identification of compounds, the collected samples and standards were also analyzed with an HP-INNOWax column (Agilent, 30 m, 0.25 mm ID, film thickness 0.25 μ m) using the GC-MS method and the same analytical parameters as the HP-5MS column. Tables 1 and 2 include information about the sources of synthetic standards used for identification. The identification of the tentatively identified compounds was based only on a match of library spectra to the mass spectra, which were measured in our samples, on both columns. The synthetic standards of (*E,E*)- α -farnesene and (*E*)-4,8-dimethyl-1,3,7-nonatriene were gifts from Roman Kaiser, Givaudan, Switzerland and Wilhelm Boland, Max Planck Institute Jena, Germany, respectively.

Electroantennogram Dose–Response Series

Dose–response series were measured by manual injection of odor standards into an air stream passed over the antennae of *C. ohridella* mounted in an electroantennogram (EAG) setup. The origins of the moths used were noted as in the GC-MS/EAD measurements. Odor standards were produced from dilution series of the respective compounds in paraffin oil (Uvasol quality, Merck/VWR). Small pieces of filter paper (2 cm²; Schleicher & Schuell, Dassel, Germany) were soaked with 100 μ l of the standard dilution. The filter paper then was put into a 10-ml glass syringe (Poulten & Graf GmbH, Wertheim, Germany). Inside the air volume of the syringe, the odorant accumulated at a concentration proportional to the concentration of the substance in the solution and its vapor pressure according to Henry’s law. A reproducible stimulus could be supplied by puffing 5 ml of air over the antenna (Schütz et al., 1999). The resulting signals were amplified (100 \times). Details (purity and source) of substances tested on insect antennae are shown in Tables 1 and 2. For the compounds (*E*)- β -caryophyllene and (*E/Z*)-linalool oxide, (furanoid) racemic mixtures were used.

Table 1 Leaf volatile compounds that appear throughout the entire year, and flower odorant compounds of *A. hippocastanum*

Parameter	Compounds	RI ^a	Purity (%) and source of synthetic standard
Leaves	(Z)-2-Penten-1-ol	795	98 ^b
	(E)-2-Hexenal	862	97 ^c
	(Z)-3-Hexen-1-ol [1]	870	98 ^c
	(E)-2-Hexen-1-ol	876	97 ^c
	(Z)-2-Penten-1-yl acetate	913	^g
	(Z)-3-Hexenoic acid methyl ester	932	^d
	(E)-2-Hexenoic acid methyl ester	967	^g
	(Z)-3-Hexen-1-yl acetate [2]	1009	98 ^d
	Hexyl acetate [3]	1012	98.5 ^d
	(E)-2-Hexen-1-yl acetate [4]	1014	98 ^b
	(Z)-2-Penten-1-yl butanoate	1086	^g
	(Z)-3-Hexen-1-yl propanoate	1094	98 ^c
	(E)-2-Hexen-1-yl propanoate	1102	^g
	(Z)-3-Hexen-1-yl butanoate	1181	98 ^d
	Hexyl butanoate	1184	98 ^d
	(E)-2-Hexen-1-yl butanoate	1187	96 ^d
	(Z)-3-hexen-1-yl 2-methyl butyrate	1224	^f
	(Z)-3-Hexen-1-yl isovalerate [5]	1229	97 ^d
	(Z)-3-Hexen-1-yl valerate [6]	1276	98 ^c
(Z)-3-Hexen-1-yl hexanoate	1373	98 ^d	
Flowers	Benzaldehyde [7]	970	98 ^b
	1,8-Cineole [8]	1033	98 ^c
	(E/Z)-Linalool oxide (furanoid) [9, 10]	1074	97 ^{d**}
	2-Phenylethanol [11]	1125	99 ^c
	Decanal [12]	1200	95 ^b
	Tridecane [13]	1285	99 ^b
	(E)- β -Caryophyllene [14]	1429	^{d*}

The numbers behind compound names label those compounds that are referred to repeatedly in the text and figures.

^a Retention indices (RI) were calculated from the chromatograms obtained with an HP-5MS column (Agilent) by using Mass Finder software (Hochmuth, 2004).

^b Acros Organics (Geel, Belgium).

^c Merck-Schuchardt (Hohenbrunn, Germany).

^d Sigma-Aldrich Chemie (Steinheim Germany).

^e TCI Europe (Zwijndrecht, Belgium).

^f Chemos (Regenstauf, Germany).

^g Tentatively identified.

*: Enantiomeric mixture.

** : Enantiomeric mixture of isomers.

Bioassays

A. hippocastanum leaves infested with various larval stages and at different degrees of browning were examined in bioassays to determine their aggregation effect on the moths.

Table 2 Leaf volatile compounds of *A. hippocastanum* caused by larval mining of *C. ohridella*

Parameter	Compounds	RI ^a	Purity (%) and source of synthetic standard
Oviposition, early larval stages (L1–L3)	1-Hexanol	877	99.9 ^d
	Benzaldehyde [7]	970	98 ^b
	1,8-Cineole [8]	1033	98 ^c
	Benzyl alcohol [15]	1048	99 ^d
	2-Phenylethanol [11]	1125	99 ^c
	Methyl salicylate [16]	1197	99 ^b
	(<i>E</i>)- β -Caryophyllene [14]	1429	^{d*}
Late larval stages, browned leaves (L4, L5)	(<i>E,E</i>)- α -Farnesene [17]	1502	80 ^c
	(<i>E,E</i>)-2,4-Hexadienal [18]	918	95 ^d
	(<i>E/Z</i>)-Linalool oxide (furanoid) [9, 10]	1074	97 ^{d**}
	Nonanal [19]	1100	98 ^c
	Decanal [12]	1200	95 ^b
	Tridecane [13]	1285	99 ^b

The numbers behind compound names label those compounds that are referred to repeatedly in the text and figures.

^a Retention indices (RI) were calculated from the chromatograms obtained with an HP-5MS column (Agilent) by using Mass Finder software (Hochmuth, 2004).

^b Acros Organics (Geel, Belgium).

^c Merck-Schuchardt (Hohenbrunn, Germany).

^d Sigma-Aldrich Chemie (Steinheim Germany).

^e Givaudan (Vernier, Switzerland).

*: Enantiomeric mixture.

** : Enantiomeric mixture of isomers.

The total leaf surface of every twig was about 1000 cm². Twigs with the following degrees of infestation were used for the bioassays:

- Twigs with leaves, 10 wk after termination of leaf development and infested by early larval stages (eggs, L1–L3): the twigs were taken from five trees treated with the systemic insecticide Confidor WG 70 (active ingredient imidacloprid, 70%; Bayer CropScience, Monheim, Germany) via soil application [0.28 g/cm diam of the stem at breast height (DBH)]. The emerging larvae bored into the leaves and started mining, but most died in the L1 stage without causing any browning on the leaves.
- Twigs with uninfested leaves, directly after termination of leaf development: the leaf material originated from seedlings dug out in February. The development of the shoots was delayed for 3 mo in a cooling chamber at 5°C. Neither eggs nor larval stages of *C. ohridella* were observed on these plants.
- Twigs with leaves that had a browning ratio of 70–90% caused by late larval stages (L4, L5) of *C. ohridella*.

For the two-choice tests, two twigs were arranged in 5-l jars filled with water within a cage (200 × 100 × 130 cm) consisting of a wooden frame and base, a polyester net (mesh size, 0.5 mm), and two glass doors in the front. The distance between the twigs was 150 cm. The lighted cage (1000 lx) was situated in a room (29°C, 45% RH) without windows or airflow. Before the experiment, adults were captured from different locations in Goettingen and

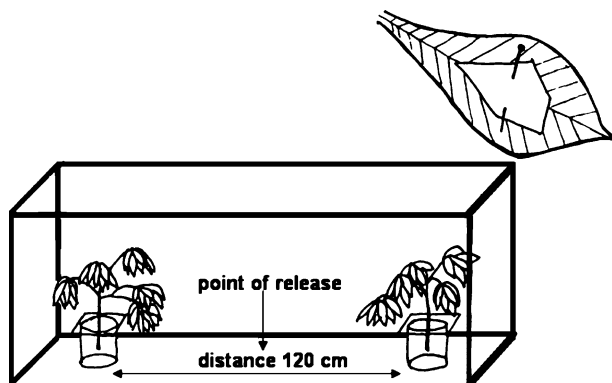
chilled in a dark chamber at 5°C overnight. In each assay, 200 moths were released in groups of 20 individuals in the middle of the cage. After an orientation phase of 20 min, all insects that were sitting on plants were counted.

In further two-choice tests with treated and control twigs, compounds identified by GC-MS/EAD were tested for influence on oviposition. The twigs originated from *A. hippocastanum* trees treated with Confidor WG 70 via soil application (0.28 g/cm DBH; see above). They were checked for fungal infection and larval mining activity. Only twigs without fungal infection or necrosis were selected for bioassays. To rule out position effects, two equally sized twigs were taken from the same branch. Filter paper covering 30–50% of the leaf surfaces was fixed to each leaflet with a needle (Fig. 1). The filter paper of one twig was soaked with a paraffin oil solution of an odorant, whereas the filter papers on the second twig were treated with paraffin oil only as a control. Thus, the volatile pattern of healthy leaves was superimposed with additional odorant compounds. The selected concentration of odorant compounds was near the inflection point of the respective EAG dose–response curves (10^{-3} v/v in paraffin oil). Fresh odor solution was added to the filter papers at the beginning of a bioassay and replenished every 6 hr (nonanal [19], decanal [12], benzaldehyde [7]) or every 12 hr (control, 1,8-cineole [8], benzyl alcohol [15], 2-phenylethanol [11], methyl salicylate [16], enantiomeric (*E*)- β -caryophyllene [14], (*E,E*)- α -farnesene [17], (*E/Z*)-linalool oxide (furanoid, enantiomeric mixture of isomers) [9, 10] and (*E,E*)-2,4-hexadienal [18]; numbered as in Tables 1 and 2) to maintain the concentration of compounds at the appropriate level. After 6 or 12 hr, before fresh odorant solution was added, the amount of the tested compound had decreased by approximately 50%. In a lighted (1000 lx) cage (180 × 75 × 75 cm), the two twigs were arranged in 5-l glass jars filled with water and 120 cm apart (Fig. 1). The cage was made out of a wooden frame, stainless steel screen (mesh size of 0.7 mm), and glass, with two glass doors in the front. The cage was situated in a dark room without air flow at 26°C and 50% relative humidity. At the beginning of each assay, 250 moths captured outdoors were released in the middle of the cage. The fresh eggs on the leaves were counted after 24 hr (nonanal, decanal, benzaldehyde) or 48 hr (all other compounds).

Statistical Analyses

The amounts of single compounds emitted from different browned leaves after mining by *C. ohridella* were analyzed with the Kruskal–Wallis *H* test ($\alpha = 0.05$; Lozán and Kausch, 1998). The data were not normally distributed ($\alpha = 0.05$; Shapiro–Wilks test; Lozán and Kausch, 1998). The analyses were carried out by using Statistica 6.1 software (StatSoft,

Fig. 1 Schematic of experimental setup for oviposition bioassay. A filter paper treated with odorant compound dissolved in paraffin oil (10^{-3}) or only paraffin oil (control) was fixed on every leaflet with a needle



Inc., 2003). To optimize the dilution of odor compounds in choice tests, a sigmoid function $y = A_2 + (A_1 - A_2)/(1 + (X/X_0)^P)$ was fitted to the dose–response curves using the Origin 7 software (OriginLab, 2002), where A is the EAG response amplitude, X is the dilution of the odor, and X_0 is the dilution at the point of inflection. In addition, the detection limit was determined via statistical analyses of EAG dose–response curves, using the Mann–Whitney U test ($\alpha = 0.05$; Lozán and Kausch, 1998; Statistica 6.1; StatSoft, Inc., 2003). In all bioassays, statistical analyses were carried out with χ^2 test 2×2 tables ($\alpha = 0.05$; Lozán and Kausch, 1998) using Statistica 6.1 software (StatSoft, Inc., 2003). Statistical comparisons were made only between the two twigs in one cage and not between the several variants.

Results

Analysis of Volatiles

In 2003, the mean annual temperature in the city was 16.0°C, 1°C higher than in the forest. A mean annual relative humidity of 69.1% was measured in the forest, which was 1% higher than in the city. In 2003, in both locations, temperatures of up to 35°C were reached. No significant differences in the profiles of volatiles were found as a result of microclimate or location. The profiles of volatiles of control trees treated with Confidor WG 70 was not different from that of untreated healthy trees.

During the spring bloom of *A. hippocastanum*, flower scent compounds such as (*E/Z*)-linalool oxides (furanoid) [9, 10] (enantiomeric composition not determined) and benzaldehyde [7] were emitted by trees and mixed with leaf odor (Table 1). The leaf odor

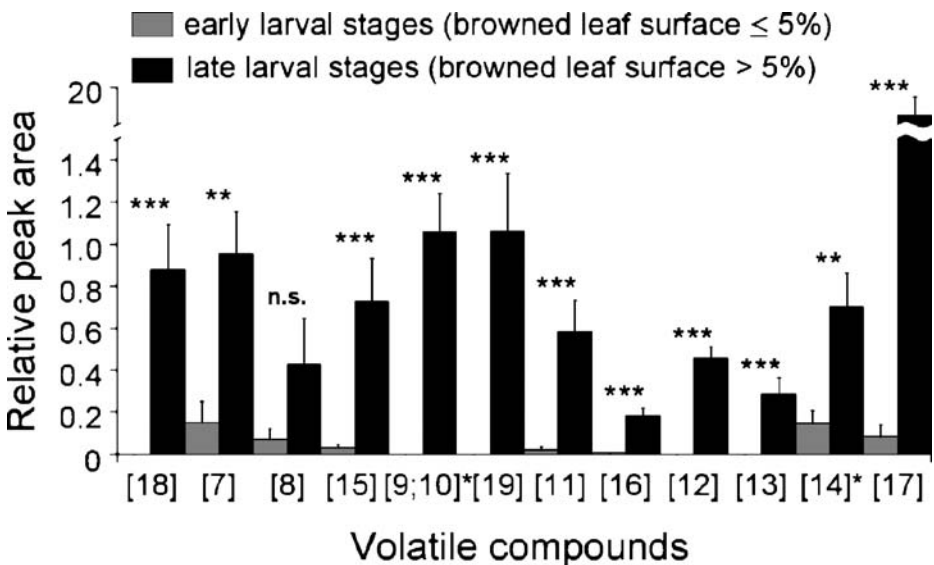


Fig. 2 Quantification of volatiles released by *A. hippocastanum* leaves infested with *C. ohridella* larvae. Compounds are shown in order of increasing retention times and labeled as in Tables 1 and 2: [7] benzaldehyde; [8] 1,8-cineole; [9,10] (*E/Z*)-linalool oxides (furanoid)*; [11] 2-phenylethanol; [12] decanal; [13] tridecane; [14] (*E*)- β -caryophyllene*; [15] benzyl alcohol; [16] methyl salicylate; [17] (*E,E*)- α -farnesene; [18] (*E,E*)-2,4-hexadienal; [19] nonanal. Twenty measurements were taken for each compound, 10 per bar (Kruskal–Wallis test, $\alpha = 0.05$; n.s. = not significant; ** $p < 0.01$; *** $p < 0.001$). Error bars indicate the standard error. Benzaldehyde (1.0×10^{-6}) [7] is equivalent to 0.22 ng/l air. *Enantiomeric composition not determined

compounds listed in Table 1 were released throughout the season by both uninfested and damaged leaves, independently of the mining degree.

Infestation by *C. ohridella* caused significant changes in the pattern of volatiles released by *A. hippocastanum* leaves. After oviposition and during development of early larval stages (L1–L3), plants started to produce additional alcohols, aldehydes, phenolics, and terpenes (Table 2). Mining by late larval stages (L4, L5) and severe loss of green leaf area caused the release of tridecane [13], (*E/Z*)-linalool oxides (furanoid) [9, 10] (enantiomeric composition not determined), and aldehydes, such as decanal [12] and (*E,E*)-2,4-hexadienal [18] (Table 2). The amount of released volatiles increased with progressive larval development (Fig. 2). Leaves infested by late larval stages and with a high ratio of browned to green area released a higher total number and amount of compounds than leaves infested by early larval stages (Fig. 2).

Electrophysiological Analyses

Because of microclimatic conditions, moths emerged 1 wk (first generation) or 2 wk (second and third generations) earlier in the city than in the forest. Moths collected from the different locations showed no differences in their olfactory responses to leaf volatiles. The response of the insects' antennae to the leaf odor of control trees treated with Confidor WG 70 was not different from the response to untreated trees.

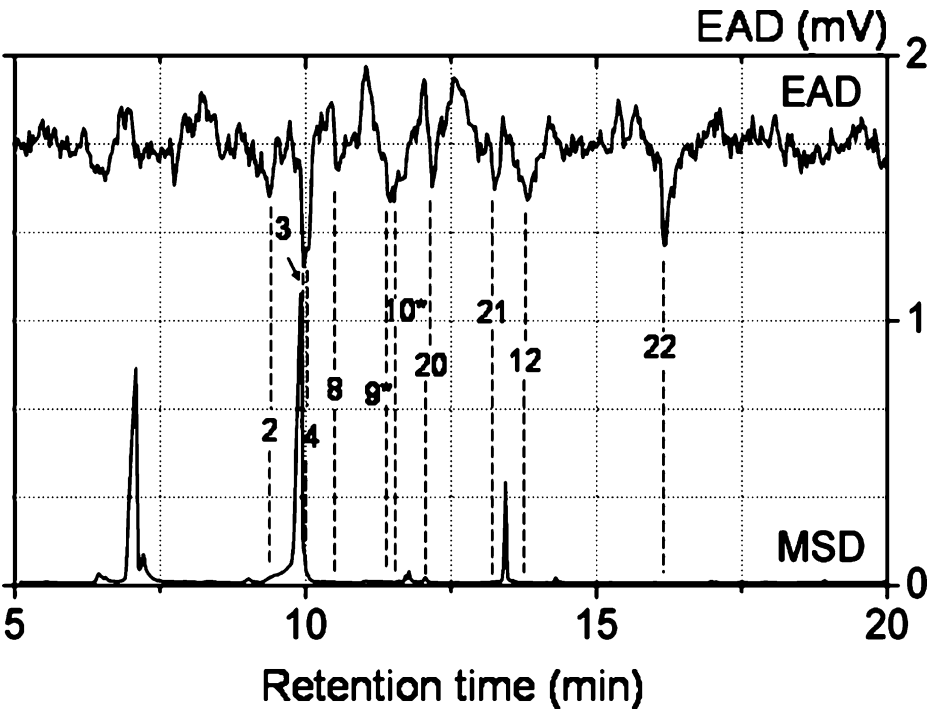


Fig. 3 GC-MS/EAD recording from antenna of *C. ohridella* in response to leaf volatiles of *A. hippocastanum* released in spring (first generation). The leaves were not browned by larval feeding. Upper, inverted trace: electroantennogram response. Lower trace: total ion chromatogram. Compounds labeled as in Tables 1 and 2: [2] (*Z*)-3-hexen-1-yl acetate; [3] hexyl acetate; [4] (*E*)-2-hexen-1-yl acetate; [8] 1,8-cineole; [9] (*E*)-linalool oxide (furanoid)*; [10] (*Z*)-linalool oxide (furanoid)*; [12] decanal; [20] (*E*)-4,8-dimethyl-1,3,7-nonatriene; [21] unknown; [22] unknown. *: Enantiomeric composition not determined

In spring, flower scent compounds were released in large amounts. These compounds were found in leaf odor samples too. These compounds included (*E/Z*)-linalool oxides (furanoid) [9, 10], 1,8-cineole [8], and decanal [12], and were detected by antennae of *C. ohridella* (Fig. 3). Green leaf volatiles of *A. hippocastanum* that were released throughout the year (Table 1), including (*Z*)-3-hexen-1-ol [1] (Fig. 4), (*Z*)-3-hexen-1-yl acetate [2] (Figs. 3 and 4), hexyl acetate [3] (Figs. 3 and 4), and (*E*)-2-hexen-1-yl acetate [4] (Figs. 3 and 4) elicited the strongest responses from *C. ohridella* antennae. (*Z*)-3-Hexen-1-yl acetate [2], hexyl acetate [3], and (*E*)-2-hexen-1-yl acetate [4] coeluted using the HP-5MS column. Other leaf volatiles that elicited antennal responses in each generation were (*Z*)-3-hexen-1-yl isovalerate [5] and (*Z*)-3-hexen-1-yl valerate [6] (Fig. 4).

Changes in leaf odor pattern induced by larval mining were detectable by moths. Additional compounds released by leaves after oviposition and during development of early larval stages (L1–L3), such as benzyl alcohol [15], 2-phenylethanol [11], and methyl salicylate [16], were detected by the moths (Fig. 4). Decanal [12], (*E,E*)-2,4-hexadienal [18], nonanal [19], and tridecane [13] were released by leaves affected by mining of late larval stages and severe loss of green leaf area. These compounds also elicited antennal responses (Fig. 4). In addition, the insect antennae responded to sesquiterpenes, such as (*E*)- β -caryophyllene [14] (enantiomeric composition not determined), and (*E,E*)- α -farnesene [17]. (*E*)-4,8-Dimethyl-1,3,7-nonatriene [20] also elicited antennal responses (Figs. 3 and

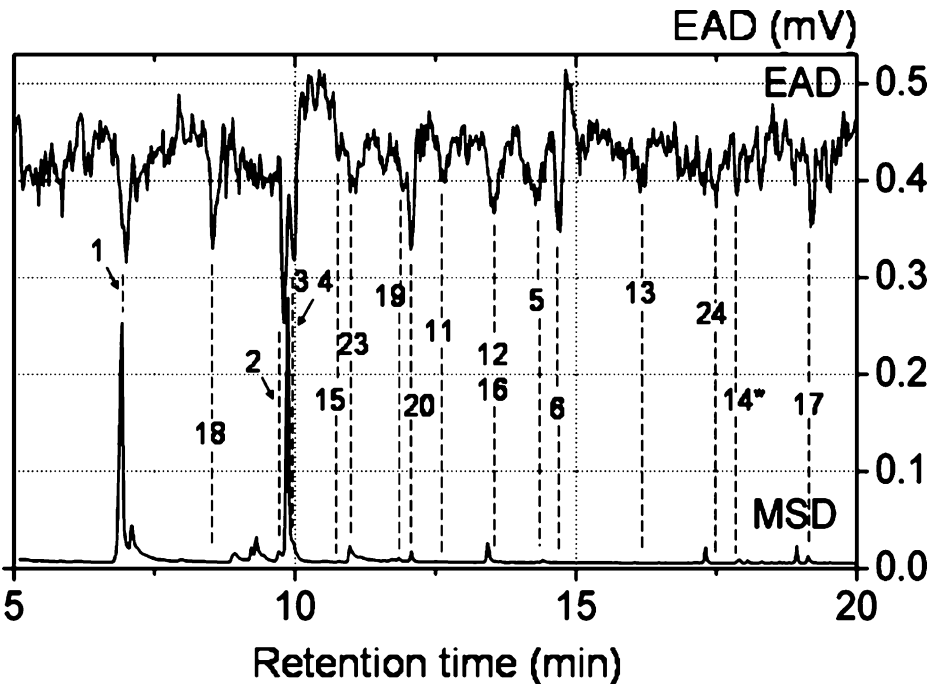


Fig. 4 GC-MS/EAD recordings of *C. ohridella* in response to leaf volatiles of *A. hippocastanum* released in summer (second generation). The leaf surface of *A. hippocastanum* was browned by mining of late larval stages. Upper, inverted trace: electroantennogram response. Lower trace: total ion chromatogram. Compounds labeled as in Tables 1 and 2: [1] (*Z*)-3-hexen-1-ol; [2] (*Z*)-3-hexen-1-yl acetate; [3] hexyl acetate; [4] (*E*)-2-hexen-1-yl acetate; [5] (*Z*)-3-hexen-1-yl isovalerate; [6] (*Z*)-3-hexen-1-yl valerate; [11] 2-phenylethanol; [12] decanal; [13] tridecane; [14] (*E*)- β -caryophyllene*; [15] benzyl alcohol; [16] methyl salicylate; [17] (*E,E*)- α -farnesene; [18] (*E,E*)-2,4-hexadienal; [19] nonanal; [20] (*E*)-4,8-dimethyl-1,3,7-nonatriene; [23] unknown; [24] unknown. *: Enantiomeric composition not determined

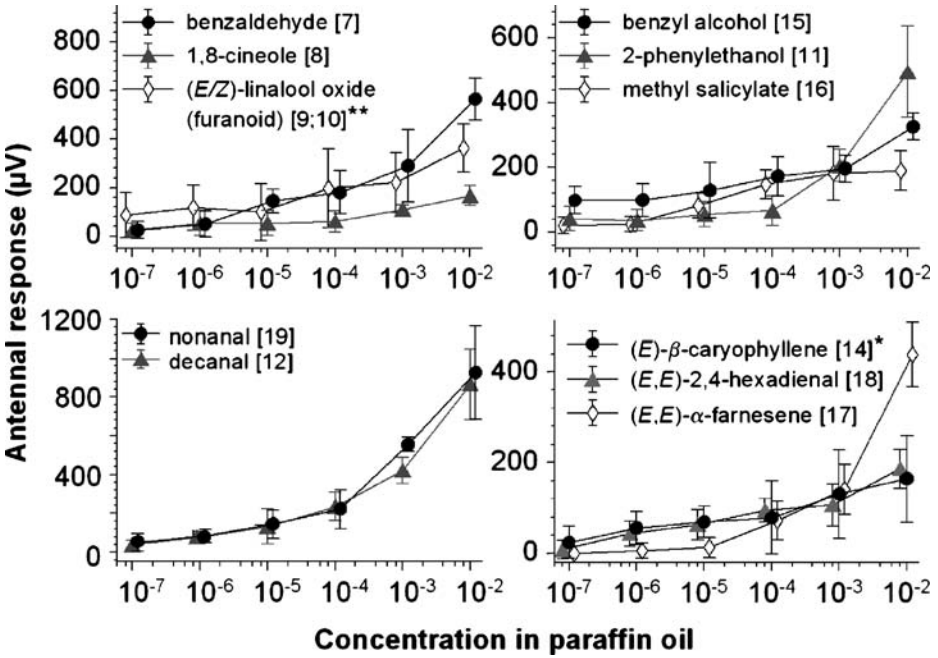
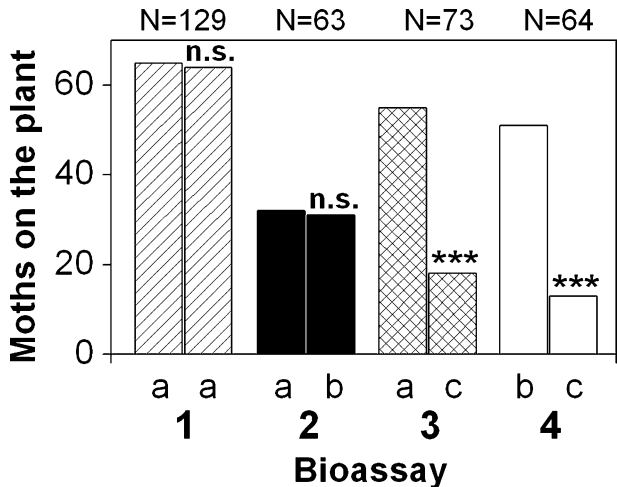


Fig. 5 Electroantennogram dose–response curves of floral odors, and compounds induced by larval mining in leaves, stimulating *C. ohridella* antennae. Compounds labeled as in Tables 1 and 2. Puffs of 5 ml of air were loaded with reference standards of plant odors (pulses 0.5 sec, minimum three animals, number of puffs = 10). Each point represents the mean of antennal response. Standard deviations are indicated as error bars. Data points are slightly shifted along the x-axis to avoid overlap of the error bars; *: racemic mixture; **: enantiomeric mixture of isomers

4), but no correlation was found between the occurrence of this compound and larval mining by *C. ohridella*.

The absolute sensitivity of the antenna toward identified compounds was determined with commercially available standards (Tables 1 and 2). (*E*)-β-Caryophyllene and (*E/Z*)-linalool oxides (furanoid) were tested as enantiomeric mixtures. The EAG dose responses

Fig. 6 Total number of *C. ohridella* adult sitting on differently infested and browned leaves of *A. hippocastanum* in two-choice tests: (a) leaves infested with early larval stages (eggs, L1–L3, destroyed leaf tissue less than 5%); (b) uninfested leaves; (c) leaves browned by late larval stages (L4, L5) of *C. ohridella* (destroyed leaf tissue 70–90%); (χ^2 test, $\alpha = 0.05$; n.s. = not significant; *** $p < 0.001$)



obtained after stimulation with reference standards in dilution series are depicted in Fig. 5, focusing on compounds emitted from flowers and leaves after leaf mining. The highest EAG responses of all compounds tested were observed in response to the aldehydes nonanal [19] and decanal [12], with amplitudes between 800 and 1000 μV for the 10^{-2} dilution. At the 10^{-3} dilution level, 2-phenylethanol [11], benzaldehyde [7], benzyl alcohol [15], (*E/Z*)-linalool oxides (furanoid) [9, 10], and methyl salicylate [16] elicited responses with amplitudes between 150 and 300 μV . In the 10^{-3} dilution series, 1,8-cineole [8], (*E*)- β -caryophyllene [14], (*E,E*)- α -farnesene [17], and (*E,E*)-2,4-hexadienal [18] evoked low responses, with amplitudes of about 100 to 150 μV . The antennal responses to (*E,E*)- α -farnesene [17] and 2-phenylethanol [11] increased strongly from the 10^{-3} dilution to 10^{-2} dilution.

Bioassays

In two-choice tests, the responses of moths to leaf odors of healthy leaves, leaves infested by early larval stages (L1–L3), and browned leaves with late larval stages (L4, L5) were

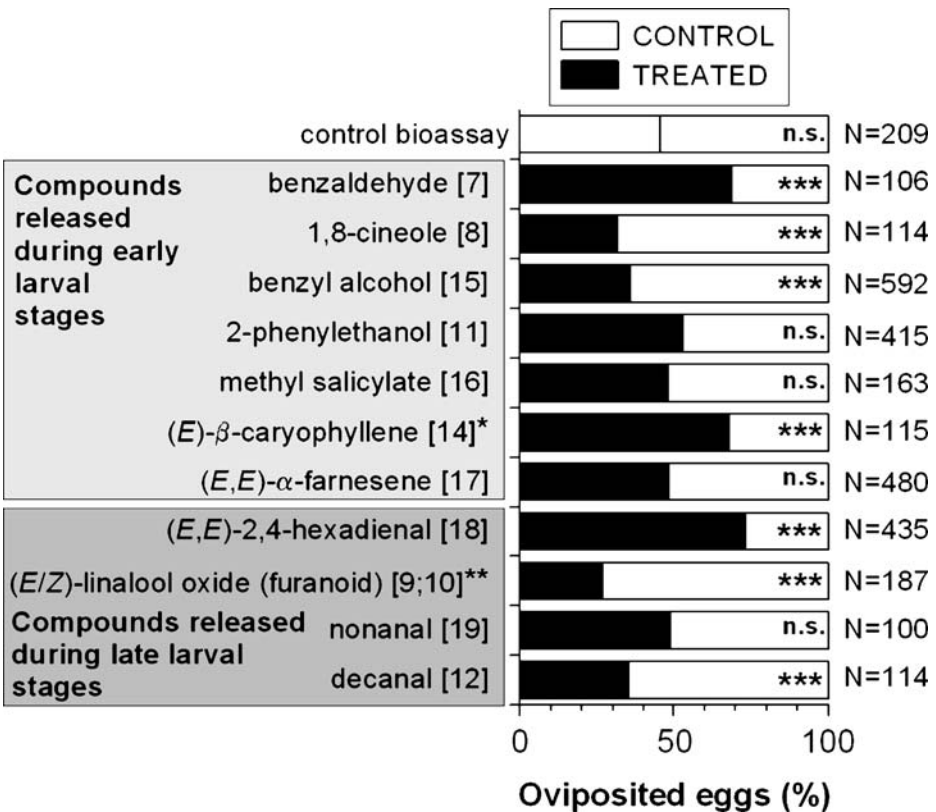


Fig. 7 Percentage of eggs laid by *C. ohridella* on leaves of *A. hippocastanum* in two-choice tests. A filter paper covering 30–50% of the leaf surface was fixed on each leaflet with a needle. The filter papers of one twig were treated with paraffin oil (Control), and the other one was treated with volatile substances in paraffin oil (solution 10^{-3} ; Treated). Compounds are listed based on their retention time, appearance during larval feeding, and are labeled according to Tables 1 and 2 (χ^2 test, $\alpha = 0.05$); n.s. = not significant; *** $p < 0.001$). *: Enantiomeric mixture; **: enantiomeric mixture of isomers

compared. A control experiment with two twigs infested by early larval stages resulted in a uniform distribution of adults ($p > 0.05$, χ^2 test; first bioassay, Fig. 6). The responses of moths to leaves infested by early larval stages and healthy leaves were not significantly different ($p > 0.05$, χ^2 test; second bioassay). In contrast, fewer moths were found on browned leaves with late larval stages than either leaves infested by early larval stages or healthy leaves ($p < 0.001$, χ^2 test; third and fourth bioassays, Fig. 6).

Single compounds were tested for their influence on oviposition. The control bioassay with paraffin oil-treated leaves resulted in a uniform dispersal of eggs ($p > 0.05$, χ^2 test; Fig. 7). Compounds induced by mining of various larval stages of *C. ohridella* could be grouped as follows: compounds without any discernable effect, compounds resulting in preferential oviposition, and compounds resulting in reduced oviposition. Benzaldehyde [7] and enantiomeric (*E*)- β -caryophyllene [14], induced by early larval stages, resulted in more oviposition than the control ($p < 0.001$, χ^2 test). In contrast, leaves treated with benzyl alcohol [15] and 1,8-cineole [8] received less oviposition than controls ($p < 0.001$, χ^2 test). Methyl salicylate [16], 2-phenylethanol [11], and (*E,E*)- α -farnesene [17] had no effect on oviposition ($p > 0.05$, χ^2 test; Fig. 7). Leaves treated with (*E,E*)-2,4-hexadienal [18], a compound that appeared during late larval stages, received more oviposition than controls ($p < 0.001$, χ^2 test). In contrast, the mixture of (*E/Z*)-linalool oxides (furanoid) [9, 10] and decanal [12] reduced the number of eggs laid ($p < 0.001$, χ^2 test), whereas nonanal [19] had no effect in comparison to controls ($p > 0.05$, χ^2 test).

Discussion

Aesculus hippocastanum trees bloom during the flight period of the first *C. ohridella* generation. Flower scent is emitted in high amounts and may serve for long-distance host plant finding (Visser, 1986; Bernays and Chapman, 1994). The bioassays presented here examined the intermediate and short distance effects on oviposition, that is, from branch to branch in one tree and from position to position on one leaf. Detection over longer distances was not examined. Besides encountering flower scent, moths of the first generation also encounter green undamaged leaves. Green leaf volatiles are important cues used by phytophagous insects in their search for host plants and selection of oviposition sites (Visser and Avé, 1978). Volatiles induced by mining do not play any important role in mediating oviposition by the first generation.

In contrast, the adults of the second and third generations are confronted with an increasing degree of mining damage and a decreasing proportion of green leaf tissue. Totally browned leaves, caused by larval mining, are frequently encountered by the third generation (Tomiczek and Krehan, 1998; Gilbert and Grégoire, 2003). Larval mining by *C. ohridella* causes significant changes in the profile of volatiles of horse chestnuts. Some of the compounds produced in infested leaves are typically found in flowers as well. Changes in the odor profiles of plants as a result of herbivore infestation can modify the responses of conspecific herbivores in different ways. For instance, the cabbage looper moth, *Trichoplusia ni*, avoids cabbage plants infested by conspecifics (reviewed by Dicke and Vet, 1999). In contrast, potato leaves infested with Colorado potato beetle larvae (Bolter et al., 1997) or adults (Schütz et al., 1997) attract host-searching Colorado potato beetles. For *C. ohridella*, the ratio of green to browned leaf surface is important, with fewer adults being found on browned leaves infested with late-stage conspecifics and a high ratio of destroyed leaf tissue. This result indicates that moths may avoid intraspecific competition for the same

food resources or poor quality hosts. The odor pattern of healthy leaves is modified by several odorant compounds produced in response to herbivore infestation; the odor pattern of leaves infested with late larval stages and a high ratio of destroyed leaf tissue shows additional compounds compared to leaves with early infestation stages. When leaves of *A. hippocastanum* were treated with single odorant compounds, these compounds elicited different effects on the number of eggs oviposited by *C. ohridella* females.

(*E*)- β -Caryophyllene, tested as the enantiomeric mixture, and benzaldehyde released by flowers and larval-damaged leaves of *A. hippocastanum*, resulted in increased levels of oviposition on treated leaves. (*E,E*)-2,4-Hexadienal, which is only emitted by leaves infested with late larval stages and a high ratio of brown to green leaf tissue, also enhanced oviposition. This compound may indicate increasing oxidative stress in leaf tissue infested with late stage larvae (Schütz, 2001). Thus, attractive compounds can be used for host location and recognition in spring (first generation, flowers). Compounds indicating the feeding activities of conspecifics may also help to identify the correct oviposition substrate (second and third generations, leaves), as was suggested for the Colorado potato beetle (Bolter et al., 1997; Schütz et al., 1997).

In contrast, 1,8-cineole and benzyl alcohol resulted in decreasing levels of oviposition on treated leaves. These compounds are known to act as defenses in plant herbivore systems. For instance, 1,8-cineole, which has antimicrobial effects (Zhu et al., 2005), is known to be induced as a plant defense against herbivores (reviewed by Langenheim, 1994). High concentrations of 1,8-cineole in plants can inhibit feeding by insects (Steinbauer and Wajura, 2002) and act as a repellent (Matsuki et al., 2001), as shown for Christmas beetles on eucalyptus. Benzyl alcohol is also known to be induced by herbivore infestation, for example, in the cases of red spotted spider mites on lima beans (Hopke et al., 1994) and psylla infestation on pear leaves (Scutareanu et al., 1997). Decanal and (*E/Z*)-linalool oxides (furanoid, tested as an enantiomeric mixture of isomers), which were released by leaves with high ratios of brown tissue because of infestation with late larval stages, also decreased the oviposition of females. Like (*E,E*)-2,4-hexadienal, these two compounds may indicate increasing oxidative stress in damaged leaf tissue due to decay processes in cells (Schütz, 2001). Decanal is known to be induced by mechanical and herbivore damage (Schütz et al., 1997; Weissbecker et al., 1999; Dicke et al., 2003) and by senescence of old leaves (Schütz, 2001). Because compounds with both positive and negative effects were emitted at the same time, further studies should focus on mixtures of these compounds on oviposition and the effect of these compounds on larval development. Furthermore, Our study investigated the chiral compounds (*E*)- β -caryophyllene and (*E/Z*)-linalool oxide (furanoid) as enantiomeric mixtures. Insects can selectively respond to different isomers and enantiomers in physiological experiments as well as in bioassays (Hobson et al., 1993; Zhang et al., 2003). Therefore, it is necessary to investigate these compounds with defined isomeric and enantiomeric purity in future experiments, and the results presented here should be interpreted with care.

The volatile compounds 2-phenylethanol, (*E,E*)- α -farnesene, and methyl salicylate, which act as defense compounds in host trees, did not influence the number of eggs laid. These compounds were recorded as present in odors released by herbivore-damaged plants and are utilized by predators (Dicke et al., 1990; Weissbecker et al., 1999; Maeda and Takabayashi, 2001) as well as parasitic wasps (De Moraes et al., 1998) to locate prey and hosts. No specialized parasitoid or predator for the leaf miner has been found to date, even in the area of first observation of *C. ohridella* (Freise et al., 2002; Grabenweger et al., 2005). 2-Phenylethanol and (*E,E*)- α -farnesene were perceived by the insect antennae, but did not affect oviposition. Methyl salicylate is involved in the induction of acquired resistance in

tobacco plants as an airborne signal (Shulaev et al., 1997). The compound was perceived by the insect antennae, but also did not affect oviposition. The observed high infestation rates produce up to 100% leaf browning in *A. hippocastanum* trees, and the defense status of leaves seems to have no effect on the host suitability for *C. ohridella*. Instead, insufficient food resources due to destroyed leaf tissue by conspecifics are probably the cause of high larval mortality, especially in autumn (Deschka, 1994; Freise, 2001; Kindl et al., 2002; Grabenweger, 2003). So far, the observed natural mortality of *C. ohridella* on *A. hippocastanum* leaves is low (Johné, 2001; Grabenweger, 2003). The lack of an effective defense by *A. hippocastanum* against infestation by *C. ohridella* might be one major cause of the invasive success of the insect.

In summary, our study demonstrated that adult *C. ohridella* were able to detect alterations in patterns of leaf volatiles induced by oviposition and larval mining by conspecifics. These alterations of leaf odor pattern affected the oviposition of the adults. Despite moths avoiding leaves highly browned by larval feeding, they did not display a reduction in oviposition to all single volatile compounds induced by larval mining. The preference for green and only slightly browned leaves ensures oviposition sites with satisfactory food resources for developing larvae, while also minimizing intraspecific competition.

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We recently reported that lepidopteran eggs and neonates contain significant amounts of JA (Tooker and De Moraes, 2005). We detected JA in each of nine lepidopteran species that we screened; this JA may have accumulated from the larval diets of the ovipositing mothers, although we found evidence that some lepidopteran species may have synthesized JA *de novo* because the unfed neonates of some species contained significantly greater amounts of JA than the eggs (Tooker and De Moraes, 2005).

The quantities of JA that we measured in lepidopteran eggs and neonates did not appear to be directly related to the amounts of JA in the larval diets of the parents—species that consumed the diets with the highest JA concentrations had relatively low levels of JA (Tooker and De Moraes, 2005). In this work, we expand our previous analyses of JA in lepidopterans and investigate the relationship between JA levels in the diets of caterpillars and in the insects themselves. We reared the generalist caterpillar *Heliothis virescens* (Fabricius) (Noctuidae) on artificial diets with differing JA concentrations, collecting frass and regurgitant and dissecting fifth instar larvae. We also collected similar samples from caterpillars reared on lines of transgenic tobacco with different capabilities of producing JA.

Methods and Materials

Insect Samples We reared the generalist herbivore *H. virescens* on three artificial diets with different concentrations of JA and on lines of transgenic tobacco that have been shown to have different capabilities of producing JA in response to insect attack. For the artificial diet experiment, we reared *H. virescens* larvae from eggs (USDA, Tifton, GA, USA) to second instar in an incubator (16:8 L:D; 25:22°C, day:night; 65% RH) on a casein-based artificial diet. When larvae reached the second instar, we starved them for 24 hr and transferred them to one of three casein-based diets that differed only in their JA concentration ($N = 30$ caterpillars per concentration). The control or “low” concentration diet was not supplemented with JA but contained $\sim 2 \mu\text{g JA/g FW}$ because diet ingredients derived from plant material (e.g., wheat germ and cellulose powder) or seaweed (e.g., agar) can contribute significant amounts of JA to diets, including our casein diet (Tooker and De Moraes, 2005, unpublished data); therefore, the use of a true “control” diet that did not contain JA did not appear possible. This concentration of $2 \mu\text{g JA/g}$ falls within the range of JA concentrations that have been reported for damaged plants (Royo et al., 1999). The medium concentration diet was supplemented to achieve $\sim 200 \mu\text{g JA/g}$, an amount similar to that used experimentally by others (Li et al., 2002). The “high” concentration diet was supplemented to $2000 \mu\text{g JA/g}$, a concentration that greatly exceeds JA levels reported for plants and might provide insight into how caterpillar process excess JA. The supplemental JA was synthesized from methyl jasmonate (Sigma-Aldrich, St. Louis, MO, USA; Farmer et al. 1992), and added to diets in an ethanol solution; an equal amount of ethanol was added to the low concentration diet.

For the transgenic tobacco study, we grew from seeds three lines of tobacco that have been shown to differ in their capability to produce JA in response to insect damage: OX-434, C17, and NahG (for details, see Felton et al., 1999). We reared *H. virescens* from eggs to the second instar (as above), then starved them for 24 hr and transferred them to diet cups containing leaf tissue. Caterpillars were monitored daily, and fresh tissue was added at least every other day.

In both experiments, we collected regurgitant with a pipette from fourth instars by gently squeezing their heads. Regurgitant was pooled and boiled for 3 min to destroy enzymatic activity and stored at -80°C until processing (see below). When larvae reached the fifth

instar (2–4 d postmolt), we collected frass and dissected caterpillars (10 per diet) on ice, collecting the labial salivary glands, gut, and remainder of the body into three separate FastPrep[®] tubes (Qbiogene, Carlsbad, CA, USA). We transferred boiled regurgitant (20 μ l/sample; 5 samples per diet) to FastPrep[®] tubes whereas frass and insect tissues were collected directly into the tubes. Tubes of known mass containing 1 g of Zirmil beads (1.1 mm; Saint-Gobain ZirPro, Mountainside, NJ, USA) were weighed to determine the mass of samples and stored at -80°C . We also collected for analysis samples of artificial diet as well as tobacco leaves that had been excised and caterpillar-damaged in diet cups for 24 and 48 hr (5 samples per line per time point; samples were processed individually, but the data were pooled to produce a representative mean per line). For the tobacco experiment, we also collected adult moths (6–10 per line) and the eggs they laid (10 samples each containing 10 eggs). We collected males and females after eggs had been laid. For the remainder of this article, we refer to the various insect tissues (e.g., salivary gland, gut, body, adults, eggs), regurgitant, and frass collectively as “tissues” to avoid awkward prose.

Extraction and Quantification of JA To extract and detect JA, we used a previously described method (Tooker and De Moraes, 2005) that was modified slightly from Schmelz et al. (2003, 2004). Briefly, we derivatized the carboxylic acid to its methyl ester, which was isolated by using vapor phase extraction and analyzed by GC-MS with isobutane chemical ionization by using selected-ion monitoring. For details on quantification and controls, see Tooker and De Moraes (2005).

To normalize the data and stabilize variance, we square-root- or natural log-transformed our data (artificial diet and tobacco data, respectively) and compared mean amounts of JA among and between samples by ANOVA, testing differences between diets and tissues as planned comparisons with the LSD means separation test (Statistix, 2003).

Results and Discussion

We detected JA in all tissues of caterpillars reared on the artificial diets and tobacco lines (Fig. 1). Retention times and mass spectra of meJA recovered from diets and tissues matched the pure standard, confirming the identity of JA in our samples. Moreover, we did not recover meJA in the absence of the derivatization agent, verifying that the methyl ester we measured was derivatized from JA and was not itself present in samples.

By adding supplemental JA, we created diets with three distinct JA concentrations (Fig. 1a; ANOVA, $F_{2,14} = 154.1$, $P < 0.001$). The final concentrations of our medium and high concentration diets exceeded our targets; however, the final concentration of diets is not as important as the differences between diets and the concentrations of JA in caterpillar tissues. Caterpillars reared on each of the three artificial diets had significant differences across their tissues (Fig. 1a). Those reared on the “low” diet had guts, bodies, frass, and regurgitant containing amounts of JA statistically similar to that found in the diet (LSD, $P > 0.05$), but the amount of JA found in salivary glands was significantly enriched above that of the diet and the other tissues (Fig. 1a; LSD, $P < 0.05$). For caterpillars reared on medium and high diets, frass contained significantly greater concentrations of JA than the diets, suggesting that JA was not metabolized but passed through the digestive tract unaltered (Fig. 1a). Moreover, guts of caterpillars reared on these high-concentration diets had significantly less JA than was found in frass and regurgitant, which includes foregut contents, indicating that JA is removed to the frass or is somehow bound while moving

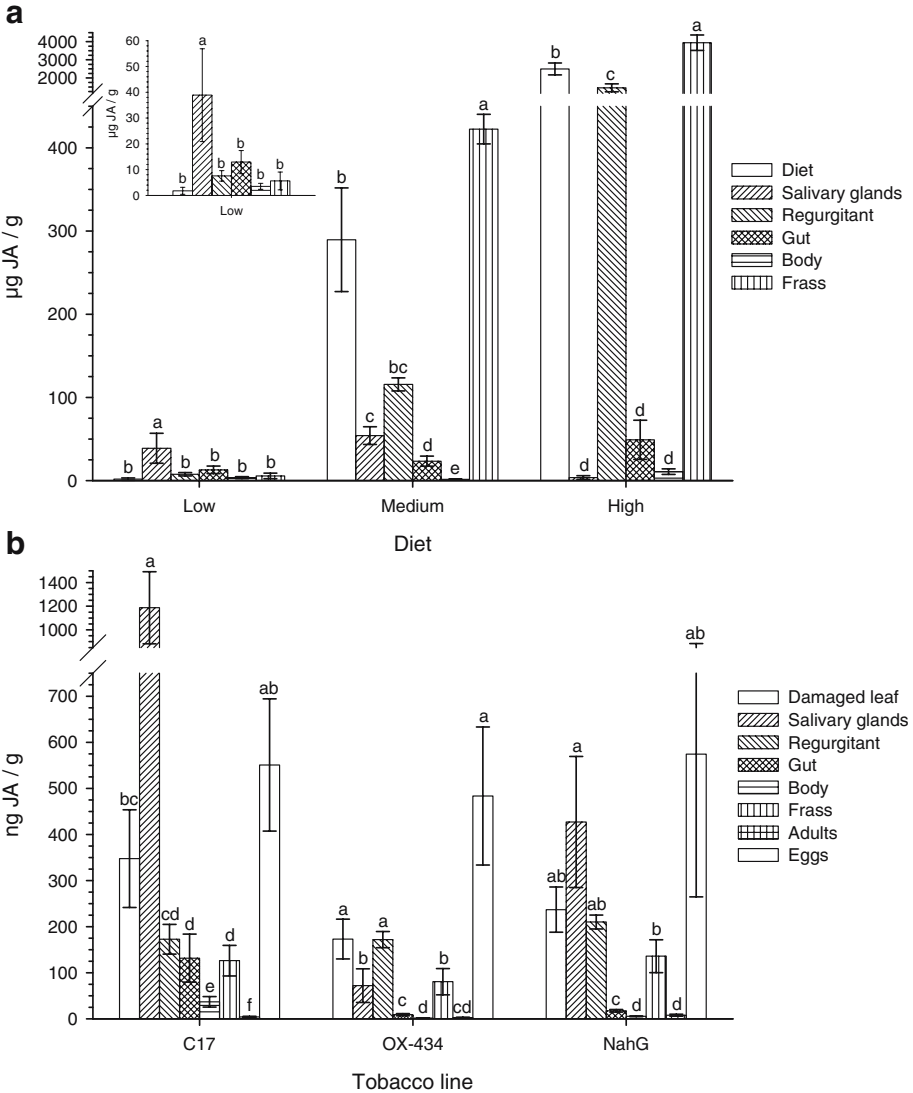


Fig. 1 Jasmonic acid (JA) detected in tissues of *H. virescens* caterpillars reared on (a) artificial diets with different concentrations of JA (inset shows details from caterpillars reared on low diet; within-diet ANOVA comparisons: low concentration— $F_{5,27} = 8.45, P < 0.001$; medium concentration— $F_{5,26} = 44.6, P < 0.001$; high concentration— $F_{5,27} = 44.3, P < 0.001$). (b) Lines of transgenic tobacco (within-line ANOVA comparisons: C17— $F_{7,76} = 23.9, P < 0.001$; OX-434— $F_{7,69} = 45.1, P < 0.001$; NahG— $F_{7,72} = 15.3, P < 0.001$). Data shown are untransformed. Within each diet, tissues marked with different letters are significantly different from each other (LSD $P < 0.05$; see text for details on statistics)

through the midgut. Caterpillars on both supplemented diets also produced regurgitant that tended to have higher concentrations of JA than those found in salivary glands and was enriched in JA significantly above gut samples, likely reflecting recently devoured diet in the foregut, which is expelled when caterpillars regurgitate. For medium and high diets, body samples (comprising mainly cuticle and fat body) contained relatively low

concentrations of JA (Fig. 1a). Caterpillars reared on the high concentration diet consumed more than those fed medium and low diets (5.8 ± 0.2 , 4.2 ± 0.3 , and 4.2 ± 0.2 g, respectively; ANOVA, $F_{2,73} = 13.9$, $P < 0.001$), indicating that the greater levels of JA in “high” larvae were a function of both diet and amount consumed. Moreover, greater consumption of this “high” diet may indicate that JA concentration alone can trigger increased consumption by insects in response to induced defenses; however, this finding should be interpreted with caution because of the unrealistic amount of JA in the high concentration diet.

The three lines of transgenic tobacco contained similar amounts of JA (ANOVA, $F_{2,29} = 2.2$, $P = 0.14$), an unexpected finding given previous work with these lines (Felton et al., 1999); however, we did not use intact plants, and the detached leaves may have had diminished capacities to respond to herbivory relative to leaves remaining on plants. Nevertheless, our results clearly indicate that caterpillars feeding upon plants with lower JA concentrations (relative to our artificial diet experiment) accumulate JA in their tissues (Fig. 1b).

Caterpillars reared on each of the three tobacco lines showed statistical differences in JA concentrations across tissues (Fig. 1b). Moreover, tissues from caterpillars reared on each diet showed similar patterns of differences across all three tobacco lines. First, C17 and OX-434 caterpillars had JA levels in frass that were significantly lower than concentrations found in damaged leaves, and NahG caterpillars showed a similar trend (Fig. 1b). These results suggest that caterpillars reared on plant tissue retained proportionally more of the JA they ingested than caterpillars reared on supplemented artificial diets whose frass had concentrations greater than those of their diet (Fig. 1a). Second, guts contained significantly less JA than the damaged leaves, again suggesting that JA is excreted in the frass or bound and made unavailable. Third and similar to the diet-reared caterpillars, levels of JA in body tissue were relatively low, implying that JA was not harbored in the exoskeleton or fat body. Fourth, adult moths (males and females were combined into one group because they had similar JA concentrations) contained relatively little JA, whereas the eggs they produced contained amounts statistically similar to those found in their tobacco diets (Fig. 1b). These results may indicate that adults provision their eggs with JA obtained from their diet, but could also indicate that moths synthesize JA and incorporate it into eggs. Because we did not collect moths upon eclosion, we can not make this distinction.

Caterpillars reared on the three tobacco lines also showed some significant differences across lines despite their similar in-plant levels of JA, suggesting that features of the individual lines influenced JA accumulation in insects. For example, significantly different amounts of JA accumulated in salivary glands of caterpillars reared on the three diets (Fig. 1b; C17 > NahG > OX-434; ANOVA, $F_{2,29} = 23.3$, $P < 0.001$). These results contrast with those from our artificial diet experiment where we found consistent amounts of JA in salivary glands across diets (ANOVA, $F_{2,14} = 1.7$, $P = 0.22$) despite large differences in the JA content of the diets (Fig. 1a). It is unclear why JA accumulates in insect salivary glands, although host plants might benefit from the incorporation of JA into insect saliva, as it is an important trigger of plant defensive responses. In this case, selection on caterpillars would be expected to favor suppressing accumulation, but JA might be retained due to physiological constraints or some countervailing benefit to the insect. The adaptive significance of JA accumulation in insect salivary glands will likely remain unclear until the physiology of accumulation and the role of accumulated JA in interactions with the host plant are better understood.

Our previous work indicated that JA could be present in lepidopteran eggs and neonates (Tooker and De Moraes, 2005). The data we present here demonstrate that JA is

consistently present in various insect tissues. When diets contained high amounts of JA, jasmonate accumulated in insect tissues, but caterpillars voided large quantities. When diet concentrations were lower as with our transgenic tobacco experiment, caterpillars appeared to retain JA and excreted relatively low amounts. Significant amounts of JA accumulated in the salivary glands, a notable result given the importance of this tissue to insect–plant interactions (Felton and Eichenseer, 1999) and the role of JA as a signaling molecule in the induction of plant defense responses. Further research on the mechanisms of JA accumulation and its physiological roles is needed to determine the adaptive significance of JA in salivary glands and other insect tissues.

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Previously, we questioned the importance of (\pm)-catechin (hereafter referred to as catechin) in spotted knapweed's invasion (Blair et al., 2005). Specifically, we (1) found published techniques to be unrepeatable, (2) found levels of catechin production more than an order of magnitude less than previously reported, (3) showed that a species described as sensitive to catechin (*Festuca idahoensis*) only exhibited slightly reduced growth at concentrations 10 times higher than previously reported to cause 100% mortality, and (4) found no measurable catechin at two spotted knapweed sites in Montana, USA. In regards to this last point, it was noted that the soil samples were collected in the late fall, when plants may be less likely to be actively producing the root exudate. This paper addresses this issue by sampling and analyzing soil from three knapweed-infested sites during the summer and fall.

Methods and Materials

Spotted knapweed is a self-incompatible Eurasian biennial or perennial that threatens grassland diversity and productivity across western North America (Roché and Roché, 1991). Spotted knapweed is commonly called *C. maculosa* in North America; however, there remains much confusion regarding the nomenclature. There are two cytotypes of the plant, which are treated either as separate species or as subspecies. *Centaurea maculosa* Lam. has been used for both the diploid and tetraploid form. The diploid form has been synonymized with *Centaurea stoebe* L. spp. *stoebe* (Ochsmann, 2000). The tetraploid has been referred to as *C. biebersteinii* DC or *C. stoebe* L. spp. *micranthos* (Gmelin ex Gugler) Hayek. Ochsmann (2000) argues that the *C. stoebe* name takes priority. The *C. stoebe* species group consists of several taxa of uncertain status that are difficult to distinguish morphologically. Herein, we refer to the plant by its common name, spotted knapweed.

Knapweed-Infested Field Sites and Sampling Protocol

Three field sites were sampled through time to assess catechin production over the course of a growing season (see Table 1 for site characteristics). The soil around five, randomly selected spotted knapweed plants was sampled from 3 to 5 cm depth using 6 cm diam cores. Three cores were taken adjacent to and directly under the knapweed crown for a total of 6 cores per plant, pooled, and mixed. Bulk soil samples (200 g) were collected from outside the three infested sites to serve as control soils. All samples were frozen within 1 h of collection and shipped on ice to Colorado State University, where they were stored at -20°C until analysis.

Quantitation of Catechin in Soil

We extracted and analyzed catechin in the soils using HPLC following protocol of Blair et al. (2005). We used field soils fortified with 5 ppm catechin to assess extraction efficiencies.

Catechin Persistence in Wet vs. Dry Soil

Because catechin was found above trace levels in August only (see results below), we conducted an additional experiment to evaluate the persistence of catechin in these field soils. Based on previous work (Blair et al., 2005), we hypothesized that catechin may have been able to build up in August because the soils were quite dry, but that it would degrade if

Table 1 Knapweed infested site and soil characteristics

Site MT, USA	Infestation Duration	Soil Type	pH	% Organic Matter	Sample Dates	Recovery Efficiency %	Catechin (ppm)	% Moisture
Nelson Gulch ^a	12 y	Windham	7.9	4.2	7/5/05 ^b	87.4	trace (1) ^f	5.1 ± 0.7
46°34.373' N		Sandy			8/4/05 ^c	+100.0	1.1 ± 0.3	2.0 ± 0.05
112°8.820' W		Loam			9/26/05 ^d	79.0	trace (1) ^f	11.2 ± 0.4
4273 m					10/27/05 ^e	90.6	0	14.1 ± 1.3
Beck Hill ^a	20 y	Tanna	7.5	3.5	9/14/05 ^a	43.6	0	9.7 ± 0.4
46°29.472' N		Sandy						
112°43.422' W		Loam			10/28/05 ^e	65.6	0	8.2 ± 0.3
4497 m								
Jens Exit ^a	>15 y	Blossberg	7.2	6.1	8/5/05 ^c	+100.0	1.3 ± 0.4	5.0 ± 0.2
46°36.359' N		Sandy			9/25/05 ^d	71.8	Trace (2) ^f	15.1 ± 0.4
113°00.832' W		Loam			10/28/05 ^e	47.4	Trace (2) ^f	12.5 ± 1.4
4136 m								

^a Plant species at Nelson and Beck include bluebunch wheatgrass (*Pseudoroegneria spicata*), Idaho fescue (*Festuca idahoensis*), and 5% scattered ponderosa pine (*Pinus ponderosa*). Species at Jen include sandberg bluegrass (*Poa secunda*) and Idaho fescue (*Festuca idahoensis*).

^b plants at bolting stage;

^c plants at flowering stage;

^d plants senescing;

^e basal regrowth (≈2 cm).

^f less than 0.1 ppm catechin; the number in parentheses refers to the number of cores with trace catechin out of 5.

the soils were moist. We tested this hypothesis using soil from the cores with the highest naturally occurring catechin concentrations from the Nelson and Jens August samples and soils from control areas of these sites fortified at 5 ppm catechin. We moistened samples of 5 g of soil ($N = 3$ per sample) with 1 ml water and allowed them to sit at room temperature in the dark for 24 h. As positive controls, un-moistened soils with natural or fortified catechin ($N = 3$ per sample) were subject to the same environment. Catechin was quantitated as above. The percent soil moisture from each field site through time was determined by drying approximately 5 g overnight at 105°C and comparing mass before and after.

Results

Quantitation of Catechin in Soil

Recovery efficiencies across the three sites through time averaged $80.3 \pm 9.4\%$ (mean \pm SE). The detection limit of our method was 0.02 ppm, well below previously reported levels of catechin found in the field, and much lower than what has been suggested to be biologically active. Catechin was found in all soil cores at the two sites sampled in August (Nelson and Jens) (Table 1). Only 14% of the other soil cores had detectable trace levels of catechin (<0.11 ppm) (Table 1).

Catechin Persistence in Wet vs. Dry Soil

The naturally occurring catechin was not detectable in the Nelson and Jens soil samples 24 h after they were moistened. Similarly, only 22% and 6% of the catechin was recovered from the moistened fortified Nelson and Jens control soils, respectively. However, the naturally occurring catechin from the same Nelson and Jens cores remained detectable in the un-moistened controls (Nelson = 1.83 ± 0.21 ppm and Jens = 2.9 ± 0.5 ppm). Fortified control soils behaved similarly; 81.4% and 74.8% of the catechin was recovered from the un-moistened Nelson and Jens control soils, respectively.

These data support our hypothesis that the amount of detectable catechin found in these soils through time can be explained by field soil moisture levels. The two sites that had detectable catechin in August 2005 were among the three with the lowest soil moisture levels of any samples (Table 1).

Discussion

We found catechin in field soils at levels three orders of magnitude lower than have previously shown to be toxic (Blair et al., 2005). Additionally, based on the research herein and our previous work, we believe that it is unlikely that catechin will persist in the field at levels necessary to influence plant distributions. Blair et al. (2005) found catechin to be highly unstable at neutral and basic pH in both water and soil, and even in acidic soils if they were moist. In this study, we found that catechin degraded rapidly in moistened soils from the low or trace amounts naturally present to undetectable (<0.02 ppm). We hypothesize that our inability to find catechin even when the soil moisture was relatively low (i.e., Nelson 7-5-2005, soil moisture $\approx 5\%$) suggests that sporadic precipitation may prevent a build-up of the compound in the soil via the chemical process of degradation and/or enhanced microbial activity.

Our finding of low or undetectable levels of catechin from field-collected soils are in sharp contrast to the recent reports of catechin found in the field at levels ranging from ~1,550 to 3,300 ppm on average, with a single high value reported at 7,100 ppm (also from Montana, USA) (Bais et al., 2003; Perry et al., 2005; Thelen et al., 2005). It is plausible that differing sites will have varying amounts of catechin, as soil characteristics and microbes will greatly influence the stability and persistence of this compound. However, we are not confident in the accuracy of the remarkably high concentrations reported in these papers. They used a methanol extraction technique that we found to yield between 0 and 17% extraction efficiency with 6 different soil types fortified with 10 ppm catechin (Blair et al., 2005). If extraction efficiency is similar for higher levels of catechin and was on the high end of this range for their soils (e.g., 20%), then their estimates would increase 5-fold to approximately 7,750 to 16,500 ppm, or 7.8 to 16.5 mg of catechin per gram of soil (Bais et al., 2003; Perry et al., 2005; Thelen et al., 2005). Given that our data here suggest catechin is not likely to build up in the soil due to precipitation, this seems like a physiologically unreasonable amount for a plant to produce, and still have carbohydrates remaining for growth and maintenance.

While catechin may be toxic to plants at high concentrations, this study adds to the case that it is unlikely to be a strong force in the successful invasion of spotted knapweed. We predict that rainfall will never allow the chemical to attain toxic levels. However, this research does not rule out the possibility that spotted knapweed is allelopathic, and we suggest that future energies be directed at assaying additional putative allelochemicals.

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Keywords Glucosinolate · Epithio-specifier protein · Nitrile · Isothiocyanate · *Arabidopsis thaliana* · *Spodoptera littoralis* · Insect development

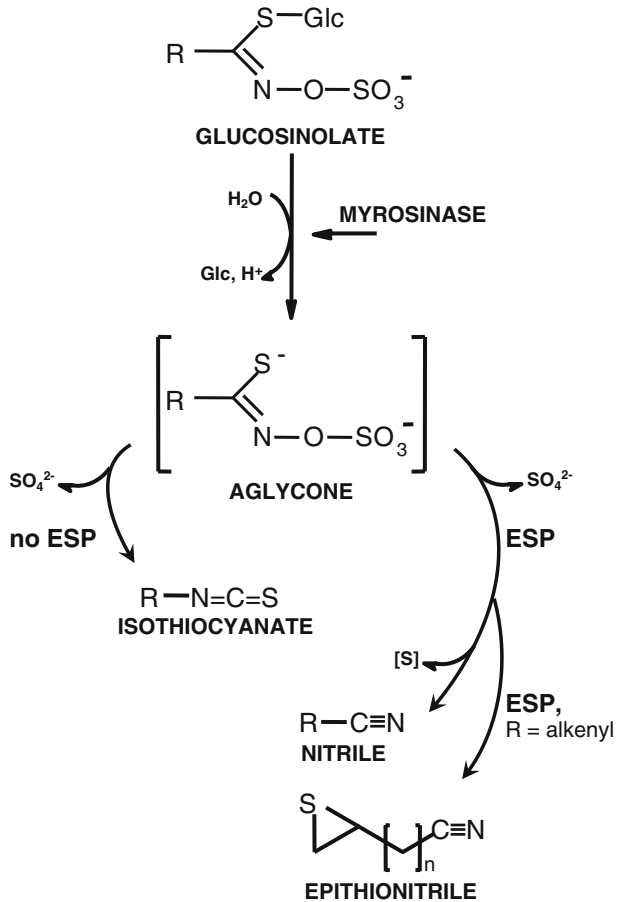
Introduction

Assessing the effects of plant defenses on insect herbivores has challenged chemical ecologists for a long time. Although numerous studies have investigated the role of chemical defenses in plant–herbivore interactions, the impact of specific compounds on insect herbivores is often hard to discern. Results obtained from insect feeding studies with intact plants that show quantitative or qualitative differences in putative defense compounds are frequently difficult to interpret, because the plants tested differ in a number of chemical and morphological parameters besides the defensive compounds of interest. These difficulties can be circumvented by the use of pure compounds added to artificial diets, but only if an artificial diet is available for the insect to be studied and the defense compounds can be isolated in sufficient quantity and purity for testing. Furthermore, the compounds must be soluble in the diet and chemically stable during the experiment. Unfortunately, artificial diet studies often do not adequately mimic the way chemical defenses are stored within the plant, and insect feeding behavior and physiology may be different on artificial diets than on intact plants (Wittstock et al., 2003). The effects of chemical defenses that are formed from inactive precursors upon insect feeding are particularly difficult to investigate in artificial diet studies, since it is hard to simulate the conversion to active forms in a way that is spatially and temporally similar to what occurs in the intact plant. In addition, if the active form is added directly to the artificial diet, the sensory response of insects may be completely different from that towards the inactive precursors the insects normally encounter when searching for a feeding site, and the insects may even completely avoid feeding on the test diet.

One of the most intensely studied examples of an activated chemical defense in plants is the glucosinolate–myrosinase system of the Brassicaceae and related plant families (Matile, 1980; Fig. 1). The defensive function of this system has been attributed to the isothiocyanates that are released from the parent glucosinolates by hydrolytic enzymes called myrosinases, upon tissue damage. However, besides isothiocyanates, other hydrolysis products can also be formed, namely nitriles, thiocyanates, and oxazolidinethiones (Schlueter and Gmelin, 1972; Hasapis and MacLeod, 1982; MacLeod and Rossiter, 1985). The formation of nitriles depends on the presence of protein factors known as epithiospecifier proteins (ESPs) and is restricted to certain plants and specific ecotypes within a species (Tookey, 1973; Petroski and Kwolek, 1985; Lambrix et al., 2001). Thus, the same glucosinolate can be hydrolyzed to different products depending on the presence or absence of ESP.

Numerous studies have been conducted to elucidate the ecological role of the different types of glucosinolate hydrolysis products (Lichtenstein et al., 1962; Chew, 1988; Stoewsand, 1995; Rask et al., 2000; Tierens et al., 2001; Buskov et al., 2002). However, these investigations have been fraught with difficulties. On the one hand, testing the effects of glucosinolate hydrolysis products by using intact plants with different profiles of hydrolysis products is problematic because plant properties other than hydrolysis products, such as leaf morphology, nutritional content, or other defensive compounds, may also vary (Nielsen et al., 2001). On the other hand, testing the effects of isolated glucosinolate hydrolysis products with artificial diets or other substrates has also proved to be a challenging task. Apart from the fact that only a few of these compounds are available in

Fig. 1 The glucosinolate–myrosinase system. Upon damage of plant tissue, glucosinolates are hydrolyzed by myrosinases yielding glucose and unstable aglycones. The aglycones spontaneously rearrange into isothiocyanates, the most common class of glucosinolate hydrolysis products. In some plants, epithiospecifier proteins (ESPs) redirect glucosinolate hydrolysis towards the formation of nitriles. If there is a terminal double bond in the amino acid derived side chain, the sulfur atom released from the thioglucosidic bond during nitrile formation is captured in a thiirane ring resulting in epithionitrile formation



pure form, they differ in their solubility and volatility as well as their potential to react with other diet components, thus making the results of comparative feeding tests difficult to interpret. A notable alternative might be the recently reported incorporation of isothiocyanates into an artificial diet by microencapsulation (Agrawal and Kurashige, 2003), a technique that should be feasible for other glucosinolate hydrolysis products as well.

The availability of molecular tools for the model plant *Arabidopsis thaliana* (Brassicaceae) has facilitated the identification of genes that control glucosinolate biosynthesis and hydrolysis (Mikkelsen et al., 2000; Wittstock and Halkier, 2000; Hansen et al., 2001; Kroymann et al., 2001; Lambrix et al., 2001; Reintanz et al., 2001). These advances have opened the possibility to specifically manipulate glucosinolate levels and to introduce biosynthetic pathways that lead to the accumulation of new defensive compounds by genetic engineering (Bak et al., 2000; Petersen et al., 2001; Tattersall et al., 2001). In this study, we generated transgenic lines of *A. thaliana* that differ from wild-type plants only in the type of glucosinolate hydrolysis that is formed upon tissue damage in order to investigate the effects of different types of hydrolysis products on insect herbivores. While isothiocyanates are highly toxic to insect herbivores (Seo and Tang, 1982) including

specialized feeders on glucosinolate-containing plants (Agrawal and Kurashige, 2003), less is known about the defensive function of nitriles. In one of the few studies that have been carried out to compare the effects of nitriles and isothiocyanates with intact plants, larvae of the generalist lepidopteran *Trichoplusia ni* preferred nitrile-producing over isothiocyanate-producing plants when they were allowed to choose among recombinant inbred lines of *A. thaliana* (Lambrix et al., 2001). However, these lines differed in their glucosinolate profile as well as the type of hydrolysis products; both factors significantly affected herbivory by *T. ni* (Lambrix et al., 2001). Therefore, additional feeding trials that use intact plants engineered to differ only in the type of hydrolysis products formed upon tissue damage are required to compare directly the toxicity of nitriles and isothiocyanates to insect herbivores. To produce such transgenic lines, we transformed plants of the ecotype Columbia-0 devoid of ESP expression with a construct containing the ESP cDNA from the ecotype Landsberg *erecta* (*Ler*) under the control of the strong constitutive cauliflower mosaic virus 35S (CaMV35S) promoter. Overexpression of ESP led to strongly enhanced formation of nitrile products at the expense of isothiocyanates upon glucosinolate hydrolysis. To test the value of these transgenic lines in insect feeding trials and to answer the question whether the presence of ESP has an impact on insect performance, we carried out bioassays with the model generalist insect herbivore *Spodoptera littoralis* (Lepidoptera: Noctuidae).

Methods and Materials

Plant and Insect Culture

Arabidopsis thaliana plants were grown on soil in a controlled environment chamber (21°C, 55% relative humidity, and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation) for 4–5 wk. The photoperiod was 10:14 hr light/dark.

Eggs of *S. littoralis* were obtained from Syngenta Crop Protection AG (Stein, Switzerland), and were kept at a temperature between 20°C and 22°C until hatching.

Intact Glucosinolates and Glucosinolate Hydrolysis Products Used as Enzyme Substrates and Standards for Analysis

Allylglucosinolate was purchased from Sigma-Aldrich, 4-hydroxybenzylglucosinolate was obtained from Bioraf Danmark (Copenhagen, Denmark). Allyl isothiocyanate (3-isothiocyanatoprop-1-ene) and allyl cyanide (but-3-enenitrile) standards were obtained from Fluka (Taufkirchen, Germany); propyl isothiocyanate (1-isothiocyanatopropane) was from Sigma-Aldrich Chemie (Schnellendorf, Germany); benzonitrile was from Merck (Darmstadt, Germany).

Cloning of ESP

To obtain the full-length ESP cDNA (GenBank, accession number AF416787), 1.3 μg RNA extracted from rosette leaves of *A. thaliana*, ecotype Landsberg *erecta* (*Ler*) were transcribed into cDNA by using Superscript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions. The ESP cDNA was amplified from the reaction by polymerase chain reaction (PCR) using the forward

primer (5'-GCAGCCATGGCTCCGAC) and the reverse primer (5'-TCATCTAGAT-TAAGCTGAATTGACCGCATAG). The PCR reaction was done in a total volume of 50 μ l PCR buffer containing 2.5 units *Pwo* DNA Polymerase (Roche Molecular Biochemicals, Mannheim, Germany), 1 mM MgSO₄, 1 μ l of the reverse transcriptase reaction, 100 μ M dNTPs, and 50 pmol of each primer. After incubation for 3 min at 94°C, 30 cycles of 94°C for 30 sec, 60°C for 50 sec, and 72°C for 60 sec were carried out. The PCR product was digested with *Nco*I and *Xba*I and ligated into the pTrc99A vector (Amersham Pharmacia Biotech, Freiburg, Germany). The correct insert sequence was verified by sequencing.

Generation of Transgenic Plants

After subcloning into the pYX223 vector (Ingenius, Abingdon, Oxon., UK) by using *Nco*I and *Sal*I, the ESP cDNA was cloned into the *Eco*RI/*Xba*I-digested pRT101 vector (Töpfer et al., 1987) downstream of the CaMV35S promoter. The *Pst*I-digested expression cassette was then transferred into the pPZP221 vector (Hajdukiewicz et al., 1994). *Agrobacterium tumefaciens* strain PGV38 c58 transformed with this construct or the empty pPZP221 vector was used to transform *A. thaliana* ecotype Col-0 by floral dip (Clough and Bent, 1998). For selection of positive transformants, seeds were germinated on MS medium containing 2% (w/v) sucrose, 0.9% (w/v) agar, and 100 μ g/ml gentamycin. Independent homozygous lines with a single T-DNA insertion were chosen based on the segregation of gentamycin resistance. Three independent lines that overexpress the ESP cDNA (35S:ESP 2.6, 37.3, 38.1) were characterized in the T3 generation, as were three independent vector control lines (pPZP221 7.1, 8.2, 36.1). Insect feeding trials were done with T4 generation plants.

Glucosinolate Analysis

For quantification of glucosinolates, 10–15 mg freeze-dried leaf material were ground to a fine powder in a paint shaker (Kliebenstein et al., 2001b) and extracted with 1.5 ml 80% (v/v) methanol for 5 min at room temperature before 50 μ l 1 mM 4-hydroxybenzylglucosinolate were added as internal standard. Insoluble material was pelleted by centrifugation at 2500 \times g for 10 min, and the supernatants were loaded on columns containing 0.4 ml of a 10% (w/v) suspension of DEAE Sephadex A25 in H₂O. Columns were washed with 1 ml 80% (v/v) methanol, 1 ml water, and 1 ml 0.02 M MES buffer (pH 5.2), before 50 μ l of sulfatase solution were applied (Hogge et al., 1988). After incubation at room temperature overnight, desulfated glucosinolates were eluted with 2 \times 0.8 ml 60% (v/v) methanol, dried at 50°C in a nitrogen stream, and then redissolved in 0.4 ml water. Samples were analyzed by high-performance liquid chromatography (HPLC) on an Agilent HP1100 Series instrument equipped with a C-18 reversed phase column (LiChrospher RP18ec, 250 \times 4.6 mm, 5 μ m particle size) by using a water (solvent A)–acetonitrile (solvent B) gradient at a flow rate of 1 ml min⁻¹ at 25°C (injection volume 50 μ l). The gradient was as follows: 1.5% B (1 min), 1.5–5% B (5 min), 5–7% B (2 min), 7–21% (10 min), 21–29% (5 min), 29–43% (7 min), 43–100% (0.5 min), 100% B (2.5 min), 100–1.5% B (0.1 min), and 1.5% B (4.9 min). The eluent was monitored by diode array detection between 190 and 360 nm (2-nm interval). Desulfoglucosinolates were identified based on comparison of retention times and UV absorption spectra with those of known standards (Reichelt et al., 2002). Results are given as μ mol (g dry weight)⁻¹ calculated from the peak areas at 229 nm relative to the peak area of the internal standard using the relative response factors 2.0 for aliphatic and 0.5 for indole glucosinolates. These relative response factors were evaluated in our laboratory to give more

accurate results than previously published relative response factors (Fiebig and Arens, 1992; Wathelet et al., 2004). The remarkable differences of relative response factors of certain glucosinolates published by different groups are likely due to considerable differences in the hygroscopic properties of the glucosinolates.

Plant Extracts for Detection of ESP Protein, Measurements of Enzyme Activities and Glucosinolate Hydrolysis Products

Crude plant extracts were prepared from rosette leaves of *A. thaliana* by grinding 500 mg of leaf material with 500 μ l of 50 mM MES buffer, pH 6.0. After incubation at room temperature for 5 min, samples were centrifuged at 10,000 \times *g* and 4°C for 10 min. The supernatants were kept on ice before they were used for measurements of ESP and myrosinase activities, hydrolysis products from internal glucosinolates and total isothiocyanates as described below. The protein content of the extracts was determined with the Pierce BCA Protein Reagent (Perbio Science Deutschland GmbH, Bonn, Germany) using bovine plasma γ -globulin (Biorad, Munich, Germany) as a standard.

ESP Assay

To assay ESP activity, 100 μ l plant extract were incubated with 1 mM allylglucosinolate in 50 mM MES buffer (pH 6.0) in a total volume of 500 μ l at room temperature for 1 hr. After addition of 50 μ l benzonitrile (100 ng/ μ l in methanol) as an internal standard, the samples were extracted with 2 \times 750 μ l dichloromethane. The combined organic phases were dried over Na₂SO₄, concentrated to about 100 μ l in a nitrogen stream, and analyzed by gas chromatography (GC) with flame ionization detection (FID) using an Agilent 6890 series gas chromatograph with an HP5MS column (30 m \times 0.25 mm \times 0.25 μ m), splitless injection at 200°C (injection volume 1 μ l), and the following temperature program: 35°C for 3 min, 12°C/min to 96°C, 18°C/min to 150°C, 60°C/min to 300°C, and 4-min final hold. Identity of the products was verified by GC-mass spectrometry (GC-MS) (Lambrix et al., 2001) in comparison with authentic standards and published MS spectra (Spencer and Daxenbichler, 1980). Products were quantified as previously described (Lambrix et al., 2001). ESP activity was expressed as the amount (nmol) of 3,4-epithiopropylcyanide formed from allylglucosinolate per min and mg protein. Under the conditions described above, 3,4-epithiopropylcyanide formation by ESP was linear with time for 2 hr using plant extracts of the ecotype *Ler*.

Myrosinase Assay

Myrosinase assays were carried out in 50 mM MES buffer, pH 6.0, with 1 mM allylglucosinolate and 25 μ l of plant extract in a total volume of 100 μ l. After incubation at 37°C for 30 min, the reaction was stopped by boiling (95°C for 5 min). Samples were centrifuged at 10,000 \times *g* for 5 min, and 90 μ l of the supernatant were used for glucose determination using the Glucose GOD/PAP Kit from Randox (Crumlin, UK). Absorbance was measured at 500 nm against the absorbance of samples incubated without allylglucosinolate. The amount of glucose formed by myrosinase was calculated based on a calibration curve obtained with a glucose standard solution. Myrosinase activity was expressed as the amount (nmol) of glucose formed per minute and mg total protein. Under standard assay conditions, glucose formation as a measure for myrosinase activity was linear with time for at least 50 min.

Analysis of Glucosinolate Hydrolysis Products in Plant Extracts

Crude plant extracts (200 μ l) to which 30 μ l benzonitrile (100 ng/ μ l in methanol) as internal standard had been added were extracted with dichloromethane and analyzed by GC-FID and GC-MS as described above for ESP assay mixtures. The temperature program used was 45°C for 3 min, 12°C/min to 96°C, 18°C/min to 150°C, 60°C/min to 270°C, and 3-min final hold. Quantification of the hydrolysis products of 4-methylsulfinylbutylglucosinolate was carried out as previously described (Lambrix et al., 2001).

Determination of Total Isothiocyanates in Plant Extracts

Total isothiocyanates were quantified after cyclocondensation with benzene-1,2-dithiol (Zhang et al., 1996). Plant extract (30 μ l), 20 μ l 1 M Tris-HCl, pH 8.5, and 150 μ l H₂O were mixed and equilibrated to room temperature before 200 μ l of 10 mM benzene-1,2-dithiol (Fluka) in methanol were added. Conversion of benzene-1,2-dithiol to benzo[1,3]dithiol-2-thione was accomplished by incubation for 1 hr at 65°C. The samples were centrifuged at 16,000 \times g for 3 min to pellet precipitate, and a 50- μ l aliquot of the supernatant was analyzed by using an Agilent 1100 series HPLC (Agilent Technologies) with an Supelcosil LC18 column (150 mm \times 2.1 mm \times 5 μ m, Supelco) and an isocratic elution with 85% (v/v) methanol. The eluates were monitored at 365 nm by a UV detector, and the area of the benzo-1,3-dithiole-2-thione peak was integrated. Total amounts of isothiocyanates were calculated based on a calibration curve done with propyl isothiocyanate.

Western Blotting

A rabbit polyclonal antibody against ESP was raised by using the synthetic peptide H₂N-RDENRNFENFRSYDTV-CO-NH₂, which corresponds to the amino acids 94–109 of the native protein N-terminally coupled with keyhole limpet hemocyanin as a carrier (Eurogentec, Seraing, Belgium). Proteins from 100 μ l plant extract were concentrated by trichloroacetic acid precipitation. The protein pellets obtained by centrifugation at 10,000 \times g and 4°C for 30 min were dissolved in SDS sample buffer, boiled for 5 min, separated on 12% Tris-SDS-PAGE gels, and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were blocked with 2% nonfat dried milk powder in TTBS [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20] for 30 min before addition of the anti-ESP antibody serum (1:10,000 diluted with TTBS, containing 2% dry milk powder). After incubation at 4°C overnight and washing with TTBS (3 \times 7 min), the secondary antibody (antirabbit IgG alkaline phosphatase conjugate; Sigma, 1:10,000) was added in TTBS, containing 2% nonfat dried milk powder. Membranes were incubated at room temperature for 1 hr, washed with TTBS (3 \times 7 min), and rinsed with detection buffer (100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl and 5 mM MgCl₂). Detection was carried out with 0.085 μ g/ml nitroblue tetrazolium and 0.17 μ g/ml 5-bromo-4-chloro-3-indolyl phosphate in detection buffer.

Insect Feeding Trials

To investigate the performance of *S. littoralis* on the ESP-expressing Arabidopsis lines (35S: ESP 2.6, 37.3, 38.1), the vector control lines (pPZP221 7.1, 8.2, 36.1) and Col-0 wild-type plants, larvae were reared from hatching to pupation on 5-wk-old plants (T4) of each line.

The parameters recorded were the larval weight gain, time until pupation, the pupal weight, the duration of the pupal stage, and the proportion of successful adult emergence.

Each experiment was started with 50 neonate larvae on 20 plants per line. Plants and insects were placed in a controlled environment chamber with a 16:8 hr light/dark photoperiod, 70% relative humidity, and a constant temperature of 22°C. The weight of each larva was recorded every day starting from day 8, when the larvae were transferred to fresh plants for the first time. To ensure sufficient food supply, larvae were transferred to new plants 5–6 additional times depending on their consumption rate. As a consequence of the enormous food consumption by larvae of later stages and for other practical reasons, we reduced the number of individuals per plant line in two steps. About 10 d after hatching, the 15 smallest and the 15 biggest individuals of each group were removed. A further reduction to 10 larvae per plant line was accomplished in the same way 2 d later. This reduced the variability within groups. Only individuals with a growth rate close to the medial growth rate of larvae on a particular plant line were investigated throughout their entire development. Two days after pupation, the pupae were removed from the soil and placed on Whatman paper in small, ventilated plastic vessels. The emergence of adults was monitored everyday for a period of 20 d. At three time points during the experiment, two uninfested plants of each transgenic line as well as two uninfested wild-type Col-0 plants grown at the same time and under the same conditions as the plants used to feed *S. littoralis* were harvested to analyze glucosinolate hydrolysis products as described above. The entire feeding experiment was carried out three times with different sets of plants. All data were analyzed using Sigmapstat (Systat Software Inc., Richmond, CA, USA). Means were tested for significant differences by one-way ANOVA with a Bonferroni test (all pairwise comparisons) or by Kruskal–Wallis ANOVA followed by Dunn's test (all pairwise comparisons) when the data did not show normal distribution.

Results

To create plants for insect testing that differ only in the type of glucosinolate hydrolysis products formed upon tissue disruption, we transformed the Col-0 ecotype of *A. thaliana*, an ecotype known to be devoid of functional ESP (Fig. 2a; Lambrix et al., 2001), with the ESP cDNA from *A. thaliana*, ecotype *Ler*, under the control of the CaMV35S promoter. Three independent ESP-overexpressing lines (35S:ESP 2.6, 37.3, and 38.1) as well as three independent control lines carrying the empty pPZP221 vector (pPZP221 7.1, 8.2, and 36.1) were characterized in comparison to the Col-0 wild-type. Plants of the transgenic lines did not differ from wild-type plants with regard to growth habit and leaf morphology. In addition, no changes in growth rate or development were observed. From each line, 10 plants of the T3 generation were analyzed and compared to Col-0 wild-type plants grown at the same time.

Transformation with the 35S:ESP construct altered the levels of ESP protein and activity. In Western blot experiments performed with an anti-ESP antibody, comparable levels of ESP were detected in plants of the three ESP-overexpressing lines and the *A. thaliana* ecotype *Ler* (Fig. 3). However, ESP was not detected in the Col-0 wild-type and in the vector control lines (Fig. 3a). To test for ESP activity, allylglucosinolate was added to plant extracts as substrate for the hydrolysis reactions. In crude extracts of Col-0 wild-type plants and vector control lines, allylglucosinolate was hydrolyzed almost exclusively to the corresponding isothiocyanate; only traces of the simple nitrile were detected (data not shown). In contrast, the hydrolysis of allylglucosinolate was redirected towards epithioni-

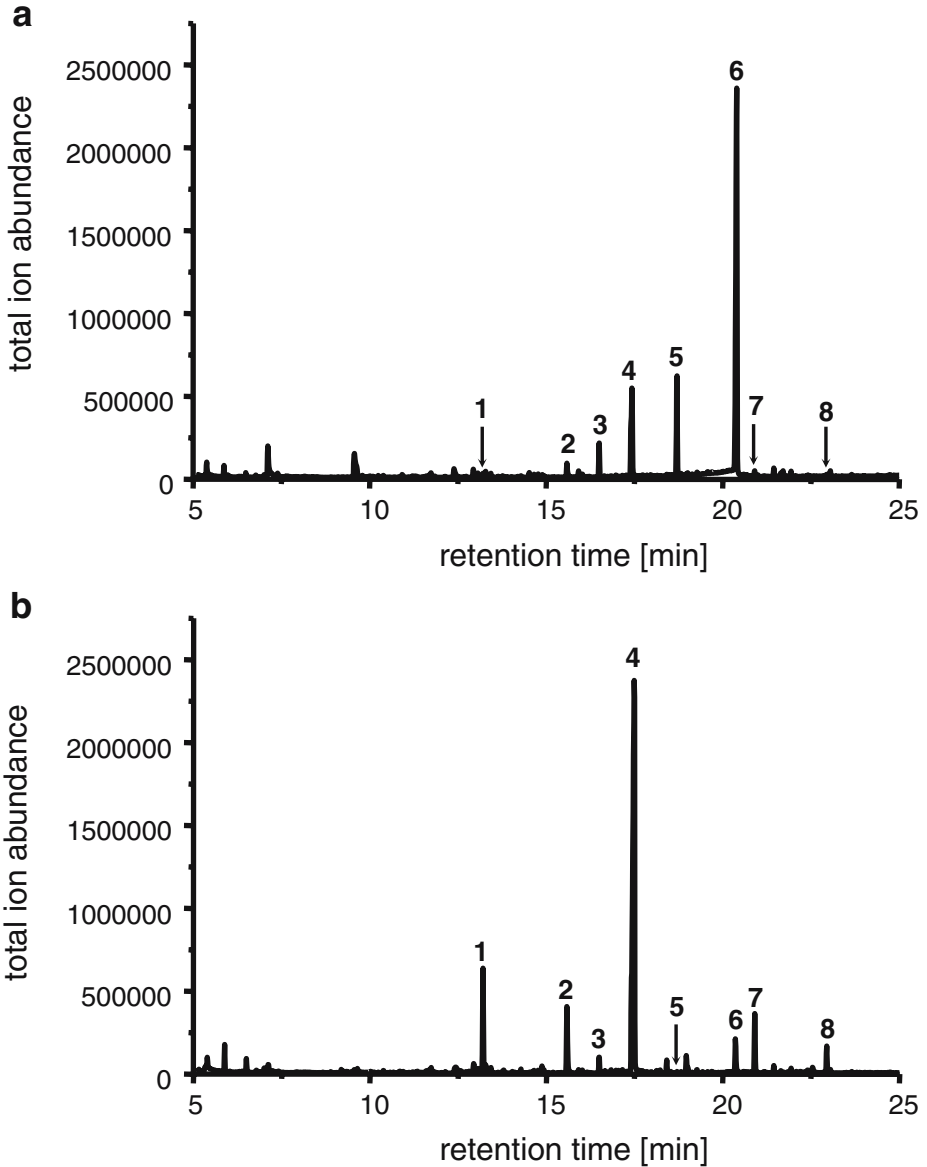


Fig. 2 Glucosinolate hydrolysis products in leaf autolysates. For qualitative analyses, aqueous leaf macerates of an *A. thaliana*, ecotype Col-0, wild-type plant (a) and a 35S:ESP plant (b) were extracted with CH₂Cl₂. Depicted are GC-MS traces of the CH₂Cl₂ phases. **1:** 5-Methylsulfinyl-pentanenitrile (nitrile product of 4MTB); **2:** 4-methanesulfinyl-butyronitrile (nitrile product of 3MSOP); **3:** 1-isothiocyanato-4-methylsulfinyl-butane (isothiocyanate product of 4MTB); **4:** 5-methanesulfinyl-pentanenitrile (nitrile product of 4MSOB); **5:** 1-isothiocyanato-3-methanesulfinyl-propane (isothiocyanate product of 3MSOP); **6:** 1-isothiocyanato-4-methanesulfinyl-butane (isothiocyanate product of 4MSOB); **7:** indole-3-acetonitrile [(2,3-dihydro-1*H*-indol-3-yl)-acetonitrile]; **8:** 4-methoxy-indole-3-acetonitrile [(4-methoxy-2,3-dihydro-1*H*-indol-3-yl)-acetonitrile]. 3MSOP: 3-methylsulfinylpropyl-glucosinolate; 4MSOB: 4-methylsulfinylbutylglucosinolate; 4MTB: 4-methylthiobutylglucosinolate

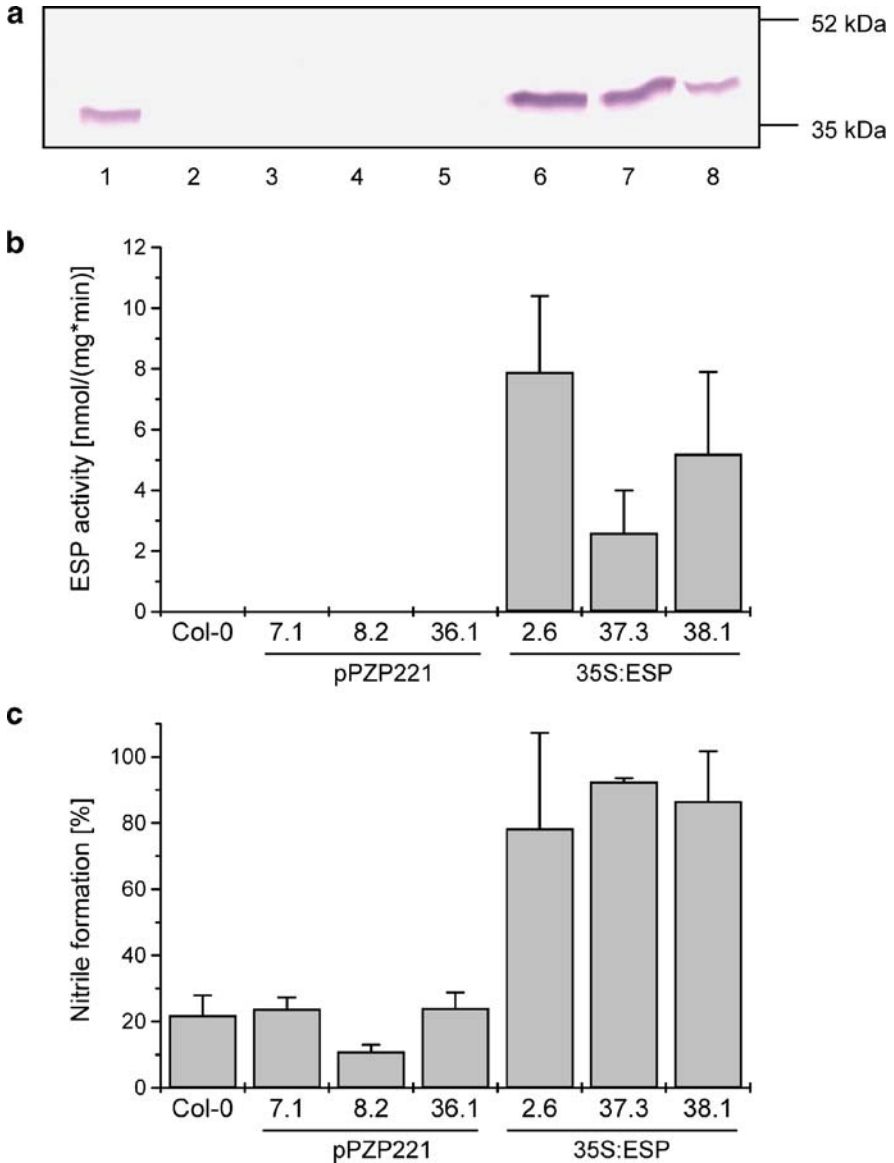


Fig. 3 ESP abundance, ESP activities and nitrile formation in the transgenic *A. thaliana* lines. (a) Proteins from crude leaf extracts were concentrated by trichloroacetic acid precipitation, separated by SDS-PAGE, and analyzed by Western blotting using a polyclonal antibody against ESP from *A. thaliana*, Ler. lane 1, *A. thaliana*, Ler; lane 2, *A. thaliana*, Col-0; lane 3, pPZP221 (7.1); lane 4, pPZP221 (8.2); lane 5, pPZP221 (36.1); lane 6, 35S:ESP (2.6); lane 7, 35S:ESP (37.3); lane 8, 35S:ESP (38.1). (b) ESP activity in leaf extracts was assayed in 50 mM MES buffer, pH 6.0, containing 1 mM allylglucosinolate. Activities of ESP are expressed as the amount (nmol) of 3,4-epithiopropylcyanide formed per minute and mg protein. Data are the mean \pm SD ($N = 7-10$). (c) Hydrolysis products formed from internal glucosinolates in crude leaf extracts of *A. thaliana* Col-0 wild-type plants and plants of the transgenic lines were analyzed by GC-FID after extraction with dichloromethane. Nitrile formation is expressed as the amount (nmol) of nitrile derived from 4-methylsulfinylbutylglucosinolate, the major leaf glucosinolate in *A. thaliana* Col-0, as a percentage of the total amount (nmol) of hydrolysis products formed from this glucosinolate. Data are the mean \pm SD ($N = 7-10$)

trile formation in the ESP-overexpressing lines (Fig. 3b). The ESP activity in the plants of the 35S:ESP lines 2.6, 37.3, and 38.1 was 7.9 , 2.6 , and $5.2 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$, respectively. These levels of ESP activity exceeded the activity of ESP in leaf extracts of *A. thaliana* ecotype *Ler* by a factor of 2–6 (data not shown).

In parallel with the *in vitro* ESP assays, the outcome of glucosinolate hydrolysis *in vivo* was tested in the *A. thaliana* lines overexpressing ESP. Col-0 wild-type plants (Figs. 2a and 3c) and pPZP221 control transformants (Fig. 3c) mainly produce isothiocyanates with a small percentage of nitriles. For example, 4-methylsulfinylbutylglucosinolate, the major glucosinolate present in the Col-0 ecotype (Kliebenstein et al., 2001a; Table 1), was approximately 80% converted into the corresponding isothiocyanate and 20% converted to the corresponding nitrile (Fig. 3c). However, in the ESP-overexpressing lines, glucosinolates were predominantly hydrolyzed to nitriles (Figs. 2b and 3c). In the case of 4-methylsulfinylbutylglucosinolate, more than 90% was converted into the corresponding nitrile (Fig. 3c). Moreover, increased amounts of indole glucosinolate-derived nitriles were detected in autolysates prepared from 35S:ESP plants compared to autolysates from Col-0 wild-type plants (Fig. 2). Measurements of the total amount of isothiocyanates present in plant extracts confirmed this trend, showing that leaf autolysates prepared from plants of the three 35S:ESP lines were significantly reduced in isothiocyanate content as compared to plants of the control lines (Table 2).

With respect to the parent glucosinolates, no quantitative or qualitative changes were detected in the ESP-overexpressing lines or the control transformant lines compared to Col-0 wild-type plants (Table 1). Myrosinase activity was also not altered in the 35S:ESP lines or the control lines (Table 2).

To investigate the differential effects of isothiocyanates and nitriles on the development of a generalist insect herbivore, larvae of *S. littoralis* were reared on wild-type *A. thaliana*

Table 1 Glucosinolate Content of the Transgenic *A. thaliana* Lines

Glucosinolate side chain	Glucosinolate content [$\mu\text{mol (g dry weight)}^{-1}$]						
	Col-0	pPZP221			35S:ESP		
		(7.1)	(8.2)	(36.1)	(2.6)	(37.3)	(38.1)
3MSOP	1.40 ± 0.52	2.12 ± 0.86	2.71 ± 0.89	2.04 ± 1.20	1.26 ± 0.49	1.94 ± 0.99	2.18 ± 0.95
4MSOB	10.62 ± 5.11	16.10 ± 7.86	12.46 ± 5.27	15.78 ± 10.68	10.66 ± 5.51	14.58 ± 9.09	15.92 ± 8.75
5MSOP	0.26 ± 0.14	0.34 ± 0.30	0.74 ± 0.68	0.27 ± 0.23	0.19 ± 0.18	0.50 ± 0.26	0.50 ± 0.37
7MSOH	0.12 ± 0.04	0.16 ± 0.07	0.12 ± 0.06	0.10 ± 0.04	0.11 ± 0.02	0.37 ± 0.83	0.14 ± 0.04
4MTB	0.56 ± 0.33	1.27 ± 0.81	1.30 ± 0.90	1.41 ± 1.12	0.55 ± 0.24	1.05 ± 0.80	1.24 ± 0.67
8MSOO	0.62 ± 0.28	0.85 ± 0.40	1.00 ± 0.70	0.76 ± 0.50	0.62 ± 0.36	0.64 ± 0.43	0.89 ± 0.56
I3M	1.84 ± 1.08	2.81 ± 1.64	3.72 ± 1.22	2.76 ± 0.90	1.87 ± 1.33	2.59 ± 1.33	4.35 ± 1.66
4MOI3M	0.25 ± 0.20	0.22 ± 0.25	0.56 ± 0.12	0.17 ± 0.20	0.29 ± 0.15	0.20 ± 0.22	0.30 ± 0.35
1MOI3M	0.12 ± 0.14	0.29 ± 0.29	0.08 ± 0.07	0.32 ± 0.31	0.22 ± 0.20	0.35 ± 0.38	0.44 ± 0.47
total	15.7 ± 6.1	24.1 ± 10.0	22.6 ± 7.3	23.6 ± 14.2	15.7 ± 6.3	22.1 ± 11.4	25.9 ± 11.6

The glucosinolate content of rosette leaves of wild-type Col-0, control lines (pPZP221), and ESP overexpressing lines (35S:ESP) was determined by HPLC of the corresponding desulfoglucosinolates. Data are the mean \pm SD ($N = 7-10$).

3MSOP: 3-methylsulfinylpropyl-; 4MSOB: 4-methylsulfinylbutyl-; 5MSOP: 5-methylsulfinylpentyl-; 7MSOH: 7-methylsulfinylheptyl-; 4MTB: 4-methylthiobutyl-; 8MSOO: 8-methylsulfinyloctyl-; I3M: indol-3-ylmethyl-; 4MOI3M: 4-methoxyindol-3-ylmethyl-; 1MOI3M: 1-methoxyindol-3-ylmethyl-.

Table 2 Total Isothiocyanate Levels and Myrosinase Activities in the Transgenic *A. thaliana* Lines

Plant line	Total isothiocyanates [nmol (mg protein) ⁻¹]	Myrosinase activity [nmol (min mg protein) ⁻¹]
Col-0	38.5 (±17.8)	13.7 (±3.7)
pPZP221 (7.1)	38.5 (±4.4)	11.2 (±1.5)
pPZP221 (8.2)	24.7 (±4.5)	14.2 (±4.4)
pPZP221 (36.1)	41.3 (±7.1)	11.3 (±1.8)
35S:ESP (2.6)	6.8 (±10.2)	18.7 (±7.7)
35S:ESP (37.3)	1.9 (±1.4)	11.5 (±2.0)
35S:ESP (38.1)	6.8 (±9.4)	11.2 (±3.0)

Total amounts of isothiocyanates formed from the internal glucosinolates were quantified in crude leaf extracts by HPLC after cyclocondensation with benzene-1,2-dithiol. Myrosinase activities in leaf extracts were measured based on the amount of glucose released from allylglucosinolate added as a substrate. Data are mean ± SD ($N = 7-10$).

Col-0, on plants of the 35S:ESP lines 2.6, 37.3, and 38.1, and on the vector control lines (pPZP221 7.1, 8.2, and 36.1). On average, larvae that were allowed to feed on plants of the 35S:ESP lines gained weight faster than the larvae on Col-0 wild-type plants or plants of the control lines during the first 10 d of development (Fig. 4). We found this effect to be significant for one and two, respectively, of the tested 35S:ESP lines in two out of three independent experiments. In the second section of the feeding experiment, larvae with growth rates close to the medial growth rate were reared until pupation on the same plant lines. The larvae that were fed on plants of the 35S:ESP lines reached the stage of pupation on average about a day earlier than on the control plants. This effect was significant for all tested lines in experiment A and for one 35S:ESP line in experiment B (Table 3). The pupal weight, the duration of the pupal stage, and the proportion of successful emergence of adults from the pupae were unaffected by the type of plant fed upon (Table 3). These results were consistent over three independent experiments conducted with the same lines, each using a different set of plants grown at different times. For each feeding trial, uninfested replicate plants of the Col-0 wild-type and the transgenic lines grown in parallel were analyzed for glucosinolate hydrolysis products. The results were consistent with those presented in Figs. 2 and 3c.

Discussion

As a damage-activated defense, the glucosinolate–myrosinase system is particularly difficult to simulate in insect feeding trials. In several studies that have been carried out to compare the impact of different types of glucosinolate hydrolysis products on insect herbivores, toxic effects have been most frequently demonstrated for isothiocyanates (Lichtenstein et al., 1962; Blau et al., 1978; Seo and Tang, 1982; Agrawal and Kurashige, 2003), whereas nitriles are generally considered to be less harmful to insects (Wittstock et al., 2003). Nevertheless, many glucosinolate-containing plant species as well as some ecotypes of *A. thaliana* possess an ESP and so form nitriles instead of isothiocyanates upon herbivore damage. To elucidate why plants should alter their glucosinolate hydrolysis product profile in this manner, more studies comparing the effects of nitriles and isothiocyanates derived from the same glucosinolates on insect herbivores are needed. Here, we used the molecular tools available for the model plant *A. thaliana* to specifically

Fig. 4 Growth rates of *S. littoralis* larvae on the different transgenic plant lines. In three independent experiments a–c, the weight of *S. littoralis* larvae reared on *A. thaliana* Col-0 wild-type plants, on the control lines pPZP 7.1, 8.2, and 36.1, or on the 35S:ESP lines 2.6, 37.3, and 38.1 was measured about 10 d after hatching. Growth rates presented are mean values \pm SD ($N = 37\text{--}47$). Mean values were tested for significant differences ($P < 0.05$) by Kruskal–Wallis ANOVA followed by Dunn’s test for all pairwise comparisons. Significant differences are indicated by different letters. n.d.: not determined

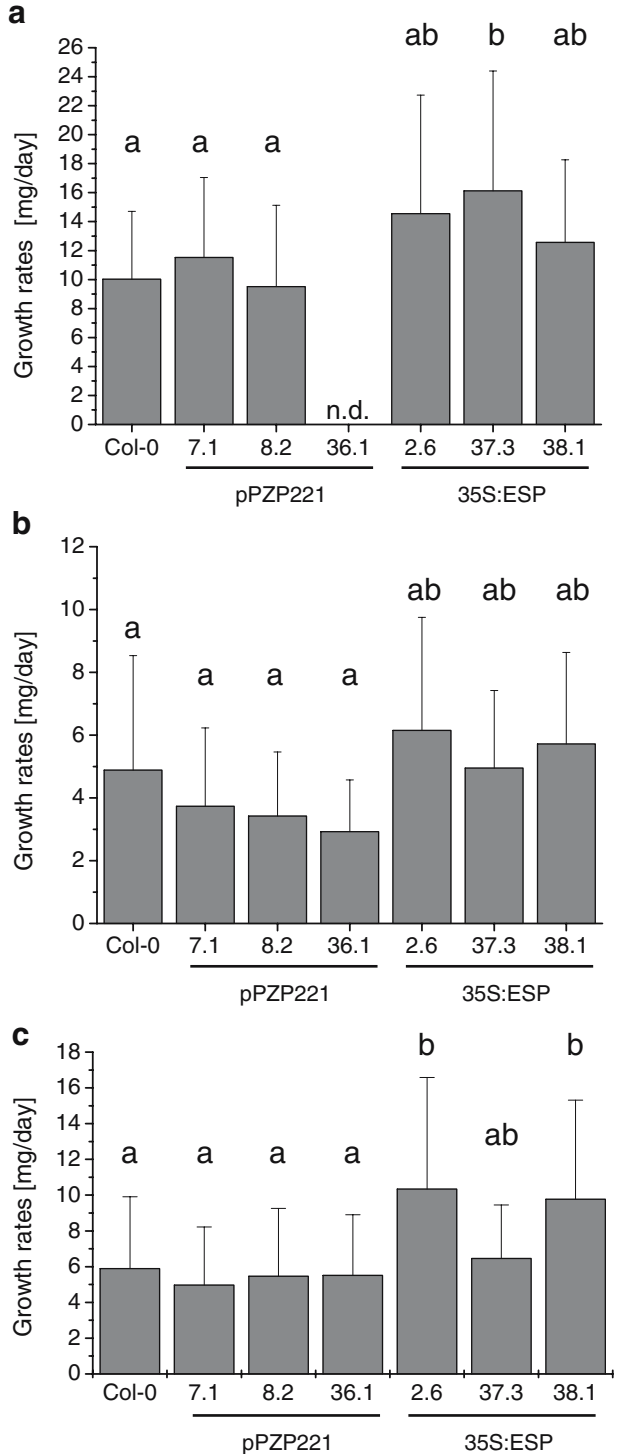


Table 3 Performance of *S. littoralis* on the Transgenic *A. thaliana* Lines

Developmental parameter	Experiment	N	Col-0	pPZP221			35S:ESP		
				(7.1)	(8.2)	(36.1)	(2.6)	(37.3)	(38.1)
Time until pupation (days)	A ¹	8–10	21.9± 0.4 ^a	22.3± 0.4 ^a	22.4± 0.5 ^a	22.7± 0.3 ^{ab}	21.0± 0.4 ^c	20.7± 0.4 ^c	21.3± 0.4 ^{cd}
	B ²	9–10	18.7± 0.5 ^a	18.6± 0.5 ^a	19.1± 0.3 ^a	18.8± 0.4 ^a	17.8± 0.4 ^{ab}	17.9± 0.3 ^{ab}	17.4± 0.5 ^b
	C ²	10	16.2± 0.6 ^a	16.4± 0.5 ^a	17.0± 0.7 ^a	16.4± 0.5 ^a	15.7± 0.7 ^a	15.6± 0.5 ^{ab}	15.5± 0.7 ^{ab}
Duration of the pupal stage (days)	A ²	6–9	14.5± 1.0 ^a	13.7± 0.8 ^a	13.9± 0.5 ^a	14.1± 0.7 ^a	15.0± 0.7 ^a	14.1± 0.7 ^a	15.0± 1.3 ^a
	B	8–10	14.6± 0.7	15.1± 0.7	14.7± 1.0	14.8± 1.0	14.9± 0.6	15.2± 1.1	15.3± 0.9
	C ²	7–10	14.7± 0.7 ^a	14.7± 0.5 ^a	14.3± 0.7 ^a	14.4± 0.5 ^a	14.9± 0.6 ^a	15.8± 0.7 ^{ab}	15.5± 0.5 ^{ab}
Pupal weight [mg]	A	8–10	364± 47	392± 12	364± 49	388± 28	358± 31	362± 44	347± 46
	B	8–10	389± 39	373± 42	386± 29	379± 37	394± 36	354± 26	385± 39
	C	8–10	353± 41	333± 52	377± 44	359± 41	357± 29	335± 29	357± 35
Successful adult emergence (%)	A	6–9	75	100	100	100	90	70	80
	B	8–10	100	100	100	90	90	100	100
	C	7–10	100	100	90	88	90	100	100

The time until pupation, the duration of the larval stage, the pupal weight, and the proportion of successful adult emergence were recorded for those larvae that had growth rates close to the medial growth rate at days 10 and 12 after hatching (for details see [Methods and Materials](#)). Data obtained in three independent experiments (A–C) are presented as means ± SD. Values followed by different letters (a–d) are significantly different ($P < 0.05$) in one-way ANOVA followed by a Bonferroni test for all pairwise comparisons (¹) or in Kruskal–Wallis ANOVA with Dunn's test for all pairwise comparisons (²). N: number of larvae.

manipulate the outcome of glucosinolate hydrolysis *in planta* in a background where the parent glucosinolate profile is unchanged, and used the genetically altered lines in feeding trials with a generalist insect herbivore.

The transgenic lines with constitutive expression of the ESP cDNA from *A. thaliana* Ler in the Col-0 background had readily detectable levels of ESP protein and activity (Fig. 3), and formed predominantly nitriles from glucosinolates instead of the corresponding isothiocyanates (Figs. 2 and 3). Although it has been questioned whether or not ESP can efficiently promote the formation of simple nitriles from nonalkenyl aliphatic glucosinolates under natural conditions (Zabala et al., 2005), nitrile formation from 4-methylsulfinylbutylglucosinolate, the dominant glucosinolate in these plants, reached 100% of the total hydrolysis products formed from this glucosinolate in some of our transformants (Fig. 3c).

The value of these nitrile-forming lines for insect bioassays would be greatest if they differed from the isothiocyanate-forming wild-type in no trait other than glucosinolate hydrolysis. The defensive function of the glucosinolate–myrosinase system against insect herbivores is influenced by changes in glucosinolate concentration and composition as well as the level of myrosinase activity in the plant tissue (Giamoustaris and Mithen, 1995; Stowe, 1998; Li et al., 2000). However, none of these parameters was changed in uninfested plants of the three ESP-overexpressing lines generated in this study compared to

wild-type plants. Furthermore, the ESP-overexpressing lines were not different from wild-type controls in growth and morphology. Therefore, these nitrile-forming transgenic lines are ideal tools to dissect the role of glucosinolate hydrolysis in plant–insect interactions away from that of other parameters of the glucosinolate–myrosinase system.

To investigate the differential effects of isothiocyanates and nitriles on a generalist herbivore, larvae of *S. littoralis* were reared on the different transgenic *A. thaliana* lines, and their development was monitored until the emergence of the adult moths. *S. littoralis* has frequently been used in toxicity tests as a model organism because its larvae are known to be extremely polyphagous and rather insensitive to a broad range of pesticides. In the present study, young larvae of *S. littoralis* gained weight faster on the ESP-overexpressing lines compared to the control lines and to Col-0 wild-type plants (Fig. 4). Faster growth in early instars affected the duration of the larval stage. Of the larvae with medial growth rates on ESP lines, control lines, and wild-type plants at days 10 and 12, those reared on nitrile-forming lines had a tendency to pupate earlier than those reared on the other plant lines (Table 3). However, these effects were only significant in up to two out of three independent experiments. The type of glucosinolate hydrolysis products formed had no impact on the average pupal weight of medial individuals, the duration of their pupal stage, or the proportion of successful adult emergence. An extended larval stage is presumed to be a disadvantage for insects due to an increased risk of attack from predators and parasitoids, unless it leads to enhanced weight or fecundity (which was not the case in our study). Our results extend previous work on the detrimental effect of isothiocyanates performed with intact plants, plant extracts, and artificial diets (Bartelt and Mikolajczak, 1989; Li et al., 2000; Agrawal and Kurashige, 2003). However, considering the large differences in hydrolysis product formation between the tested control and 35S:ESP lines (Fig. 3), the effects of the different plant diets on the growth of *S. littoralis* larvae were surprisingly weak and were, moreover, only observed for young larvae. These findings might be explained by the induction of detoxification pathways for isothiocyanates in young larvae that may counteract the defensive potential of these compounds. The alternative hypothesis, that herbivory triggers plant responses that balance the effect of the transgene, will be tested in future investigations.

Because the presence of ESP and the subsequent formation of nitriles did not have negative effects on the performance of *S. littoralis*, a role for ESP in direct defense against generalist insect herbivores seems unlikely. Nevertheless, the effects of isothiocyanates vs. nitriles need to be further investigated in comparative bioassays with other generalist insect herbivores as well as with insect species that are specialized feeders on glucosinolate-containing plants. Moreover, the transgenic lines created here will allow the investigation of several alternative functions for ESP, including the possibility that nitriles act as defensive compounds against pathogens, as inhibitors of insect oviposition, or as attractants to herbivore enemies.

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Introduction

Complex phenotypes that contribute to plant fitness in natural and agricultural environments, such as herbivore and pathogen defense, can be dissected by using quantitative genetic and molecular methods. Perhaps more than in any other plant family, studies of defense in the Brassicaceae have identified mechanisms, quantitative trait loci (QTL), and candidate genes that contribute to resistance, tolerance, and avoidance of enemies in *Arabidopsis thaliana* and *Brassica* (Rask et al., 2000; Wittstock and Halkier, 2002; Koornneef et al., 2004). Two herbivore defense characteristics that have been intensively investigated are glucosinolate (GS) composition and trichome density (Mauricio 1998; Agrawal 1999; Traw 2002; Traw and Dawson, 2002a; Strauss et al., 2003). While it is often true that more GS and more hairy leaves confer greater resistance against insect herbivory, these functional relationships are not found for all plant–herbivore interactions. To understand the ecology and evolution of plant defense, the contribution to resistance of phenotypic and genetic variation in candidate defense traits must be assessed for relevant ecological contexts.

In the model *A. thaliana*, differences among accessions in GS, trichomes, and herbivore resistance have been functionally dissected in laboratory and common garden experiments (Mauricio and Rausher, 1997; Mauricio 1998; Jander et al., 2001; Kliebenstein et al., 2001b, 2002; Lambrix et al., 2001; Kroymann et al., 2003; Weinig et al., 2003a,b; Symonds et al., 2005). In contrast, little is known about the ecological interactions of different accessions in their native, usually human-disturbed environments (Arany et al., 2005). Recently, attention has turned to closely related nonmodel *Arabidopsis* species in which many of the tools developed for *A. thaliana* can be applied, but which differ in their biological characteristics (Nasrallah et al., 2002; Bert et al., 2003; Clauss and Koch, 2006). Comparative studies within the Brassicaceae also enable us to ascertain whether findings in the model are more generally true. In *A. lyrata*, the genetic basis of variation in trichome production (glabrous or hairy) and trypsin proteinase inhibitors has been investigated (Karkkainen and Agren, 2002; Clauss and Mitchell-Olds, 2003; Karkkainen et al., 2004), and GS diversity has been surveyed (Windsor et al., 2005). Because populations of perennial and self-incompatible *A. lyrata* are geographically well-defined, persistent, and subject to little human disturbance, the ecological and evolutionary context of plant–herbivore interactions can be established *in situ*. An appropriate scale at which to study species interactions and adaptive evolution of plant defense in *A. lyrata* is within local, polymorphic populations (Clauss and Mitchell-Olds, 2003, 2006; Clauss and Koch, 2006).

Quantitative genetic experiments can be used to estimate the genetic architecture of defense. From one central European population of *A. lyrata*, we collected 51 naturally occurring genotypes for the parental generation of a sires-nested-within-dam crossing design. Because genetic architecture can change with plant age and environmental conditions, we estimated heritabilities and genetic correlations from phenotypic measurements of GS and trichome density for the F1 generation in years 1 and 3. Furthermore, we estimated the heritability of herbivore resistance and the contribution of candidate defense traits to resistance for *A. lyrata* in four herbivory contexts. In growth chamber resistance assays, three lepidopteran herbivores (*Plutella xylostella*, *Pieris brassicae*, and *Trichoplusia ni*) were employed that differ in their digestive physiologies, and are thus expected to respond differently to chemical and morphological defenses. All three taxa are common herbivores on Brassicaceae, and *P. xylostella* is a dominant herbivore on *A. lyrata* in Scandinavia (Løe 2006). We also assessed herbivore resistance in a common garden where 3-yr-old plants were subject to feeding by the chrysomelid *Phyllotreta cruciferae* and diverse other herbivores.

Methods and Materials

Study Species and Focal Population *Arabidopsis lyrata* is a self-incompatible perennial herb found in the temperate biomes of Eurasia and North America. The North American and European subspecies were previously called *Arabis lyrata* (L.) and *Arabis petraea* (L.) Lam. or *Cardaminopsis petraea* Hill, respectively. We follow O’Kane and Al-Shehbaz (1997) and Koch et al. (2001) in referring to these species as *Arabidopsis*, and focus on the European subspecies *A. lyrata* subsp. *petraea*, hereafter *A. lyrata*. We conducted our investigation on a population of *A. lyrata* collected near Plech, Bavaria, Germany ($N > 1000$; 49°54’99”N; 11°30’64”E; same as KOCH, Pfaffenhofen b. Neuhaus, Bavaria, Germany, leg. KOCH; Koch et al., 2001) for which all tested individuals were diploid ($2N=16$; sample size=60). Analyses of multilocus diversity patterns indicate that the Plech population is genetically diverse, largely random mating, and significantly differentiated from nearby populations (Clauss et al., 2002; Clauss and Mitchell-Olds, 2003, 2006). In the course of these studies, seeds were collected from maternal plants distributed throughout the Plech population (see Clauss and Mitchell-Olds, 2006 for details). In September 2000, seeds from 51 of the maternal seed families were germinated, and one seedling per family was grown to reproductive maturity in a growth chamber.

Quantitative Genetics Study Year 1: Laboratory Fifty-one reproductively mature *A. lyrata* plants were randomly assigned to one of 17 triads, in which two plants designated as sires were each crossed to one designated dam plant. This resulted in 17 pairs of full sibships, each sharing a dam. The F1 seeds were placed into Petri dishes, subject to 4°C chilling for 4 wk, germinated, and 30 single plants per cross were transferred into 7-cm pots in February 2002 (34 full sibships × 30 plants = 1020). Plants were grown in the growth chamber under short day conditions (light: 11 hr, 22°C, 60% RH; dark: 13 hr, 15°C, 50% RH). Plants were rerandomized within the same growth chamber every 6 wk, fertilized ca. every 3 mo [1.2 ml Compo Sana (NPK, 5-5-6) fertilizer/l], and periodically cleaned of senescent leaves. For traits measured on these F1 plants throughout the next 3 yr, we estimated genetic variance components, broad-sense heritability, and genetic correlations (see below). We employed a sires-within-dams mating design (Pilson 1996) for several reasons. (1) We obtain an estimate of genetic variation with lower sampling variance than paternal half-sib experiments of equal size. (2) This design examines variation among sires that is not contaminated by maternal effects, which are a genuine concern in most designs. (3) In random mating populations, the covariance of parent and offspring actually includes a portion of the epistatic variance, hence epistasis contributes to short-term evolutionary responses to natural selection. (4) Finally, partitioning of genetic variance into additive, dominance, and epistatic components is entirely contingent on allele frequencies, which will fluctuate whenever variation is determined by a finite number of loci. For these reasons, we chose a design that provides optimal control for maternal effects at a given sample size and estimates broad-sense genetic variance of ecologically important traits from a large random sample of the natural population.

At 8 wk, leaves were harvested to determine constitutive GS composition, trichome density, specific leaf area (SLA), and leaf dry matter content (LDMC). Glucosinolate extraction and purification followed the basic sephadex/sulfatase *Arabidopsis* protocol previously described (Hogge et al., 1988), with adjustment for sampling in a 96-well format (Kliebenstein et al., 2001b), for ca. 0.1 g of fresh leaf tissue harvested from the most recent fully expanded leaves on each plant. Plants were uninduced, and leaves were harvested

directly into liquid nitrogen to minimize breakdown of GS by the enzyme myrosinase. Each 96-well plate represented a block to which 94 *A. lyrata* plants were randomly assigned, together with one sample from a single *A. thaliana* Ler-0 plant (0.1 g wet weight) as a positive control and one empty negative control. Forty microliters of the GS extract were run on a reverse-phase column (LiChroCART 250-4 RP19e; Hewlett-Packard, Waldbronn, Germany) on a Hewlett-Packard 1100 series high-performance liquid chromatography (HPLC) machine. Compounds were detected at 229 nm and separated using the 60-min program described by Kliebenstein et al. (2001b). All GS have previously been identified by MS and NMR analysis (Kliebenstein et al., 2001b; Reichelt et al., 2002). HPLC peaks were called based on retention time and UV absorption spectrum. For ca. half of the samples, we added a known amount of an internal standard (4-hydroxybenzyl GS=sinalbin) before extraction, which allowed us to calculate the concentrations for all GS compounds in micromole per gram dry weight. The concentration was calculated from HPLC peak areas using response factors computed for pure desulfo-GS standards at 229 nm (Brown et al., 2003), and recalculated for sinalbin. The relationship between leaf wet and dry weights was ascertained from the measurement of LDMC below. GS concentrations were log-transformed before analyses.

For analysis of ratios of GS compounds, peak areas from all samples, with and without internal standard, were used after correction for response factors. Ratios among compound classes were calculated to reflect enzyme function at key steps in GS biosynthesis involving side-chain modification and side-chain elongation (Kliebenstein et al., 2001b). Three ratios were calculated to reflect aspects of aliphatic side-chain modification (RMT, RMS, RPropenyl; Table 1). RMT is the ratio of all aliphatic compounds other than methylthio GS over methylthio GS, regardless of chain length. RMS is the ratio of the sum of 2-propenyl and 3OHP GS over all methylsulfinyl GS. RPropenyl is the ratio of 3OHP over 2-propenyl GS. Three ratios were calculated that quantify variation in aliphatic side-chain elongation for which the *MAM* locus (containing *methylthioalkylmalate synthase* genes) has been identified in *A. thaliana* and proposed as a candidate in *A. lyrata*: (1) R3MAM is the ratio of all >3 carbon length GS over C3 chain length GS; (2) R6MAM is the ratio of all >6 carbon length GS over C6 chain length GS, and (3) R7MAM is the ratio of 8 over 7 carbon length GS (Table 1; Kliebenstein et al., 2001b; Kroymann et al., 2001, 2003; Heidel et al., 2006). These ratios are closely related to product/substrate ratios, and high values indicate that the conversion from shorter to longer carbon chain length by the *MAM*-encoded enzymes is more efficient. The following two ratios reflect indole GS modifications: (1) I3MR1 is the ratio of 4MO13M plus 1MO13M over I3M, and (2) I3MR2 is the ratio of 4MO13M over 1MO13M (Table 1). Finally, we calculated the ratio of total aliphatic to total indole GS, which reflects the ratio of methionine over tryptophan amino acid precursors in the GS skeleton (aliphatic/indole; Table 1). GS ratios were not transformed before analysis.

For three fully expanded, nonsenescent leaves per individual, we measured the number of trichomes in the first full field of view below the leaf tip at 5× magnification with a dissecting microscope (area=0.1257 cm²). For each of these leaves, one leaf disk with an area of 0.1963 cm² was then taken from just below the leaf tip, weighed immediately, dried at 50°C for 48 hr, and weighed again to calculate SLA (dry weight/area) and LDMC (dry weight/wet weight).

Insect feeding bioassays were separated by ca. 6 mo, allowing the plants to grow new uninduced leaves. We conducted resistance bioassays using three lepidopteran herbivore species known to feed on *A. thaliana* and other Brassicaceae, with well-established laboratory populations. Performance of two specialist insect species, *Plutella xylostella* (Yponomeutidae) (PX) and *Pieris brassicae* (Pieridae) (PB), and the generalist *Trichoplusia*

Table 1 Variables measured for *A. lyrata* ssp. *petraea* (PLECH)

Name	Description	Year	Number of samples	Mean ^a	SD	Heritability (H^2)	<i>P</i> values ^b
LDMC	Leaf dry matter content (dry wt/wet wt)	1	711	0.10	0.01	0.15	0.076
SLA	Specific leaf area (grams dry wt/cm ²)	1	711	0.00	0.00	0.21	0.004
Trichomes_Yr1	Number trichomes per cm ²	1	714	26.64	13.16	0.33	0.027
Trichomes_Yr3	Number trichomes per cm ²	3	788	127.08	87.02	0.03	0.308
PX emergence	Number of <i>P. xylostella</i> adults emerging of 5	1	793	2.50	1.57	0.30	<0.001
PB larval growth	Proportional growth of <i>P. brassicae</i> larvae	2	504	3.16	1.01	0.00	0.500
PB plant damage	Leaf damage by <i>P. brassicae</i> (0, 1, 2)	2	585	0.88	0.40	0.12	0.156
TN larval growth	Proportional growth of <i>T. ni</i> larvae	2	193	1.57	0.72	0.07	0.313
TN plant damage	Leaf damage by <i>T. ni</i> (0, 1, 2)	2	430	1.13	0.71	0.08	0.271
TN host acceptance	<i>T. ni</i> host choice (1 = staying, 0 = leaving)	2	405	0.52	0.50	0.18	0.002
Field herbivore damage	Leaf damage by herbivores (0, 1, 2)	3	799	0.92	0.34	0.04	0.307
Glucosinolates							
TotalGS_Yr1	Sum of all GSC	1	360	14.69	4.67	0.54	0.080
Aliphatic_Yr1	Sum of methionine-derived aliphatic GS	1	360	14.06	4.55	0.52	0.082
Indole_Yr1	Sum of tryptophan-derived indole GS	1	360	0.63	0.29	0.38	0.039
3MTP_Yr1	3-Methylthiopropyl	1	360	0.07	0.11	0.40	0.061
3MSOP_Yr1	3-Methylsulfinylpropyl	1	360	0.05	0.17	0.17	0.141
2-Propenyl_Yr1	2-Propenyl	1	360	0.05	0.02	0.59	0.089
3OHP_Yr1	3-Hydroxypropyl	1	360	12.88	4.34	0.58	0.063
6MSOH_Yr1	6-Methylsulfinylhexyl	1	360	0.08	0.06	0.29	0.076
7MTH_Yr1	7-Methylthioheptyl	1	360	0.07	0.07	0.67	0.008
7MSOH_Yr1	7-Methylsulfinylheptyl	1	360	0.83	0.59	0.24	0.079
8MSOO_Yr1	8-Methylsulfinyloctyl	1	360	0.03	0.03	0.34	0.057
I3M_Yr1	Indol-3-ylmethyl	1	360	0.53	0.25	0.37	0.048
4MO13M_Yr1	4-Methoxyindol-3-ylmethyl	1	360	0.08	0.07	0.27	0.080
1MO13M_Yr1	1-Methoxyindol-3-ylmethyl	1	360	0.02	0.01	0.58	0.046
Aliphatic/Indole_Yr1	Ratio of aliphatic to indole GS	1	748	25.61	11.15	0.36	0.002
RMT_Yr1	Ratio of (3OHP + 2-propenyl + MS) to MT ^d	1	748	172.42	159.52	0.66	0.003

Table 1 Continued

Name	Description	Year	Number of samples	Mean ^a	SD	Heritability (H^2)	<i>P</i> values ^b
RMS_Yr1	Ratio of (3OHP + 2-propenyl) to MS ^c	1	748	20.96	15.58	0.54	0.025
RPropenyl_Yr1	Ratio of 3OHP to 2-propenyl	1	748	345.46	578.83	0.02	0.368
R3MAM_Yr1	Ratio of (C6 + C7 + C8) to C3 ^f	1	748	0.08	0.05	0.53	0.028
R6MAM_Yr1	Ratio of (C7 + C8) to C6	1	748	11.50	3.47	0.40	0.004
R7MAM_Yr1	Ratio of C8 to C7	1	748	0.04	0.02	0.64	0.016
I3MR1_Yr1	Ratio of (4MO13M + 1MO13M) to 13M	1	748	0.21	0.14	0.28	0.038
I3MR2_Yr1	Ratio of 4MO13M to 1MO13M	1	748	5.72	7.03	0.44	0.011
Total GS_Yr3	Sum of all GS	3	799	6.66	2.84	0.09	0.146
Aliphatic_Yr3	Sum of methionine derived aliphatic GS	3	799	6.43	2.82	0.08	0.175
Indole_Yr3	Sum of tryptophan derived indole GS	3	799	0.23	0.15	0.33	0.023
3MTP_Yr3	3-Methylthiopropyl	3	799	0.02	0.03	0.00	0.498
3MSOP_Yr3	3-Methylsulfinylpropyl	3	799	0.01	0.06	0.04	0.283
2-Propenyl_Yr3	2-Propenyl	3	799	0.04	0.03	0.15	0.080
3OHP_Yr3	3-Hydroxypropyl	3	799	6.18	2.78	0.07	0.183
6MSOH_Yr3	6-Methylsulfinylhexyl	3	799	0.02	0.02	0.07	0.187
7MTH_Yr3	7-Methylthioheptyl	3	799	0.01	0.01	0.30	0.026
7MSOH_Yr3	7-Methylsulfinylheptyl	3	799	0.15	0.14	0.13	0.086
8MSOO_Yr3	8-Methylsulfinyloctyl	3	799	0.00	0.00	0.20	0.053
I3M_Yr3	Indol-3-ylmethyl	3	799	0.19	0.14	0.31	0.024
4MO13M_Yr3	4-Methoxyindol-3-ylmethyl	3	799	0.03	0.02	0.30	0.028
1MO13M_Yr3	1-Methoxyindol-3-ylmethyl	3	799	0.01	0.01	0.14	0.087
Aliphatic/indole_Yr3	Ratio of aliphatic to indole GS	3	799	42.84	45.44	0.26	0.032
RMT_Yr3	Ratio of (3OHP + 2-propenyl + MS) to MT ^d	3	799	501.09	464.40	0.14	0.087
RMS_Yr3	Ratio of (3OHP + 2-propenyl) to MS ^c	3	799	90.76	130.46	0.07	0.164
RPropenyl_Yr3	Ratio of 3OHP to 2-propenyl	3	799	196.79	203.38	0.03	0.349
R3MAM_Yr3	Ratio of (C6 + C7 + C8) to C3 ^f	3	799	0.03	0.04	0.06	0.201
R6MAM_Yr3	Ratio of (C7 + C8) to C6	3	799	8.79	6.22	0.27	0.033
R7MAM_Yr3	Ratio of C8 to C7	3	799	0.05	0.07	0.08	0.156

Table 1 Continued

Name	Description	Year	Number of samples	Mean ^a	SD	Heritability (H^2)	<i>P</i> values ^b
I3MR1_Yr3	Ratio of (4MO13M + 1MO13M) to 13M	3	799	0.28	0.58	0.16	0.065
I3MR2_Yr3	Ratio of 4MO13M to 1MO13M	3	799	6.45	8.94	0.28	0.025

^a Mean and standard deviation were calculated from untransformed data.

^b Heritabilities significant after sequential Bonferroni adjustment with family-wise alpha=0.05 are indicated in bold for several groups of traits: nonglucosinolate traits, yr 1 glucosinolate concentrations, yr 1 glucosinolate ratios, yr 3 glucosinolate concentrations, and yr 3 glucosinolate ratios.

^c Amounts of glucosinolates given in $\mu\text{mol/g}$ dry wt.

^d MT=3MTP + 7MTH.

^e MS=3MSOP + 6MSOH + 7MSOH + 8MSOO.

^f C3=3MTP + 2MSOP + 2-Propenyl + 3OHP; C6=6MSOH; C7=7MTH + 7MSOH; C8=8MSOO.

ni (Noctuidae) (TN) was assessed on each *A. lyrata* plant. Differences in the performance of individual insects feeding on different *A. lyrata* individuals reflect the plant's resistance to herbivory in a way that integrates many characteristics. Differences among plant sibships in insect performance, host choice, and plant damage reflect the degree to which these resistance-related traits are genetically based.

In June 2002, 897 plants were divided into three randomized blocks for the *P. xylostella* (diamondback moth) assay. The *Plutella* larvae originate from line G88 (Cornell University, USA) and were reared on artificial diet (Shelton et al., 1991), from which they were removed 16 hr prior to the experiment. On d 1, for each plant in block 1, four leaves were cut and placed into a Petri dish (diam 9 cm) together with five third instars of *Plutella*. The petri dishes were kept in the growth chamber with short day conditions, and larvae had no additional food source. Four fresh leaves from the same plant were added to each Petri dish every 2 d until the last larva pupated. Feeding trials for blocks 1, 2, and 3 were each offset by 1 d. After pupation was complete, Petri dishes were maintained at 50% RH with a day/night cycle of 11/13 hr, 25% full light, and 26°C/19°C until *Plutella* adults emerged. We recorded the number of adults out of five that emerged when feeding on each plant. Plants from which fewer *Plutella* adults emerged were considered more herbivore-resistant.

The assay of *P. brassicae* (cabbage white butterfly) performance was conducted by allowing one larva to feed on each *A. lyrata* plant for 7 d in February 2003. A total of 751 plants were divided into four randomized blocks reflecting the bench position in the growth chamber. Second instars, ordered from Seritech (Warwickshire, UK), were allowed to develop for 5 d on *Brassica oleraceae* leaves before being starved overnight, individually weighed, and placed on *A. lyrata* plants. Plants were widely spaced to prevent *Pieris* from moving between individuals, and soapy water in the trays below the plants prevented other excursions. Larvae were frozen immediately after removal from the plant for later weighing. For each plant, we measured the growth rate of the resident larvae (ratio of biomass after to before feeding). The amount of leaf damage due to feeding by *Pieris* was estimated in gross categories reflecting no, little, and extensive tissue removal (0, 1, and 2; PB damage). The architectural complexity of the 1-yr-old *A. lyrata* plants with many leaves and side rosettes prevented a more exact quantification of feeding damage. Plants on which *Pieris* larvae grew less or which were less damaged were more herbivore-resistant.

The assay of *T. ni* (cabbage looper) performance was conducted in July 2003. Eggs of *Trichoplusia* were ordered from Benzon Research Inc. (Carlisle, PA, USA), and larvae were raised on cabbage looper diet (BioServe, Frenchtown, NJ, USA) for 1 wk. Second instars were starved overnight, weighed, and then placed on 546 *A. lyrata* plants divided into three randomized blocks in the growth chamber. Plants were widely spaced, and soapy water in the trays below the plants prevented larvae from reaching other plants. Larvae were removed from the plants after 3 d of feeding, immediately frozen, and weighed. *Trichoplusia* larvae were mobile, with ca. 50% leaving their resident plant. Each plant was scored as having a larva that remained and accepted the host (acceptance=1) or rejected the host and left (acceptance=0). Larval growth rate was calculated only for those accepting the host (departing larvae entered the soapy water below the plants and could not be accurately weighed). Finally, we scored the amount of leaf damage for each plant in gross categories as above (0, 1, and 2). Plants that were accepted less as hosts, had lower *Trichoplusia* larval growth, or experienced less plant damage were more resistant.

Quantitative Genetics Study Year 3: Common Garden In October 2003, we transplanted all F1 plants (now 21 mo old) into four randomized blocks in an experimental field plot near Jena, central Germany, located approx. 180 km north of the original *A. lyrata* Plech population in southern Germany (Clauss and Mitchell-Olds, 2006). Winter survival was >98%, and we measured trichome density and GS composition of new growth in May–June 2004 as described above with the following modifications. Tissue for GS extraction (ca. 0.1 g wet weight) was weighed after freeze-drying. Because GS concentration was lower in these 3-yr-old plants, we doubled the injection volume to 80 μ l. Furthermore, we shortened the HPLC program to 44 min to eliminate only the later minutes of the run where no GS peaks are observed in *A. lyrata* (8 min gradient from 1.5% to 5.0% [v/v] acetonitrile, 2 min gradient from 5.0% to 7.0% [v/v] acetonitrile, 25 min gradient from 7.0% to 42.0% [v/v] acetonitrile, 1 min gradient from 42.0% to 92.0% [v/v] acetonitrile, 5 min at 92.0% [v/v] acetonitrile, and 1 min gradient from 92.0% to 1.5% [v/v] acetonitrile), and a final 1 min at 1.5% (v/v) acetonitrile).

We counted the number of trichomes within only 0.25 \times of the leaf area described above because trichome density was greater for the plants in the common garden. On June 16, 2004, we scored the extent of unmanipulated herbivore damage for each plant (0, 1, and 2, as above).

Analyses All analyses were based on the same set of 34 sibships (17 dams each crossed with two sires), with some variation in the number of offspring measured per cross (average $N=20$). Phenotypic correlations were calculated among GS compounds and trichome density with Spearman correlation coefficients. The relationship between defense-related phenotypes and *A. lyrata* resistance to *Plutella*, *Pieris*, *Trichoplusia*, and naturally occurring herbivores in the common garden was quantified by using multiple regression analysis with the logarithms of aliphatic and indole GS amounts, the logarithm of trichome density, and SLA as independent variables. For each analysis, we employed the subset of individuals for which standard-corrected GS concentrations were available, and which had no missing data. Further, the analysis of *Trichoplusia* larval growth rate included only plants on which larvae remained resident and accepted the host.

Genetic variance components were estimated within an analysis of variance framework for each variable separately, using raw data or data transformed by log, square root, or arcsine square root as appropriate to give rise to an approximately normal distribution of

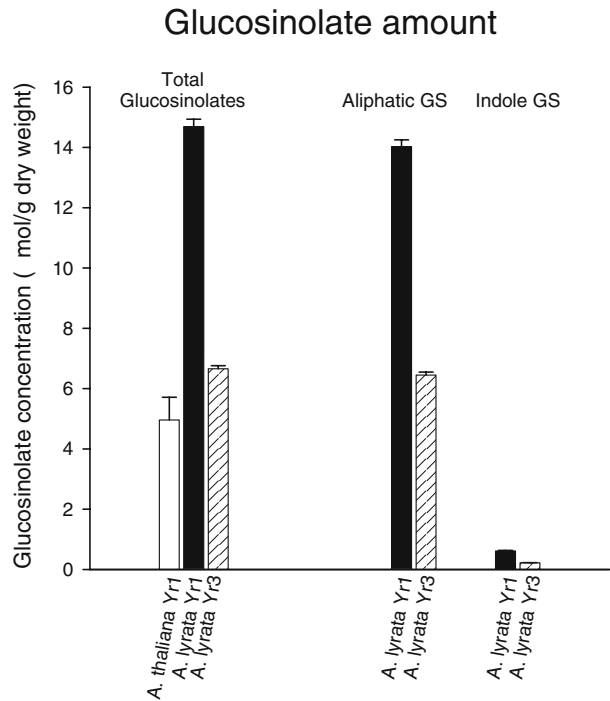
residuals. For each variable, we used the residuals of a model including block effects to estimate the magnitude and significance of the variance components associated with dams and sires nested within dam from PROC MIXED with restricted maximum likelihood estimation (SAS Institute Inc., Cary, NC, USA; see Table 1 for sample sizes). For PB larval growth, PB plant damage, TN larval growth, TN host acceptance, TN plant damage, and Field damage, we used a noniterative unbiased estimator (MIVQUE0 in PROC MIXED; SAS Institute Inc.). We report broad sense heritabilities (H^2) calculated as four times the sire variance component. Sire-based estimates of genetic correlation are free of maternal effects but may include dominance and epistatic effects as well as additive genetic variance (see above). For PX emergence, we report significance of H^2 from a random effects analysis of dam and sire nested within dam that treated PX emergence as Poisson distributed response variable using PROC GENMOD with a log link function (SAS Institute Inc.). For TN acceptance, we report significance from a similar random effects analysis that treated TN acceptance as binomially distributed response variable using PROC GENMOD with a logit link function.

Genetic correlations among pairs of traits with nonzero sire variance components were calculated as follows. In the context of the variance component analysis for each trait above, we estimated breeding values for each dam and sires within each dam (sireA and sireB), using empirical best linear unbiased prediction (BLUP; Littell et al., 1996). BLUP estimates are more accurate than sire family means because they use all information available, and thus are not biased by environmental effects, as are family means (e. g., Agrawal et al., 2002). We then calculated the difference between the breeding values of sireA and sireB (sireA–sireB) for each dam. Genetic correlations among traits were estimated by Spearman rank correlation of the sire breeding value differences (hereafter sire-based correlations). We also calculated genetic correlations among the dam breeding values of selected traits using Spearman rank correlation analysis (dam-based correlations). Dam-based genetic correlations are calculated from a larger number of offspring per family ($N=40$ on average), may sample unique genotypes from the original populations not compared in the sire variance analysis, and may include maternal effects.

Results

Phenotypic Variation The sample size, mean, standard deviation, and units for the raw data of all traits measured while plants were in the growth chamber (yr 1 and 2) and after they were transplanted to the common garden (yr 3) are given in Table 1. The glucosinolate phenotype of *A. lyrata* was composed of aliphatic GS derived from methionine (>96%) and indole GS derived from tryptophan amino acid precursors (4%) (Fig. 1). All compounds identified in *A. lyrata* have also been found in natural accessions of *A. thaliana*, and comparison with 8-wk-old rosettes of *A. thaliana* Ler-0 demonstrated that young *A. lyrata* plants had a slightly higher overall concentration of GS (Fig. 1, but see Kliebenstein et al., 2001b for variation among accessions). The single most dominant GS compound in 8-wk-old *A. lyrata* rosettes was the aliphatic 3OHP, followed by 7MSOH, and the indole I3M (Table 1). Phenotypic correlations among different GS compounds were generally positive (Table 2). The total GS concentration decreased from 14.7 $\mu\text{mol/g}$ dry weight in yr 1 to 6.7 $\mu\text{mol/g}$ dry weight when plants were remeasured in the common garden in yr 3 (Fig. 1 and Table 1). Overall GS profiles were correlated between years ($r_{\text{spearman}}=0.812$, $P<0.001$, $N=11$; Table 1; Fig. 1), and individual GS compound amounts and ratios were positively

Fig. 1 GS concentrations measured at 8 wk in the growth chamber (yr 1, solid bars) and 28 mo in a common garden (yr 3, striped bars) for *A. lyrata* subsp. *petraea* from Plech, Germany. GS content was also assessed for 8-wk-old *A. thaliana* (Ler-0) in the laboratory (open bar)



correlated for 612 individuals measured in both yr 1 and 3 (r_{spearman} ranged from 0 to 0.378, significant in 17 out of 23 cases; Table 3).

The distribution of trichome counts was continuous and unimodal, with no completely glabrous plants. Whereas rosette leaves on young plants in yr 1 had, on average, 27 trichomes/cm², plants in yr 3 growing in the common garden had 127 trichomes/cm². The phenotypic correlation in trichome density among years was positive ($r_{\text{spearman}}=0.149$, $P<0.001$, $N=585$).

The relationship between defense-related phenotypes and *A. lyrata* resistance was quantified using multiple regression analysis. Less than 5% of the phenotypic variance in resistance traits was explained by four independent defense-related variables (aliphatic GS, indole GS, trichome density, and SLA; Table 4). Trichome density, but not GS characteristics, significantly reduced adult emergence in the specialist *Plutella* (Table 4). Resistance to *Pieris* was not explained by any of the independent variables tested. For the generalist herbivore, *Trichoplusia*, indole GS negatively affected larval growth and reduced leaf damage by feeding (Table 4). Interestingly, plants with higher aliphatic GS experienced greater feeding damage by *Trichoplusia*. In the common garden, plants with more trichomes and more aliphatic GS experienced less leaf damage by herbivores (Table 4). The most common leaf herbivore in the common garden experiment was the crucifer specialist flea beetle, *Phyllotreta cruciferae* (Chrysomelidae, Coleoptera; $N=458$ during 30 min of herbivore scans conducted eight times between May 15 and September 30, 2004; Claus, unpublished data).

Single Trait Heritability Total indole GS concentration was significantly heritable in yr 1 and 3 (Fig. 2). In contrast, variation in total aliphatic GS had no significant genetic basis.

Table 2 Phenotypic and genetic correlations in leaf glucosinolate amounts for *A. lyrata* spp. *petraea* (PLECH) at 8 wk

	3MTP_Yr1	3MSOP_Yr1	2-Prop_Yr1	3OHP_Yr1	6MSOH_Yr1	7MTH_Yr1	7MSOH_Yr1	8MSOO_Yr1	I3M_Yr1	4MOI3M_Yr1	IMO13M_Yr1
3MTP_Yr1	0.226		0.248	0.417	-0.066	0.483	-0.145	-0.275	0.181	-0.235	0.512
3MSOP_Yr1	0.366		0.321	-0.029	0.375	-0.042	0.380	0.108	0.201	-0.076	-0.081
2-Prop_Yr1	0.296	0.166		0.615	0.135	-0.272	0.015	0.000	0.593	0.471	-0.093
3OHP_Yr1	0.387	0.271	0.729		-0.427	-0.167	-0.400	-0.223	0.228	0.324	-0.064
6MSOH_Yr1	0.134	0.209	0.315	0.368		-0.032	0.853	0.664	0.353	0.336	0.262
7MTH_Yr1	0.333	0.110	-0.006	-0.021	-0.016		-0.130	-0.125	-0.130	-0.238	0.576
7MSOH_Yr1	0.085	0.179	0.208	0.240	0.888	0.091	0.873	0.890	0.419	0.392	0.157
8MSOO_Yr1	0.049	0.124	0.095	0.078	0.658	0.155	0.497	0.507	0.431	0.618	0.120
I3M_Yr1	0.066	0.100	0.273	0.296	0.455	0.033	0.497	0.507		0.657	0.311
4MOI3M_Yr1	-0.058	0.007	0.161	0.216	0.193	-0.078	0.221	0.160	0.446		0.150
IMO13M_Yr1	0.190	0.140	0.290	0.272	0.490	0.062	0.410	0.309	0.173	-0.005	

Phenotypic (lower left; N=794) and genetic (upper right; N=17) correlations.

Spearman correlation coefficients significant after sequential Bonferroni adjustment with family-wise alpha=0.05 are indicated in bold.

Table 3 Cross-year phenotypic and genetic correlations in leaf glucosinolate amounts and ratios for *A. lyrata* spp. *petraea* (PLECH)

	Phenotypic	Genetic sire-based	Genetic dam-based
<i>N</i>	612	17	17
GS amounts			
Total GS	0.109	0.272	0.223
Aliphatic	0.111	0.328	0.191
Indole	0.327	0.436	0.713
3MTP	0.233	−0.103	0.787
3MSOP	0.074	0.069	0.458
2-Propenyl	0.148	0.537	0.341
3OHP	0.149	0.404	0.218
6MSOH	0.222	0.409	0.358
7MTH	0.016	0.216	0.079
7MSOH	0.317	0.343	0.583
8MSOO	0.149	0.145	0.230
I3M	0.343	0.351	0.672
4MO13M	0.194	0.270	0.000
1MO13M	0.083	0.674	0.064
GS ratios			
Aliphatic/Indole	0.346	0.373	0.753
RMT	0.203	0.213	0.630
RMS	0.378	0.370	0.858
RPropenyl	0.001	0.110	0.000
R3MAM	0.370	0.262	0.870
R6MAM	0.278	0.346	0.613
R7MAM	0.227	−0.066	0.463
I3MR1	0.190	0.000	0.326
I3MR2	0.147	0.409	0.363

Spearman correlation coefficients significant after sequential Bonferroni adjustment with family-wise alpha=0.05 are indicated in bold.

When GS were broken down into their individual compounds and ratios among compound classes, 3 of 11 GS concentrations and 8 of 9 GS ratios were significantly heritable in yr 1 (Fig. 2). After sequential Bonferroni corrections (Holm, 1979), one GS concentration (7MTH) and three ratios (RMT, R6MAM, and aliphatic/indole) remained significant (Table 1). Although there was an average decline in GS heritability of 50% from yr 1 to 3, indole GS were less affected than aliphatics (Fig. 2). Two indole GS showed significant heritability in yr 3, as did two long-chain aliphatic GS (7MTH, 8MSOO) and three ratios (R6MAM, I3MR2, and aliphatic/indole). After sequential Bonferroni correction, no GS heritabilities in yr 3 were significant (Table 1).

Trichome density was significantly heritable in yr 1, but not in yr 3 (Table 1). Specific leaf area, but not LDMC, was heritable in yr 1 (Table 1).

Resistance bioassays demonstrated significant genetic variation in one aspect of resistance to *Plutella* (adult emergence) and to *Trichoplusia* (host acceptance) (Table 1). There was no significant genetic basis to variation in *Pieris* larval growth or to the amount of tissue damage inflicted by *Pieris* and *Trichoplusia* (Table 1). In the common garden in

Table 4 Multiple regression of phenotypic variation in defense-related traits in *A. lyrata* spp. *petraea* (PLECH) on herbivore resistance bioassays

Dependent variable		Number of samples	F value	P value	Model R ²	Independent variable	df	Parameter estimate	t value	P value
<i>Plutella</i>	Adult emergence	264	2.81	0.026	0.03	Aliphatic GS_Yr1	1	-0.18	-1.29	0.197
						Indole GS_Yr1	1	-0.19	-1.77	0.078
						Trichome_Yr1	1	-0.17	-2.06	0.040
<i>Pieris</i>	Larval growth	152	0.51	0.727	0.00	SLA_Yr1	1	38.09	0.38	0.704
						Aliphatic GS_Yr1	1	-0.20	-0.77	0.445
						Indole GS_Yr1	1	-0.09	-0.44	0.664
<i>Pieris</i>	Plant damage	152	0.14	0.967	0.00	Trichome_Yr1	1	0.06	0.43	0.669
						SLA_Yr1	1	-131.59	-0.72	0.473
						Aliphatic GS_Yr1	1	-0.03	-0.56	0.580
<i>Trichoplusia</i>	Larval growth	60	5.10	0.028	0.02	Indole GS_Yr1	1	0.01	0.27	0.786
						Trichome_Yr1	1	-0.01	-0.34	0.733
						SLA_Yr1	1	16.74	0.45	0.654
<i>Trichoplusia</i>	Plant damage	123	3.00	0.021	0.06	Aliphatic GS_Yr1	1	0.13	0.50	0.622
						Indole GS_Yr1	1	-0.45	-2.26	0.028
						Trichome_Yr1	1	0.00	0.02	0.986
<i>Trichoplusia</i>	Host acceptance	123	2.37	0.057	0.04	SLA_Yr1	1	74.55	0.42	0.676
						Aliphatic GS_Yr1	1	0.24	2.14	0.035
						Indole GS_Yr1	1	-0.30	-3.28	0.001
Various herbivores	Field plant damage	784	6.16	< 0.001	0.02	Trichome_Yr1	1	-0.01	-0.22	0.825
						SLA_Yr1	1	115.08	1.35	0.181
						Aliphatic GS_Yr1	1	-0.11	0.81	0.420
Various herbivores	Field plant damage	784	6.16	< 0.001	0.02	Indole GS_Yr1	1	0.18	1.61	0.109
						Trichome_Yr1	1	0.14	-1.70	0.092
						SLA_Yr1	1	138.91	-1.32	0.191
Various herbivores	Field plant damage	784	6.16	< 0.001	0.02	Aliphatic GS_Yr3	1	-0.05	-1.88	0.051
						IndoleGS_Yr3	1	0.02	1.46	0.15
						Trichome_Yr3	1	-0.04	-3.60	<0.001

Values in bold are significant at $P < 0.05$

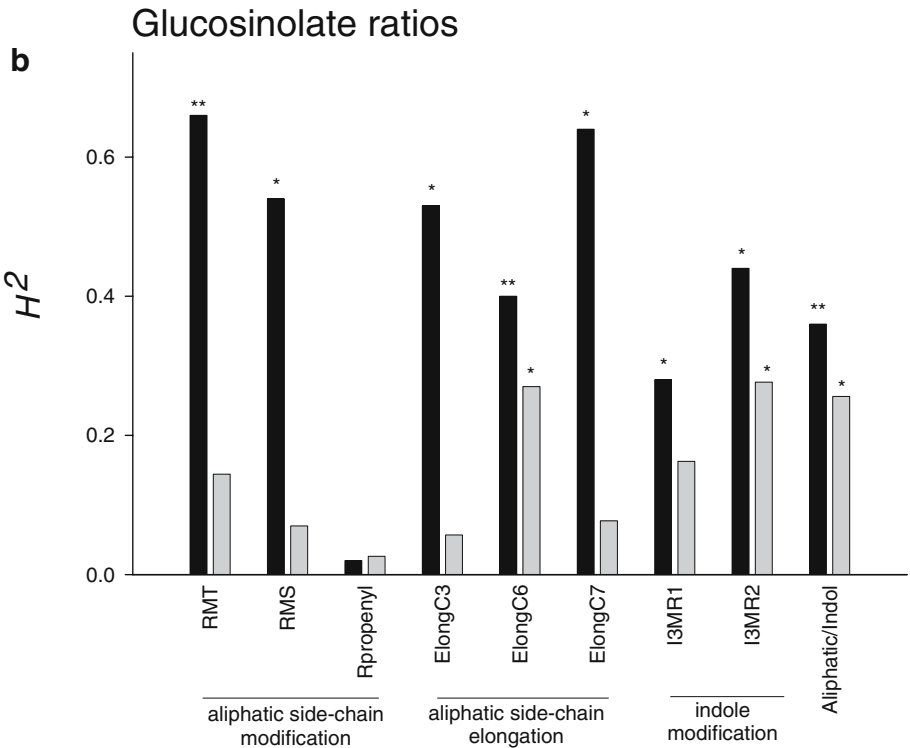
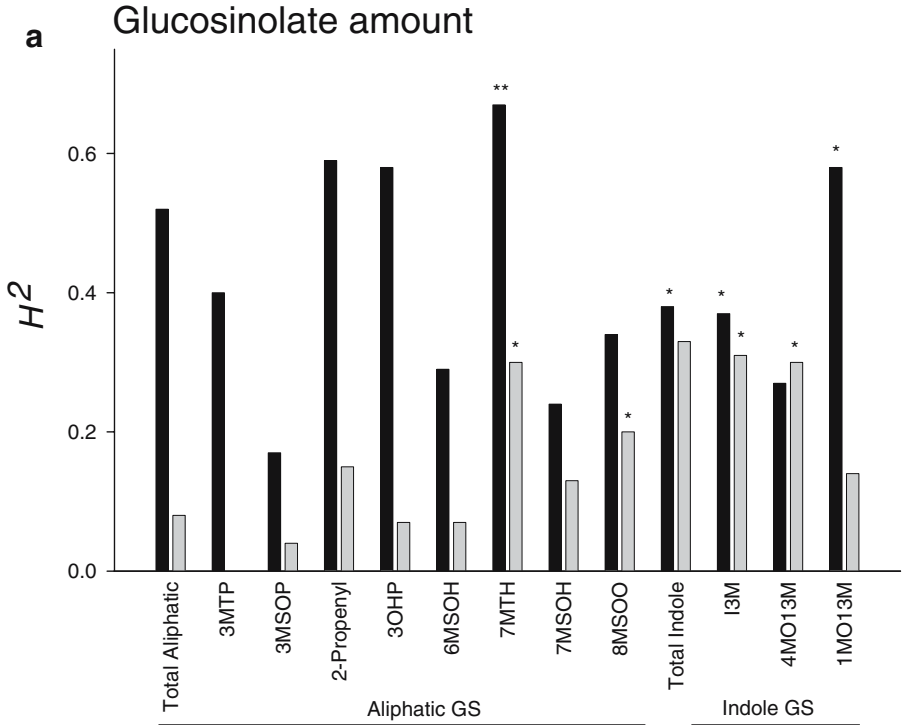


Table 5 Phenotypic and genetic correlations in leaf glucosinolate ratios for *A. lyrata* spp. *petraea* (PLECH)

(A) Year1	R3MAM_Yr1	R6MAM_Yr1	R7MAM_Yr1
R3MAM	1.000	0.429	-0.613
R6MAM	0.579	1.000	0.164
R7MAM	-0.548	-0.210	1.000
(B) Year3	R3MAM_Yr3	R6MAM_Yr3	R7MAM_Yr2
R3MAM	1.000	0.250	-0.770
R6MAM	-0.03	1.000	0.049
R7MAM	-0.85	0.09	1.000

For phenotypic (lower left) and genetic (upper left) correlations, sample sizes were $N=794$ and 14 in (A), and $N=805$ and 17 in (B), respectively.

Spearman correlation coefficients significant after sequential Bonferroni adjustment with family-wise $\alpha=0.05$ are indicated in bold.

yr 3, damage to plants by herbivores was pervasive but did not show a significant genetic basis (Table 1).

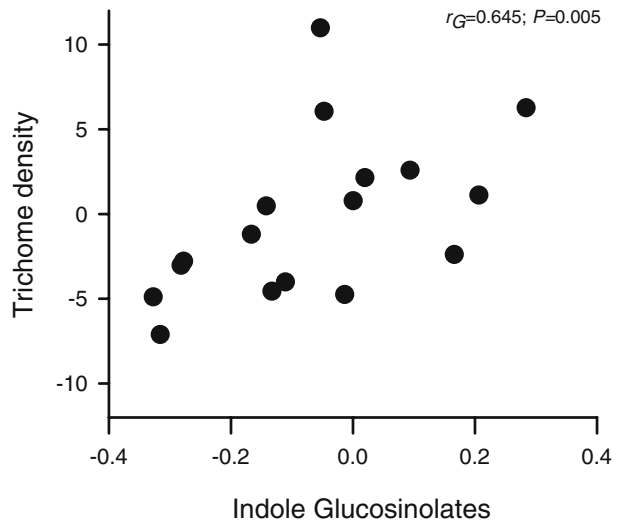
Genetic Correlations Significant positive genetic correlations among GS compounds were observed for 6MSOH, 7MSOH, and 8MSOO in yr 1 (Table 2) and between 7MSOH and 8MSOO in yr 3 ($r_{\text{spearman}}=0.811$, $P<0.001$). Genetic correlations among aliphatic chain length ratios in yr 1 showed that while R3MAM and R6MAM were positively correlated, both varied negatively with R7MAM (Table 5A). A strong negative genetic correlation was also seen between R3MAM and R7MAM in yr 3 (Table 5B). Between yr 1 and 3, genetic correlations among GS amounts and ratios were generally positive, but not significant (Table 3). Dam-based estimates of genetic correlations were more strongly positive (Table 3). Cross-year genetic correlations were not significant for trichome density (sire-based: $r_{\text{spearman}}=0.353$, $P=0.165$, $N=17$; dam-based: $r_{\text{spearman}}=0.348$, $P=0.171$, $N=17$). A significant genetic correlation between trichomes and GS was seen only for trichome density and indole GS in yr 3 ($r_{\text{spearman}}=0.645$, $P=0.005$, $N=17$; Fig. 3). Although biochemical and physical defenses can be correlated as the result of common induction (e.g., Agrawal 2000; Traw and Dawson, 2002b), we found no evidence of positive phenotypic correlations between trichomes and GS in yr 3 ($r_{\text{pearson}}=-0.019$, $P=0.582$, $N=784$).

Resistance to different herbivores was genetically correlated with different defense mechanisms. Here, we focus on selected GS compounds and ratios that were highly and/or consistently heritable across years, and also included total aliphatic and total indole GS to facilitate comparison with other studies. *Plutella* emergence was negatively genetically correlated with SLA (Table 6) and trichome density ($r_{\text{dam-based}}=-0.596$, $P=0.012$, $N=17$). Host-plant damage due to feeding by *Pieris* was significantly correlated with one ratio of aliphatic GS chain lengths (R6MAM), but not with the amount of GS (Table 6). *Trichoplusia* host acceptance showed a significant negative genetic correlation with indole



Fig. 2 Heritability of GS amounts (A) and ratios (B) for *A. lyrata* subsp. *petraea* Plech in the laboratory at 8 wk (yr 1, dark bars) and in the common garden at 28 mo (yr 3, gray bars). Abbreviations and calculation of ratios are given in Table 1 ($*P<0.05$, $**P<0.01$)

Fig. 3 Genetic correlation between indole GS concentration and trichome density for *A. lyrata* subsp. *petraea* Plech in yr 3 in the common garden



GS, primarily reflecting the main indole compound, I3M (Table 6). The growth rate of *Trichoplusia* larvae remaining on their host plant was also negatively correlated with indole GS ($r_{\text{spearman}} = -0.529$, $P = 0.029$, $N = 17$). Considering only the two resistance assays with significant heritabilities, there was a significant positive genetic correlation between resistance to the specialist *Plutella* and to the generalist *Trichoplusia* ($r_{\text{spearman}} = 0.520$, $P = 0.032$, $N = 17$). However, genetic correlations have notoriously large standard errors (Falconer, 1989, p. 317), and all tests reported for resistance were no longer significant after sequential Bonferroni correction.

Table 6 Genetic correlations of defense-related traits in *A. lyrata* spp. *petraea* (PLECH) and herbivore resistance bioassays

	<i>Plutella</i> adult emergence	<i>Pieris</i> plant damage	<i>Trichoplusia</i> host acceptance	Field plant damage
Aliphatic GS ^a	-0.260	0.154	-0.333	0.289
7MTH	0.130	-0.047	0.277	-0.299
Indole GS	-0.101	0.422	-0.581*	-0.101
I3M	-0.049	0.333	-0.552*	-0.130
RMT	-0.284	0.287	-0.235	0.517*
R6MAM	0.216	-0.537*	0.150	-0.647**
Aliphatic/Indole	-0.017	-0.223	0.338	0.287
I3MR2	0.375	0.248	0.069	-0.059
TrichNo	0.108	-0.056	0.066	-0.029
SLA	-0.510*	0.270	-0.306	

*Significant at $P=0.05$ without Bonferroni correction.

**Significant at $P<0.005$ without Bonferroni correction.

^a Defense traits measured in yr 1 except for Field plant damage (yr 3).

Discussion

The diverse GS profiles observed in *A. thaliana* and other species in the Brassicaceae arise largely from biosynthetic modifications in type and length of GS side chains and in regulatory steps that determine the amino acid precursors for the GS skeleton (Wittstock and Halkier, 2002; Kliebenstein et al., 2005; Grubb and Abel, 2006). Although *A. lyrata* spp. *petraea* is closely related to the model *A. thaliana* and the subject of an increasing number of comparative investigations, little is known about glucosinolates in this out-crossing perennial herb. We report 11 aliphatic and indole GS compounds in *A. lyrata*, all of which have previously been identified in natural accessions of *A. thaliana* and other *Arabidopsis* species (Table 1; Kliebenstein et al., 2001b; Windsor et al., 2005). The single most abundant compound in the *A. lyrata* GS profile was the short chain aliphatic 3-hydroxypropyl GS (>86%). GS profiles remained similar across years and environmental conditions, even though the overall concentration declined twofold in 3-yr-old plants (Fig. 1; Tables 1 and 3). Age-dependent decreases in GS concentration are also seen in *A. thaliana* and *Brassica* (Fahey et al., 2001; Brown et al., 2003; Lambdon et al., 2003). Within a single population of *A. lyrata*, we found significant and independent genetic variation for amounts and ratios of GS compounds (Table 1, Fig. 2). Studies of GS in *A. thaliana* have so far focused exclusively on natural genetic variation among populations or accessions (e.g., Kliebenstein et al., 2001b). Somewhat unexpectedly, heritabilities for indole GS in *A. lyrata* were high and less affected by differences in plant age and environment than aliphatic GS (Fig. 2). Indole GS are considered to be the dominant inducible compounds in the Brassicaceae and generally have lower heritabilities (Agrawal et al., 1999; Kliebenstein et al., 2001a; Traw 2002; Mikkelsen et al., 2003). Below, we address (1) the genetic basis of GS variation in *A. lyrata* in relation to candidate enzymes and genes identified in *A. thaliana*, and (2) the contribution of GS composition and trichome density to herbivore resistance in *A. lyrata*.

Elongation of the carbon side chain of the base amino acid is the first step in biosynthesis of *Arabidopsis* aliphatic GS. The allelic state of the *GS-Elong* locus determines the distribution of carbon chain lengths observed in methionine-derived aliphatic GS, and contains two or three directly repeated paralogous sequences in *A. thaliana* encoding MAM (Kroymann et al., 2003). The chain elongation phenotype can be quantified by ratios of longer to shorter chain GS (Kliebenstein et al., 2001b), and all three ratios reflecting the putative function of the MAM enzyme in *A. lyrata* were significantly heritable (Fig. 2). Indeed, a cosegregation test for the F1 progeny from several of the same families used in this study suggests that the *MAM* locus is responsible for segregating variation in chain length ratios in *A. lyrata* (Heidel et al., 2006). Interestingly, both the phenotypic and genetic correlations among MAM ratios are positive between R3MAM and R6MAM, but negative for R7MAM (Table 5). This suggests that while overall the amounts of long chain GS are positively correlated to one another (Table 2), a high conversion of 3-carbon and 6-carbon GS was associated with a low conversion of 7-carbon GS. This negative correlation has also been observed in *A. thaliana* (Kliebenstein et al., 2001b).

Genetic control of side-chain modifications converting methylthioalkyl to methylsulfanylalkyl GS has been attributed to the *GS-OX* locus (Giamoustaris and Mithen, 1996; Kliebenstein et al., 2001c). The GS ratio quantifying this modification step in *A. lyrata* was significantly heritable (RMT; Fig. 2, Table 1). Although *GS-OX* is variable in *A. thaliana* and has been subject to QTL mapping, a single underlying gene has not been unambiguously identified (D. Kliebenstein, personal communication). Ratios corresponding

to the carbon chain modification functions of *AOP2* and *AOP3* candidate loci (Kliebenstein et al., 2001c) did not vary genetically in the Plech population of *A. lyrata* (RMS and RPropenyl in Table 1). In contrast, the heritability of the ratio of methionine to tryptophan-derived GS was significant (aliphatic/indole; Fig. 2), and the corresponding QTL in *A. lyrata* appears to contain *CYP79F1* and *CYP79F2* (Heidel et al., 2006). Mutants in these two cytochrome P450 genes affect the ratio of indole to aliphatic GS and are involved in chain elongation for aliphatic GS biosynthesis (Chen et al., 2003).

Our investigation of the natural quantitative genetic variation for GS in *A. lyrata*, and a companion paper showing a molecular genetic basis for variation in GS carbon chain elongation (Heidel et al., 2006) exemplify the possibilities for functional genetic research in nonmodel systems. Beyond the natural variation observed among accessions of *A. thaliana*, populations of closely related species represent further biological complexity and the opportunity to understand the causes and consequences of variation in function (Clauss and Koch 2006).

GS compounds are an important plant defense against many insect herbivores. However, the strength of resistance conferred by GS, the most effective GS compounds, and the role of other defense-related traits can vary when different plants and herbivore species interact. Below, we infer defense function from the phenotypic covariance structure between defense-related traits and plant resistance in four different herbivory experiments. Given equivalent exposure to herbivores, plants with less feeding damage or plants on which insects had reduced performance were considered more resistant. Future experiments manipulating individual defense traits are necessary to confirm the conclusions from our correlative study.

The specialist herbivore *P. xylostella* is restricted to feeding on Brassicaceae and possesses a detoxification mechanism for GS–myrosinase defenses (Ratzka et al., 2002). Commensurate with the detoxification physiology of this insect, performance of *Plutella* was not related to GS composition but significantly reduced on *A. lyrata* individuals with greater trichome density (Table 4). Leaf morphological characteristics, and in particular trichome density, have been previously shown to contribute to resistance to herbivores in *A. thaliana* and *Brassica* spp. (Agren and Schemske, 1993; Mauricio, 1998). Defenses outside the GS–myrosinase system are expected to play an important role in resistance to herbivores specialized on Brassicaceae.

Larvae of butterflies in the genus *Pieris* feed primarily on plants in the Brassicaceae and related families, and have been the focus of a large number of ecological and functional investigations of plant–insect interactions (Renwick and Lopez, 1999; Agrawal and Kurashige, 2003). Although *Pieris rapae* larvae can respond to GS-containing diet by redirecting enzymatic breakdown in the gut to favor formation of less toxic products (Wittstock et al., 2004), larval performance can be reduced by the presence of GS and their breakdown products (e.g., isothiocyanates) under some conditions (Agrawal and Kurashige, 2003; Rotem et al., 2003). GS and isothiocyanates can also serve as stimulants for feeding and oviposition in *Pieris* (Renwick and Lopez, 1999; Rask et al., 2000; Fahey et al., 2001; Renwick, 2002). For *A. lyrata*, we observed no phenotypic relationships between GS, isothiocyanates, or leaf morphological traits with *Pbrassicae* larval growth or plant damage (Table 4; PB plant damage vs. total isothiocyanates; $r_{\text{spearman}} = 0.005$, $P = 0.911$, $N = 493$; Clauss, unpublished data).

Trichoplusia ni is a generalist herbivore that feeds on a variety of plant species, including members of the Brassicaceae, but is sensitive to the particular composition of GS and their breakdown products (Jander et al., 2001; Lambrix et al., 2001; Kliebenstein et al., 2002). A higher indole GS phenotype in *A. lyrata* was associated with in reduced *Trichoplusia* larval growth and feeding damage (Table 4). There was no effect of trichome

density on resistance to the generalist *Trichoplusia*. A role for indolic GS in defense against pathogens and insect herbivores has been previously suggested (Hopkins et al., 1998; Brader et al., 2001; Reymond et al., 2004). It was unexpected that rather than confer resistance against *Trichoplusia* (Raybould and Moyes, 2001; Kliebenstein et al., 2002), the dominant aliphatic GS in *A. lyrata* had a weak *positive* phenotypic relationship with feeding damage (Table 4).

In the common garden, the crucifer flea beetle, *P.cruciferae*, was the most common herbivore, and *A. lyrata* plants with more trichomes and more aliphatic GS experienced less leaf damage (Table 4).

In summary, we found that *A. lyrata* resistance was conferred by both indole GS amount and trichome density, and that aliphatic GS amount had a defensive as well as a stimulatory role. The contribution of these defense traits to resistance was dependent on the experimental herbivory context.

Phenotypic variance and covariance are shaped by both genetic and environmental factors. Although variation in trichome density had a genetic basis when measured in the laboratory ($H^2=0.33$), there was a fivefold increase in density when the experiment was transferred to the common garden. Plants of the same age maintained in the growth chamber showed no change in trichomes (Clauss, personal observation). The environmental conditions in the common garden caused not only an increase in the mean trichome phenotype, but also an increase in the environmental variance component, which resulted in a dramatic decline in trichome heritability ($H^2=0.03$, where $H^2 = \text{Var}_{\text{Gen}}/(\text{Var}_{\text{Gen}} + \text{Var}_{\text{Env}})$). Interestingly, density in the common garden (127 trichomes/cm²) was more representative of plants growing in the natural Plech field population (111 trichomes/cm², SD=69.9, N=45). The high trichome density in the Plech population is in contrast with the observation that *A. lyrata* individuals of many Swedish populations are lacking any hairs and are completely glabrous both under field and greenhouse conditions (Karkkainen et al., 2004). A single locus with simple Mendelian inheritance (GLABROUS1) has been proposed for the glabrous/trichome-producing polymorphism (Karkkainen and Agren, 2002). However, when trichomes are present in *A. lyrata*, trichome density appears to be under polygenic control as indicated by the significant heritability observed for trichome density in the laboratory for Plech, and the continuous unimodal distribution of F1 phenotypes (results not shown). Regardless of the genetic basis, the dramatic changes in phenotype for trichomes and GS seen over the course of development under different environmental conditions (Tables 1 and 3) emphasize the importance of ecologically relevant experimental conditions when interpreting the role of trait variation in biological interactions (Conner et al., 2003).

Considering the genetic components of variation, the heritability of individual traits and genetic correlations among traits reflect past evolutionary processes such as natural selection, drift, migration, recombination, and mutation. For the *A. lyrata* Plech population, we found genetic variation in GS and trichome defense traits as well as in resistance to *Plutella* and *Trichoplusia* (Table 1 and Fig. 2). Although balancing selection, frequency-dependent selection, and heterozygote advantage can contribute to the maintenance of genetic variation in some interaction-related traits (Stahl et al., 1999; Mauricio et al., 2003; Charlesworth, 2006), the importance of these selective processes for herbivore defense within populations of perennial plants such as *A. lyrata* remains to be shown. Because genetic variation in defense can be hidden from selection by high environmental variance and age-dependent changes in heritability (Fig. 2; Charmantier and Garant, 2005; Charmantier et al., 2006), future studies in *A. lyrata* must also address the possibility that substantial nearly neutral diversity in functional characteristics might segregate in old and stable glacial relict populations (Clauss and Mitchell-Olds, 2003, 2006).

When genetic polymorphisms in defense traits cause variation in resistance, we expect genetic correlations to reflect this functional relationship. One aspect of leaf morphology, biomass per leaf area (SLA), had a negative genetic correlation with *Plutella* emergence (Table 6). Although not primarily involved in defense, traits affecting leaf toughness can influence herbivore feeding and thus resistance to herbivory. Sire-based genetic correlations between trichome density and *Plutella* emergence were not significant, suggesting that the significant relationships we observed between trichomes and resistance in phenotypic and dam-based analyses were not due to genetic differences. Indole GS concentration was negatively genetically correlated with host acceptance by *Trichoplusia* and the growth rate of larvae (Table 6), confirming the defensive role for indole GS observed in the phenotypic data. Two GS ratios were significantly correlated with resistance, suggesting that the relative GS composition, in addition to the amount, can be of ecological importance (Table 6). Such genetic correlations in a population-level survey motivate further functional investigations employing crosses of divergent genotypes, QTL mapping, and testing of candidate genes underlying ecologically important traits (e.g., Kroymann et al., 2003; Heidel et al., 2006).

Genetic correlations can also affect the future response to selection across generations. Models of multispecies interactions predict that positively correlated resistances contribute to a “diffuse” coevolutionary dynamic where plant defense evolves in response to a whole suite of attackers (Hougen-Eitzman and Rausher, 1994). A weak positive genetic correlation between resistance to the specialist *Plutella* and to the generalist *Trichoplusia* was observed in *A. lyrata*. In the natural Plech population of *A. lyrata*, single long-lived plants (>10 yr) experience attack by many different herbivore species throughout their lifetime (Clauss, unpublished data). Correlated resistance against multiple herbivores could be beneficial when individual herbivores species are rare and the composition of the herbivore community varies among months and years (Clauss, unpublished data). Interestingly, the observed correlation in resistance was not mediated via a shared response to GS, but rather might reflect an underlying positive genetic correlation between trichome density and indole GS concentration (Fig. 3). If trichomes and GS defend *A. lyrata* plants against multiple herbivores and are positively genetically correlated, why has natural selection not swept all genetic variation in these traits to fixation? In the common garden, we observed a negative correlation between plant size and total GS concentration (Clauss, unpublished data), which might indicate a resource-related trade-off that imposes limits on selection for increased defense (Bergelson and Purrington, 1996; Mauricio and Rausher, 1997; Mauricio 1998). In *A. lyrata*, we can now test hypotheses concerning the ecological and evolutionary processes that shape functional genetic variation within stable and persistent populations of a perennial plant species.

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achieved by wounding the plants and applying herbivore regurgitant or elicitors such as jasmonates (JA) to the damaged sites (Turlings et al., 1993a; Hopke et al., 1994; Halitschke et al., 2001). Induced volatile blends vary considerably in quantity and quality with plant species or variety (Takabayashi and Dicke, 1996; Krips et al., 2001; Fritzsche-Hoballah et al., 2002; Degen et al., 2004; Van Den Boom et al., 2004; Bukovinszky et al., 2005), which may reflect in differences in attractiveness to the natural enemies of herbivores (Krips et al., 2001; Fritzsche-Hoballah et al., 2002; Bukovinszky et al., 2005). However, little to nothing is known about the consequences of these differences among plant genotypes for the effectiveness of the natural enemies in the field.

Nilaparvata lugens is one of the most important rice pests. It feeds on the phloem and causes a decrease in leaf area, plant height, dry weight, leaf and stem nitrogen concentration, chlorophyll contents, and photosynthetic rate (Rubia-Sanchez et al., 1999; Watanabe and Kitagawa, 2000), which subsequently results in yield loss. Previous studies have shown that *N. lugens* attack or JA treatment alters the volatile profiles of rice plants, and these volatiles are strongly attractive to the egg parasitoid *Anagrus nilaparvatae* (Lou et al., 2005a,b), a major natural enemy of the rice planthoppers (Cheng and He, 1996). Moreover, the parasitism of *N. lugens* eggs by *A. nilaparvatae* on plants that were surrounded by JA-treated plants is more than twofold higher than on control plants in the greenhouse and field (Lou et al., 2005a), implying that augmenting the release of rice-produced attractants has the potential to enhance the effectiveness of the parasitoid in the control of *N. lugens*. This should be testable, as the volatiles released from different rice varieties in response to *N. lugens* infestation show distinct differences in attraction of the parasitoid (Ma et al., 2004). In this work, we performed such tests, whereby we compared the volatile emissions of various rice varieties after JA induction, as well as their attractiveness to *A. nilaparvatae* in the laboratory and field.

Methods and Materials

Plant Growth

Rice varieties used in this study were TN1, IR26, IR64, Xiushui 63, Bing 97-34, and Bing 97-59, whose genetic backgrounds are described in Table 1. Of these varieties, both IR26 and IR64 contain the *Bph1* gene (Zhu et al., 2004), a resistance-related gene to biotype one of *N. lugens*, and share several parents in their pedigrees; the other four are all susceptible to *N. lugens*, and three (Xiushui 63, Bing 97-34, and Bing 97-59) have a similar genetic background (Table 1). Pregerminated seeds were sown in a greenhouse, and after 20–25 d,

Table 1 Genetic background of six rice varieties

Variety	Genetic background
TN ₁	DGWG/Tsai Yuan Chan
IR26	IR24/TKM6; containing <i>Bph1</i> gene
IR64	...IR24/TKM6...IR5657-33-2-1/IR2061-465-1-5-5; containing <i>Bph1</i> gene
Xiushui 63	Shan 41r/Bing 8961//Bing 8961(M)
Bing 97-34	Bing 8961/Bing 93390
Bing 97-59	Bing 9147/Bing 9117//Bing 88122(M)/3/Xiushui 63(F)

“/,” “//,” and “/3/” indicate the first, second, and third hybridization, respectively. For each cross, the female parent is listed first unless otherwise specified. F, female parent; M, male parent.

seedlings were transplanted into small clay pots [8 cm (diam) × 10 cm (height)] each with one plant, or big clay pots (16 × 10 cm) each with three plants. In the latter, plants were arranged in an equilateral triangle 8 cm apart. Plants were watered daily, and each pot was supplied with 10 ml of nutrient solution ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.5 g/l; $\text{K}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.125 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.125 g/l; K_2HPO_4 , 0.125 g/l; FeCl_2 , 0.005 g/l) every 3 d. All plants were placed in a controlled climate room that was maintained at $23 \pm 2^\circ\text{C}$, 70% r.h. and 18 hr photophase (25,000 lx). Twenty-five to 30 d after potting, plants were used for experiments. Plantings were continued at regular intervals so that enough of suitable age were available for all experiments.

Insects

The *N. lugens* culture was originally obtained from the China National Rice Research Institute (CNRRI), Zhejiang, and maintained on Shanyou 63 rice plants (susceptible to *N. lugens*) in a greenhouse. Late instar nymphs of *N. lugens* were captured from the greenhouse and reared on potted Shanyou 63 rice plants, which were confined in plastic cages (11 × 40 cm). Caged rice plants were maintained in a controlled climate room at $28 \pm 2^\circ\text{C}$, 12 hr photophase, and 70–80% r. h. Newly emerged adults of *N. lugens* were collected daily and fed on potted fresh Shanyou 63 rice plants. With this procedure, *N. lugens* adults of uniform age were obtained.

A laboratory colony of the egg parasitoid *A. nilaparvatae* was started from individuals trapped in rice fields in Hangzhou, using Shanyou 63 rice plants with *N. lugens* eggs as bait. The colony was propagated on *N. lugens* eggs in rice shoots enclosed in glass tubes (2.5 × 20 cm), which were kept in a controlled climate room at $28 \pm 2^\circ\text{C}$, 12 hr photophase, and 70–80% r.h. Each day, newly emerged wasps were collected into clean glass tubes (2.5 × 20 cm) with access to both water and honey solution, and held for at least 2 hr to ensure mating. From the second generation onwards, female parasitoids were used in experiments less than 24 hr after emergence.

Plant Treatment

Potted plants (1 or 3 per pot) were washed with running water. They were individually damaged with a needle at the lower and upper positions of the rice stems, each with 200 pricks. They were subsequently treated by applying 20 μl of 10 mM jasmonic acid in 50 mM sodium phosphate buffer (titrated with 1 M citric acid until pH = 8) at each damage site (JA). In a previous study, we found that JA treatment induces the release of volatiles in rice, resulting in the attraction of the parasitoid (Lou et al., 2005a). Plants were treated at 17:00 hr, and then the plants were placed in a controlled climate room that was maintained at $28 \pm 2^\circ\text{C}$, 12 hr photophase, and 80% r.h. Fifteen hours after treatment, i.e., at 8:00 hr the next day, plants were used for experiments.

Collection, Isolation and Identification of Volatile Compounds

The volatile collection system has been described in detail (Turlings et al., 1998). It basically consists of six vertically placed cylindrical glass tubes (9.5 cm i.d., 54 cm high). A split Teflon plate with a hole in the center at the base of a cylinder was filled loosely around the plant stem, allowing the separation of the aerial part of the plant, in the cylinder, from the pot, which remained outside (Turlings et al., 1998). Purified and humidified air was pushed into each cylinder at a rate of 1 l/min and passed over the plant. Around the base of

each cylinder, just above the Teflon disk, eight openings served as ports that could hold the collection traps. Only one port was used during an experiment. For collections, air was pulled (0.8 l/min) through a Super-Q adsorbent trap (Heath and Manukian, 1992), whereas the rest of the air was vented out through the hole in the bottom, thus preventing outside, impure air from entering. The automated part of the collection system (Analytical Research System, Gainesville, FL, USA) controlled the flow through the trap. The climate chamber (CMP4030; CONVIRON, Winnipeg, Manitoba, Canada) in which the collection cylinders were housed was kept at 17.5°C; due to heat irradiation, the temperature inside the cylinders was $23 \pm 3^\circ\text{C}$. During the light cycle, light intensity was about 20,000 lumen/m².

Volatiles emitted from plants of the six varieties that were wounded and treated with JA were collected. Collections started immediately after the lights were turned on, 15 hr after treatment, and lasted 4 hr. After each collection, traps were extracted with 150 µl of methylene chloride (Lichrosolv.; Merck, Whitehouse Station, NJ, USA) and 200 ng of *n*-octane and nonyl acetate (Sigma, Buchs, Switzerland) in 10 µl of methylene chloride were added to the samples as internal standards. Collections were replicated six times for each variety.

Analyses were done with an HP 6890 series gas chromatograph equipped with an automated on-column injection system (HP G1513 A) and a flame ionization detector. A 3-µl aliquot of each sample, was injected onto an apolar SE-30 capillary column (30 m, 0.25 mm i. d., 0.25 µm film thickness; Alltech, Deerfield, IL, USA) that was preceded by a deactivated retention gap (5 m, 0.25 mm i.d.) and a deactivated precolumn (30 cm, 0.530 mm). Helium (24 cm/sec) was used as carrier gas. After injection, the column temperature was maintained at 40°C for 3 min, increased to 230°C at 8°C/min, and held at 230°C for 9.5 min. The detector signal was processed with HP GC Chemstation software. Compounds were identified by comparison of GC retention times with those from our previous studies (Lou et al., 2005a) and those of authentic standards.

Olfactometer Bioassay

Responses of *A. nilaparvatae* females to rice volatiles were measured in a Y-tube olfactometer. The olfactometer consisted of a Y-shaped glass tube (1 cm diam). The base and the two arms of the Y tube were all 10 cm in length. Each arm was connected to an odor source container (a glass cylinder, 6 × 46 cm). An airstream was generated and was divided in two, and each secondary airstream was led through a flowmeter, a tube with active charcoal, a humidifier bottle, and one of the odor containers. Subsequently, the two airstreams were led through the two arms of the Y-tube olfactometer at 150 ml/min. The Y-tube olfactometer was placed in a box painted white with an artificial light source consisting of two 20-W fluorescent lamps placed in front of the box. All bioassays were conducted between 09:00 and 17:00 hr. During experiments, the temperature in the room was maintained at 25–28°C.

The six varieties were divided into three groups on the basis of the volatiles profiles that they emitted after JA treatment (Table 2; Fig. 2): group 1 (TN1), group 2 (IR26 and IR64), and group 3 (Bing 97-34, Bing 97-59, and Xiushui 63). To compare attractiveness among and within these groups, we selected the following five pairs for olfactometer tests: IR26 vs. IR64, Bing 97-59 vs. Xiushui 63, TN1 vs. IR26, Bing 97-59 vs. IR26, TN1 vs. Bing 97-59. Fifteen hours after JA treatment, 10 individually potted plants of each variety were cut off at soil level, their cut stems wrapped in wet cotton, and the plants of one variety were placed into one of the odor source containers. Mated female parasitoids were introduced individually into the base tube of the Y-shaped olfactometer and given 10 min to walk towards the end of one of the arms. A choice for an odor source was defined as a female crossing a line 7 cm after the division of the base tube and remaining there for at least 1 min.

Table 2 Comparison of volatile compounds emitted from Ja-treated plants of various rice varieties^a

Chemical	TNI	IR64	IR26	Bing 97-34	Bing 97-59	Xiushui 63
1 (2-Heptanone)	776.75±259.34 a	118.39±29.65 b	201.37±111.00 b	5.53±1.99 c	5.50±0.88 c	5.60±1.22 c
2 (2-Heptanol)	471.79±119.64 a	222.10±37.88 ab	212.23±72.46 b	5.31±2.33 c	5.62±2.26 c	10.21±4.40 c
3 (Unknown 1)	25.83±6.05 a	11.04±1.15 b	5.52±1.32 c	5.03±1.07 c	3.79±0.59 c	4.01±0.91 c
4 (Unknown 2)	40.01±10.69 a	14.44±1.26 b	8.62±2.02 c	7.95±1.67 c	6.11±0.88 c	6.11±1.29 c
5 (Limonene)	243.26±57.54 a	102.61±8.92 b	44.75±7.87 c	55.30±7.34 c	42.47±6.05 c	41.37±8.65 c
6 (Unknown 3)	134.02±44.88 a	48.29±8.24 ab	29.67±12.67 bc	19.51±6.11 bc	11.36±2.37 c	12.94±4.47 c
7 (Unknown 4)	219.54±88.17 a	37.17±7.87 b	28.52±14.44 b	4.86±0.71 c	5.43±1.11 c	6.33±2.21 c
8 (Linalool)	1731.30±489.07 a	597.56±149.02 b	611.53±134.16 b	843.42±173.41 b	633.65±95.84 b	719.98±125.97 b
9 (C ₁₁ H ₁₈) ^b	146.91±29.30 a	76.45±13.35 ab	39.46±10.19 bc	35.30±5.06 bc	24.81±3.51 c	27.37±5.73 c
10 (Methyl salicylate)	194.45±55.45 a	113.96±27.43 a	46.26±16.66 ab	11.78±4.81 bc	7.15±1.84 c	15.54±7.99 bc
11 (β-Caryophyllene)	52.60±13.37 b	27.11±6.41 b	15.70±2.20 b	201.94±29.39 a	240.77±33.82 a	149.74±31.91 a
12 ((E)-α-bergamotene)	7.86±2.15 bc	6.09±1.11 cd	2.86±0.70 d	27.11±3.60 a	29.44±6.17 a	15.48±2.70 ab
13 (Unknown 5)	23.50±4.18 ab	9.68±0.87 b	11.15±1.86 b	43.23±7.56 a	52.74±9.73 a	32.27±7.84 a
14 (Unknown 6)	20.72±5.59 bc	11.87±3.41 cd	5.56±1.57 d	91.41±13.00 a	102.75±21.61 a	55.97±9.92 a
15 (Unknown 7)	37.47±8.89 b	13.89±1.78 b	13.15±2.28 b	82.00±14.18 a	121.65±13.04 a	80.93±18.87 a
16 (n-Heptadecane)	18.42±3.94 bc	11.93±2.72 cd	5.42±2.00 d	39.27±4.43 a	36.04±9.90 a	13.88±4.48 cd
17 (Unknown 8)	22.16±5.25 b	10.30±1.21 c	5.33±1.84 d	46.74±7.54 a	51.88±10.95 a	28.02±3.57 ab
18 ((E)-nerolidol)	272.06±46.98 a	36.23±5.62 b	25.11±7.74 b	36.89±3.63 b	40.59±5.82 b	32.95±4.42 bc
19 (C ₁₆ H ₃₀) ^c	46.99±7.24 a	20.44±2.43 b	11.07±2.64 c	10.06±1.55 c	6.84±0.84 c	7.60±1.52 c
Total	4495.85±1119.35 a	1494.24±248.70 b	1333.78±367.65 b	1580.86±247.05 b	1433.98±201.13 b	1275.12±203.00 b

^aData represent the mean amount (% of IS peak area) of six replications. Different letters in the same row indicate significant differences among treatments ($P < 0.05$, Fisher LSD *post-hoc* tests).

^b(E)-4,8-Dimethyl-1,3,7-nonatriene.

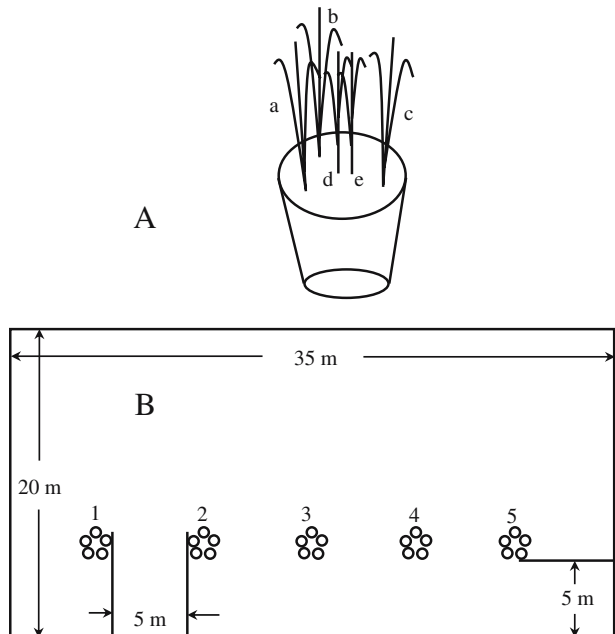
^c(3E,7E)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene.

If a parasitoid did not make a choice within 10 min, this was recorded as “no response.” After two females were tested, the olfactometer tube was washed with 98% alcohol and heated at 80°C for several minutes. To eliminate the effects of asymmetrical bias, connections of the two arms of the olfactometer to the odor source containers were exchanged after testing of two females, and the odor source containers were exchanged and supplied with a new set of plants after testing of eight females. For each odor source combination, at least 48 females were tested. All females that were tested made a choice.

Field Experiment

To obtain plants with *N. lugens* eggs, plants of the variety Shanyou 63 were individually infested for 1 d with 10 gravid *N. lugens* females that were confined to the plants in two parafilm bags at the upper and lower positions of the stems. Of these plants, two were chosen with about 80–100 1-d-old *N. lugens* eggs, and they were transplanted to a pot into the center of a triangle of three plants of a particular variety, which had been wounded and treated with 10 mM JA 15 hr earlier (Fig. 1A). Five pots for each of the varieties—TN1, IR26, IR64, Bing 97-59, and Xiushui 63—were placed at five locations in a rice field (20 m wide, 35 m long; rice variety Jialeyou 2) as shown in Fig. 1B. Hence, each location included five pots, each of them with JA-treated plants of a different variety. Two days after the plants were introduced into the rice field, they were transferred to the controlled climate room at $28 \pm 2^\circ\text{C}$, 12 hr photophase, and 80% r.h. Each pot of plants was confined to an 11×40 cm plastic cage (after herbivores, predators, and parasitoids had been removed). Five days later, the plants were cut off at soil level and dissected under a microscope to record the total and parasitized *N. lugens* eggs by using the method described in Lou et al. (2005a). This experiment was done two times (July 6 and September 27, 2005).

Fig. 1 Design of potted plants for field experiment (A) and arrangement of plants in a rice field (B). Letters a, b, and c represent three plants that were arranged in an equilateral triangle 8 cm apart and were wounded and treated with $40 \mu\text{l}$ of 10 mM JA in 50 mM sodium phosphate buffer (pH = 8); d and e are two plants of variety Shanyou 63, each with 80–100 *N. lugens* eggs, which were planted into the center of the triangle. Numbers indicate locations, and each location included five pots of plants, each with JA-treated plants of one of the five varieties: TN1, Bing 97-34, Xiushui 63, IR26 or IR64



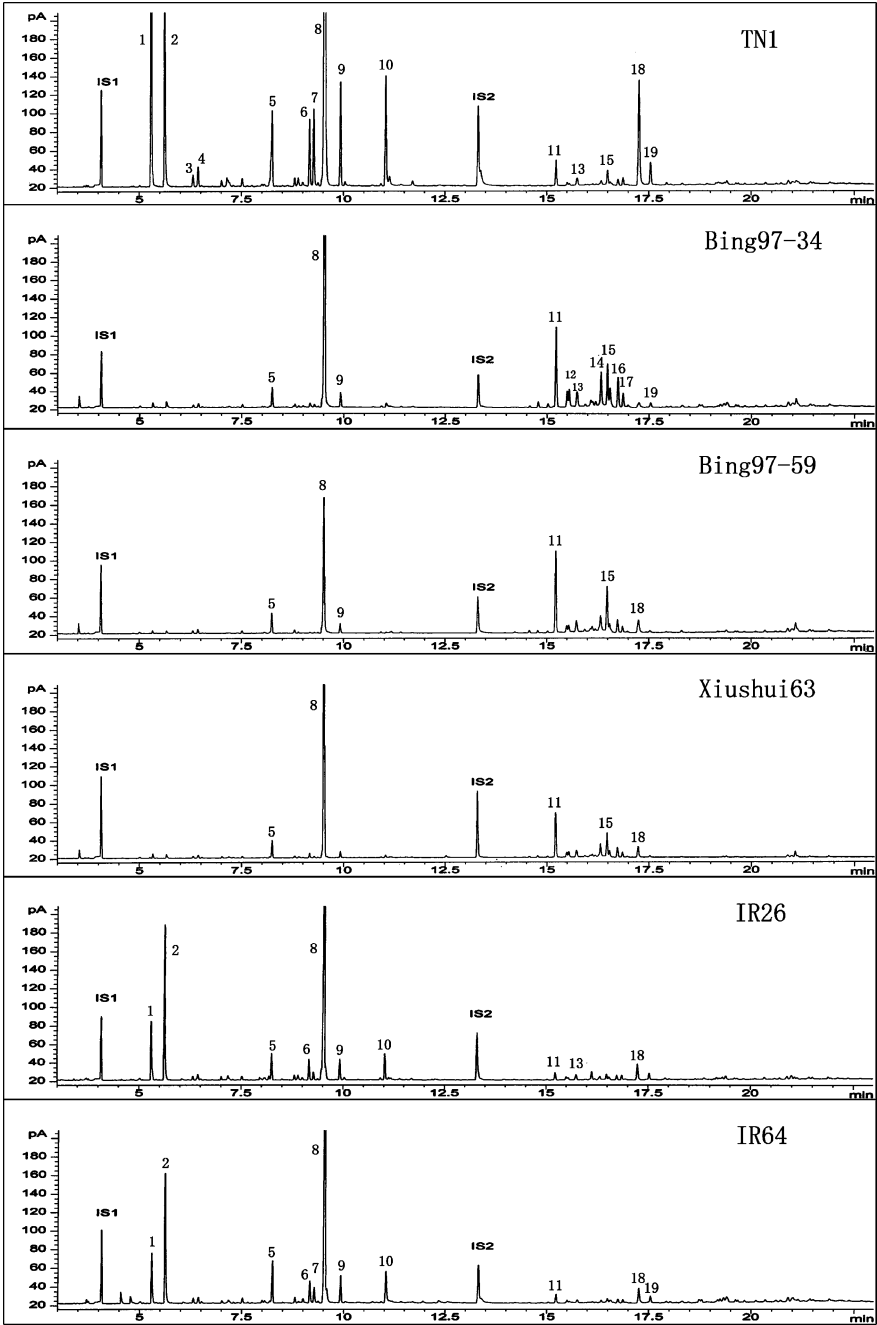


Fig. 2 Typical chromatograms obtained from headspace collections from JA-treated rice plants of rice variety TN1, Bing 97-34, Bing97-59, Xiushui 63, IR26 and IR64. 1, 2-Heptanone; 2, 2-heptanol; 3, unknown 1; 4, unknown 2; 5, limonene; 6, unknown 3; 7, unknown 4; 8, linalool; 9, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 10, methyl salicylate; 11, β -caryophyllene; 12, (*E*)- α -bergamotene; 13, unknown 5; 14, unknown 6; 15, unknown 7; 16, *n*-heptadecane; 17, unknown 8; 18, (*E*)-nerolidol; 19, (*3E,7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

Data Analysis

Differences in behavioral responses of the parasitoid to pairs of JA-induced rice volatiles of various varieties were determined by χ^2 tests. To test for differences in parasitism among varieties, we used the Friedman rank sum test. Comparison of the data on plant volatiles were analyzed by analysis of variance (ANOVA) after the data were log-transformed. If the ANOVA analysis was significant ($P < 0.05$), Fisher LSD *post-hoc* tests to detect significant differences among groups were conducted. Data were analyzed with Statistica (Statistica, SAS, Institute Inc., Cary, NC, USA).

Results

Volatiles Comparison

The analyses revealed significant differences in volatile profiles emitted from JA-treated plants of the six varieties (Table 2; Fig. 2). The total amount emitted from TN1 was significantly (from 2.84 to 3.53 times) higher than those from the other five varieties, whereas no differences in total amounts were found among the remaining varieties. The volatile profiles of the six varieties could be roughly divided into three groups. The first group represented the volatiles released from TN1 plants, which, in addition to the total amount, had the highest amount of compounds 2-heptanone, 2-heptanol, limonene, linalool, (*E*)-4,8-dimethyl-1, 3, 7-nonatriene, (*E*)-nerolidol, (*3E,7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and unknowns **1**, **2**, **3**, and **4** (Table 2; Fig. 2). The second group consisted of varieties IR64 and IR26 plants. In this group, plants usually released intermediate amount of 2-heptanone, 2-heptanol, and unknown **4**, and the lowest levels of (*E*)- α -bergamotene, *n*-heptadecane, and unknowns **6** and **8**. The third group included Bing 97-34, Bing 97-59, and Xiushui 63 (Table 2; Fig. 2). They usually had the highest levels of β -caryophyllene, (*E*)- α -bergamotene, *n*-heptadecane, unknowns **5**, **6**, **7**, and **8**, and the lowest levels of 2-heptanone, 2-heptanol, unknown **4**, methyl salicylate and (*3E,7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Table 2; Fig. 2).

Olfactometer Bioassay

We compared the choices of female *A. nilaparvatae* when offered pairs of odors within groups and between groups. When the parasitoid was exposed to volatiles from pairs of

Fig. 3 Number of *Anagrus nilaparvatae* female adults attracted by volatiles released from JA-treated plants of pairs of varieties: plants were used 15 hr after they were wounded and treated with 40 μ l of 10 mM JA in 50 mM sodium phosphate buffer (pH = 8). Asterisks indicate significant differences between members of a pair ($P < 0.05$, χ^2 test)

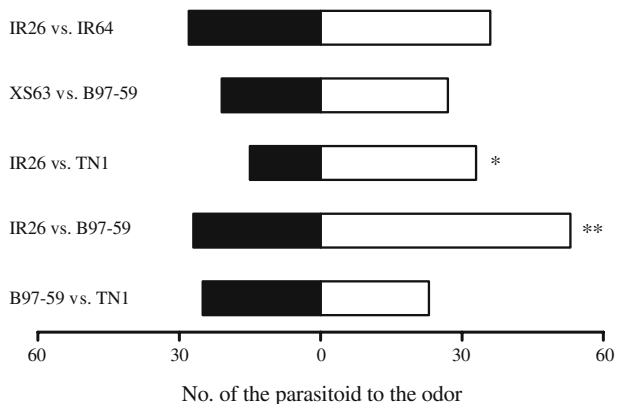
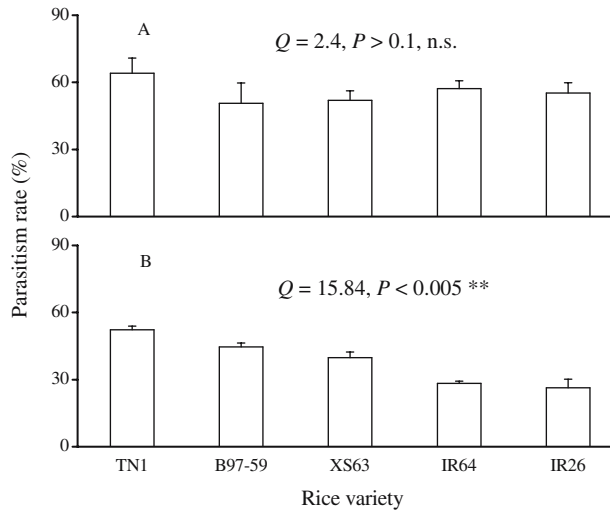


Fig. 4 Mean (\pm SE) parasitism rates (%) of *Nilaparvata lugens* eggs by *Anagrus nilaparvatae* in the field on Shanyou 63 rice plants of that were surrounded by JA-treated plants of variety TN1, Bing 97-59 (B97-59), Xiushui 63 (XS63), IR26, or IR64. Plants were placed in the field for 2 days on September (A, $N = 5$) or October (B, $N = 5$), 15 hr after they were wounded and treated with 40 μ l of 10 mM JA in 50 mM sodium phosphate buffer (pH = 8). The differences in parasitism among the varieties were determined by Fieldman rank sum test



varieties within a particular group (IR64 vs. IR26 or Bing 97-59 vs. Xiushui 63), they showed no preference (Fig. 3). However, in two cases, significant preferences were observed between varieties from different groups (Fig. 3). Parasitoids preferred TN1 (group 1) and Bing 97-59 (group 3) over IR26 (group 2), but were equally attracted to TN1 and Bing 97-59 (Fig. 3).

Field Experiment

In the first field experiment, no significant difference was found in parasitism of *N. lugens* eggs on Shanyou 63 plants that were surrounded by JA-treated plants of different varieties (Fig. 4A). However, in the second experiment, there were significant differences (Fig. 4B). These reflected the differences in attractiveness of the varieties that had been observed in the olfactometer experiments (Fig. 3). Parasitism was highest on plants that were surrounded by TN1, followed by those on plants that were surrounded by Bing 97-59 (85.3% of that surrounded by TN1) and Xiushui 63 (76.0% of TN1), and lowest on plants surrounded by IR26 (50.4% of TN1) and IR64 (54.2% of TN1) (Fig. 4B).

Discussion

Varietal or genotypic differences in herbivore-induced volatiles have been recorded for several plant species (Loughrin et al., 1995; Turlings et al., 1998; Krips et al., 2001; Degen et al., 2004). Here, we show such differences in induced profiles among rice varieties (Table 2; Fig. 2). The assignment of the varieties to different groups on the basis of the induced volatile profiles (Table 1; Fig. 2) is consistent with the genetic backgrounds of these varieties (Table 1). For instance, varieties Bing 97-34, Bing 97-59, and Xiushui 63, all of which have a Bing 8961 parent in their pedigrees, shared similar profiles (Fig. 2). The sharing of parents such as IR24 and TKM6, which is the case for IR26 and IR64, also resulted in similar profiles, confirming that the volatiles emitted from varieties are genetically determined.

Volatiles released from both TN1 and Bing 97-59 were more attractive to the parasitoid than those from variety IR26, whereas TN1 and Bing 97-59 were equally attractive (Fig. 3). Moreover, the parasitoid did not distinguish between the volatiles from IR26 and IR64, and those from Bing 97-34 and Bing 97-59 (Fig. 3). A comparison of their profiles (Table 2) does not provide much insight into which compounds may have been responsible for the observed differences in attractiveness. Linalool is a candidate that is attractive to *A. nilaparvatae* (Lou, 1999). 2-Heptanone, 2-heptanol, and methyl salicylate seem less likely to be important because they were released in considerable amounts by some of the least attractive varieties. The importance of specific plant volatiles for attraction of natural enemies is documented. For example, the lacewing (*Chrysopa carnea*), a predator of small insects, was caught more in traps containing β -caryophyllene than in traps that contained caryophyllene oxide, bisabolene, or limonene (Flint et al., 1979). When exposed to individual chemicals in an olfactometer, the predatory mite *Phytoseiulus persimilis* was attracted to linalool, methyl salicylate, (*E*)- β -ocimene, and (3*E*)-4,8-dimethyl-1,3,7-nonatriene (Dicke et al., 1990). (*E*)- α -Bergamotene increased *Manduca sexta* egg predation rates on *Nicotiana attenuata* by a generalist predator *Geocoris pallens* (Kessler and Baldwin, 2001). Recently, Kappers et al. (2005) demonstrated that the predatory mite *P. persimilis* is attracted by (*E*)-nerolidol or (3*E*)-4,8-dimethyl-1,3,7-nonatriene released from transgenic *Arabidopsis thaliana* plants. Similarly, transformation of *Arabidopsis* with a sesquiterpene synthase gene from maize resulted in the constitutive release of a blend of sesquiterpenes, which made the transformed plant attractive to a parasitoid of lepidopteran larvae, but the parasitoid had to learn the volatiles during an oviposition experience (Schnee et al., 2006). In general, the identities of key attractants for parasitoids remain largely unknown (D'Alessandro and Turlings, 2006), and common herbivore-induced compounds may not be as important for attraction as usually assumed (D'Alessandro and Turlings, 2005).

We have previously shown that parasitism rates of *N. lugens* eggs by *A. nilaparvatae* on plants surrounded by JA-treated plants are significantly higher than those surrounded by control plants (Lou et al., 2005a). Here, we compared the effect of JA-treated plants of various rice varieties on parasitism. The clear and significant differences found in the second field experiment nicely matched the differences in attraction of the parasitoid that were found in the olfactometer bioassay. The average percentage parasitism (55.82 ± 2.63) in the first field experiment was significantly higher than that (38.29 ± 2.27) in the second time ($t = 5.05$, $df = 48$, $P < 0.001$). This suggests a higher population density of the parasitoid in the field during the first field experiment. Parasitoids may interfere with each other intraspecifically or interspecifically, and the degree of interference increases with the increase in population density. Parasitoids and predators may also use volatiles to avoid intraspecific or interspecific competition (Janssen et al., 1995, 1997; Fatouros et al., 2005). Therefore, stronger interference among parasitoids under high population density might explain why there was no difference in parasitism rates among varieties in the first field experiment. From the results of the second experiment, however, it is clear that rice varieties with appropriate volatile profiles might enhance the effectiveness of the parasitoid in the field.

Many parasitoids have a keen ability to learn to respond to a specific odor by associating the odor with hosts or host feces (Lewis and Tumlinson, 1988; Vet and Groenewold, 1990; Turlings et al., 1993b). The parasitoids used here had emerged from *N. lugens* eggs on plants of variety Shanyou 63, and they had been in contact with this host plant before they were tested. The field experiments with potted plants were conducted in fields with the variety Jialeyou 2, on which the parasitoids would also have had experiences during the experiment. It is possible that the observed differences in attraction and parasitism were due to similarities or differences in volatile profiles between the *N. lugens*-infested rice varieties

that the wasps experienced and the JA-treated experimental plants. We did not compare the volatile profiles of these varieties, and an effect of learning cannot be ruled out. This does not interfere, however, with the general conclusion that differences in volatile profiles and preferences for certain odors can be used to enhance the parasitism rate of *A. nilaparvatae*.

Only few studies have demonstrated the importance of herbivore-induced odor emissions outside the laboratory (Scutareanu et al., 1997; De Moraes et al., 1998; Thaler, 1999; Kessler and Baldwin, 2001; Lou et al., 2005a; Rasmann et al., 2005). One of those studies (Rasmann et al., 2005) also shows that the effectiveness of the natural enemies in the field can be enhanced by planting crop varieties that emit the appropriate volatile signal(s) from their roots. The results reported here represent a first example of how varietal differences in leaf volatiles affect tritrophic interactions in the field, implying that induced rice volatile emissions can be manipulated to enhance the control potential of egg parasitoids.

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Key words *Adelges tsugae* · chemical indicators · GC/MS · eastern hemlock · hemlock woolly adelgid · phenology · plant resistance · SPME · terpenes · terpenoids · *Tsuga canadensis*

Introduction

The genus *Tsuga* (hemlock trees) consists of nine species, two in eastern North America, two in western North America, and five in Asia (Farjon, 1990). The Asian and western North American hemlock species are considered resistant to the hemlock woolly adelgid, *Adelges tsugae* Annand, whereas the eastern North American species are susceptible to feeding by the adelgid, resulting in a slow decline in tree health and eventual tree death (McClure et al., 2001). The hemlock woolly adelgid (HWA) is an introduced pest to North American hemlock. The predominant eastern North American species is *T. canadensis* (L.), with pockets of *T. caroliniana* Engelm. in the Southern Appalachians. We previously examined the terpenoid profiles of seven species [*T. caroliniana*, *T. canadensis*, *T. chinensis* (Franch.) E. Pritz., *T. diversifolia* (Maxim.) Mast., *T. heterophylla* (Raf.) Sarg., *T. mertensiana* (Bong.) Carriere, and *T. sieboldii* (Carriere)] by solid-phase microextraction (SPME) sampling with gas chromatography/mass spectrometry (GC/MS) analysis in the headspace of a single needle of mature foliage (Lagalante and Montgomery, 2003). Data reduction by principal component analysis (PCA) revealed a “species” component and a “resistance/susceptibility” component. Examination of factor loadings of individual terpenoids within the resistance/susceptibility component revealed 13 terpenoids with statistically significant ($P < 0.05$) factor loadings.

While our previous study provided a foundation for understanding the role of terpenoid chemistry in species level resistance to HWA, this study focused on how the patterns of terpenoids vary in time and space within the susceptible species, *T. canadensis*. Although the needle is the most convenient and consistent place to sample for comparing terpenoid variation among hemlock species, it is not the exact location where the adelgid feeds. The adelgid crawler (a newly hatched nymph that is the only mobile stage on eastern hemlock) settles at the base of the needle and inserts a stylet bundle (a feeding tube three times its body length) just below the leaf abscission layer into the leaf cushion. It technically feeds on nutrients in the stem wood and the xylem ray parenchyma tissue rather the leaf needle (Young et al., 1995). The needle possesses a single resin canal along the ventral side of the midrib; however, defined resin canals are absent in the woody tissue (Farjon, 1990). Therefore, it is important to compare the terpenoid chemistry in the needle and the leaf cushion.

The lifecycle of the HWA consists of two parthenogenetic generations per year on hemlock. The spring generation (progrediens) develops between March and June, and the overwintering generation (sistens) develops between June and March, with overlap of the two generations in the late spring. Between June and mid-July, hatched crawlers of the sistens generation settle preferentially at the base of the current year’s growth needles and insert their stylet bundles into the immature leaf cushion. They do not feed during this period; instead, they enter summer diapause (estivation). In October, when the weather cools, estivation is broken and feeding commences, and the sistens’ nymphs mature over the winter to produce the progredien generation the following spring (McClure, 1987). The progredien generation crawlers settle preferentially on the same age tissue on which their sistens’ parents fed. Generally, both generations of the adelgid feed on foliage that is mature, but less than 14 mo old at the tip of the branch. In the late spring and early summer, the sistens crawlers

move onto the new foliage, settle, and enter diapause. Thus, there are “winter” and “spring” feeding periods on mature foliage separated by a “summer” nonfeeding period on immature foliage.

The current study examined how the terpenoids in the foliage of *Tsuga canadensis* vary: (1) temporally during three feeding periods of the HWA, (2) spatially between the new growth and the previous year’s growth, and (3) spatially between the needle and leaf cushion tissues. The spatial and temporal results obtained are interpreted in relation to the settling preferences and feeding of the HWA on eastern hemlock.

Methods and Materials

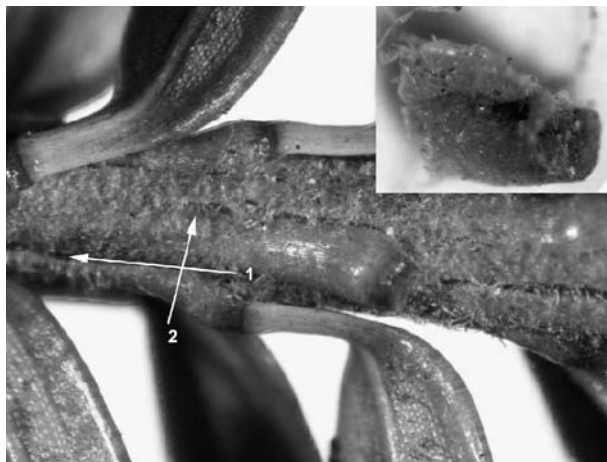
Plant Material Samples were collected from June 2003 to May 2004 from a hemlock stand located at Lake Scranton (41.3858°N, 75.6286°W) in Scranton, PA, USA. The area around Lake Scranton possesses hemlock stands that span the entire range of HWA population densities, from dense conspicuous infestations where branches are covered with white ovisacs to stands that are visually uninfested. A stand of healthy hemlock with a light infestation (possessing only a few visible ovisacs scattered among trees in the stand) on the eastern shore of the lake was selected as a sampling site. This particular stand was selected because the vast majority of branches were devoid of adelgid or other pests, yet a neighboring stand (approximately 0.5 km closer to the lakeshore) possessed moderate to heavy levels of adelgid where we could observe the settling behavior of the crawlers of the 1st instar nymph stage. Thus, our analytical results are based on the terpenoid present in healthy hemlock, presumably devoid of induced host-plant responses from adelgid wounding.

Hemlock trees, approximately 10 m in height, were tagged within the sampling site, and the peripheral 10 cm of foliage was periodically clipped from branches lying approximately 2 m above ground level. Due to time constraints imposed by the analytical method, one of the six trees was sequentially sampled each day to provide a single branch clipping for analysis. The clipped branch was immediately placed in a polyethylene bag in a cooler on ice packs and taken to the laboratory. Dissection and analysis were initiated <1 hr postsampling. Although the buds started opening in mid-May 2003, needle dissections were not possible on the tiny (3 mm), pliant, bright green foliage. Thus, sampling was delayed until June when the shoot had elongated to an average of 2 cm and the needle length had reached 10 mm. Initially, about 5 samples were collected each week, with the sampling frequency decreasing in November when the 2003 foliage had visually matured (turned dark green). Sampling was terminated when bud break occurred for the 2004 year’s growth in May.

Sample Preparation On each sampling date, 6 samples were analyzed from one hemlock twig; 2 samples were needles representing new growth (2003), 2 samples were needles representing the previous year’s growth (2002), 1 sample comprised 5 combined, new growth (2003) leaf cushions, and 1 sample comprised 5 combined, previous year’s growth (2002) leaf cushions. The samples were collected, prepared, and analyzed for terpenoids on the same day. This method minimized extraneous influences on comparisons between the two types of tissue and the two foliage ages.

We define the twig as a branch tip consisting of the linear length from the tip back to the start of the previous year’s growth, ignoring any lateral shoots. The needle and leaf cushion (pulvinus) are separated by an abscission layer (Fig. 1). In hemlocks, as in most conifers,

Fig. 1 Photograph indicating the cuts performed in the leaf cushion dissection. Arrows indicate the direction of cuts and numbers are referred to in the text. A removed leaf cushion is shown in the inset picture



the leaf or needle consists of the blade and a very short petiole. During the sampling period, the abscission layer was distinct in both young and mature tissue. The leaf cushion is the thickened base below the abscission layer that remains after the leaf falls off the stem. The leaf cushion is technically part of the woody stem tissue and contains the nutrient laden ray parenchyma cells that the adelgid penetrates with its stylet bundle during feeding.

Under a stereoscope at 40 \times , 2 needles from the 2002 growth and 2 from the 2003 growth were broken cleanly away from the leaf cushion at the leaf abscission layer with a stainless steel scalpel. Using the same shoot from which the needles were taken, 5 needles representing the previous year's growth (2002) and 5 representing new growth (2003) were selected for leaf cushion dissection. The needles were removed at the leaf abscission layer as described above. The needle portion was discarded, and two cuts were made with a stainless-steel scalpel (Fig. 1). The first cut (arrow 1) was made along the twig from the distal to the basal end of the leaf cushion. The second (arrow 2) was made perpendicular to the first cut at the bend denoting the base of the leaf cushion. On the mature previous year's growth, the leaf cushion is distinctly darker brown than the light-tan stem, thus allowing a visual cue for the second cut. On immature needles, the distinct visual color difference does not exist, and so the placement of the cut was made at the same relative angle and position used for dissecting mature tissue. The five leaf cushions from new growth (2003) and five cushions from previous year's growth (2002) were collected in two separate 4-ml screw-top vials and capped with PTFE/silicone septa (VWR Scientific, Pittsburgh, PA, USA). Five leaf cushions were necessary to produce a measurable terpenoid profile, owing to the reduced amount of plant volatiles in the leaf cushion as compared to the needle that contains a foliar canal where the resins are concentrated.

SPME/GC/MS Analysis We used a headspace SPME sampling procedure for both the needle and leaf cushion analyses (Lagalante and Montgomery, 2003). The GC/MS conditions were identical except a splitless injection was used for the individual sample containing 5 leaf cushions. The same 51 compounds previously quantified by Lagalante and Montgomery (2003) were identified from a mass spectrum database search (Varian NIST MS database, 1998, and IMS terpene library, 1992), and on the basis of their measured retention indices as compared to the retention indices reported using an equivalent DB-5 column (Adams, 2001). The area under an identified peak was integrated

by using a single m/z fragment from the total ion spectrum for each compound. The m/z fragment was the most intense ion in the mass spectrum that was provided from our previously published work. Relative, as opposed to absolute, amounts (area percent) of an individual compound were calculated from the ratio of the peak area for an individual compound, relative to the total peak area for all identified compounds in a chromatogram.

On a given day, the SPME/GC/MS analyses were performed on 6 samples from a single twig as described above. Fifty-one terpenoids were measured for each of the 6 samples on each sampling day. Of the 51 terpenoids, 28 were present in amounts that averaged <0.1%, and these were not included in the statistical analysis.

Statistical Analysis There were 11 sampling days during the first “spring” period (June 10 to July 14, 2003), 10 sampling days during the second “summer” period (July 15 to October 14, 2003), and 15 sampling days during the third “leaf-off” period (October 15, 2003 to May 10, 2004). This division of periods is made to correspond to distinct aspects of the adelgid’s life history on hemlock: spring, summer, and leaf-off. Spring is when the progrediens generation crawlers select feeding sites, generally settling on the current year’s growth and immediately feeding and completing development in about 2 mo. Summer is when the sistens generation crawlers settle on the new, current year growth but enter diapause. Leaf-off is when the sistens generation feeds and develops.

We used a three-way ANOVA in the general linear model (GLM) procedure of Systat 11 (Systat Software, Richmond, CA, USA) to examine influence of the three factors: (1) *tissue* (needle or leaf cushion), (2) *age* [current year (2003) or previous year (2002) growth], and (3) *period* (spring, summer, leaf-off) and their interactions. Each twig sample within the seasonal periods was treated as a block, and, thus, twig is nested within tissue. *F*-tests were performed on the main effects and the interactions and *P* values were evaluated at the 0.05 significance level (95% confidence interval). Systat Software was also used to conduct PCA on the symmetric correlation matrix of the terpenoids in order to collapse the variability into a few components so that meaningful relationships could be identified.

Classical multidimensional scaling (CMDS) was used to calculate distances among the four “spatial” components (two ages and two tissues) for each twig sample by using all the terpenoids listed in Table 1. The distance matrix was calculated by using a cityblock measure (Manhattan distance) and the *pdist*, *eigval*, and *cmdscale* commands with Matlab® Statistics Toolbox 5.2 software (MathWorks, Natick, MA, USA). This analysis was then separated into the period components to show how the “spatial” relationships change temporally. The scaling analysis was repeated by using global nonmetric multidimensional scaling (GNMDS) and Bray–Curtis coefficient distance to ensure that the obtained results were consistent with the CMDS procedure, and not dependent on the type of MDS or distance variable used. Since the results of the GNMDS agreed with those of the CMDS, they are not included here.

Results

Visually, the new growth progressed from a bright green, pliant needle in May to a dark green, hardened needle in November. By November, the new, current year’s growth of both the needle and leaf cushion were visually identical to the previous year’s growth needles and leaf cushion. The twig also had matured and hardened, although it was not nearly as hard as the previous year’s growth. This visual maturation coincides with the sistens generation crawler breaking estivation and commencing feeding.

Table 1 Terpenoid percentages (Least squares means and Standard errors) and significance tests of the three factors: Age (Previous or Current year's growth), Period of the year (Spring, Summer, Leaf-off) and Tissue (Needle or Leaf cushion)

Period	Spring		Summer		Leaf-off		Spring		Summer		Leaf-off		Age × Period × Tissue <i>F</i> (<i>P</i>)	Age × Period × Tissue <i>F</i> (<i>P</i>)	Age × Period × Tissue <i>F</i> (<i>P</i>)			
	Needle (<i>N</i> =11)	Leaf cushion (<i>N</i> =11)	Needle (<i>N</i> =10)	Leaf cushion (<i>N</i> =10)	Needle (<i>N</i> =15)	Leaf cushion (<i>N</i> =15)	Needle (<i>N</i> =11)	Leaf cushion (<i>N</i> =11)	Needle (<i>N</i> =10)	Leaf cushion (<i>N</i> =10)	Needle (<i>N</i> =15)	Leaf cushion (<i>N</i> =15)						
Age	Previous year's growth foliage												<i>df</i> =1	<i>df</i> =2	<i>df</i> =2	<i>df</i> =2		
Tissue	Current year's growth foliage												<i>df</i> =1	<i>df</i> =2	<i>df</i> =2	<i>df</i> =2		
Tricyclene	3.47 (0.36)	1.56 (0.36)	4.06 (0.38)	1.58 (0.38)	3.60 (0.31)	1.57 (0.33)	5.13 (0.36)	3.07 (0.36)	4.93 (0.38)	2.50 (0.41)	4.90 (0.33)	1.69 (0.36)	1.33 (0.268)	25.80 (0.000)	1.68 (0.192)	1.05 (0.308)	1.70 (0.199)	0.89 (0.415)
α-Pinene	10.04 (0.98)	13.04 (0.98)	12.22 (1.03)	10.90 (1.03)	11.46 (0.84)	6.48 (0.88)	15.04 (0.98)	8.45 (0.98)	15.07 (1.03)	6.94 (1.10)	13.02 (0.88)	4.10 (0.97)	11.26 (0.654)	0.20 (0.654)	0.16 (0.850)	36.33 (0.000)	7.48 (0.001)	2.29 (0.107)
Camphene	8.48 (0.50)	2.98 (0.50)	9.68 (0.52)	3.15 (0.52)	8.97 (0.43)	3.85 (0.45)	10.09 (0.50)	5.68 (0.50)	11.07 (0.52)	4.49 (0.56)	9.66 (0.45)	3.43 (0.49)	1.59 (0.000)	18.25 (0.000)	4.68 (0.012)	0.00 (0.979)	2.44 (0.093)	1.34 (0.266)
Sabinene	0.61 (0.17)	0.36 (0.17)	0.66 (0.18)	0.36 (0.18)	0.40 (0.15)	0.66 (0.16)	0.45 (0.17)	0.09 (0.17)	0.36 (0.18)	0.42 (0.19)	0.24 (0.16)	0.54 (0.17)	0.27 (0.107)	2.65 (0.107)	1.19 (0.767)	0.09 (0.28)	3.34 (0.625)	0.44 (0.040)
β-Pinene	0.81 (0.15)	1.24 (0.15)	1.35 (0.16)	1.32 (0.16)	1.48 (0.13)	0.87 (0.14)	2.47 (0.15)	0.55 (0.15)	2.13 (0.16)	0.67 (0.17)	1.69 (0.14)	0.94 (0.15)	0.70 (0.011)	8.82 (0.011)	2.06 (0.133)	55.41 (0.000)	0.06 (0.938)	14.67 (0.000)
Myrcene	3.00 (1.40)	21.07 (1.40)	2.53 (1.47)	19.81 (1.47)	1.79 (1.20)	2.72 (1.27)	2.09 (1.40)	0.48 (1.40)	1.80 (1.47)	2.59 (1.58)	1.12 (1.26)	0.61 (1.40)	19.25 (0.000)	76.44 (0.000)	51.78 (0.000)	60.54 (0.000)	12.98 (0.000)	13.43 (0.000)
α-Phellandrene	0.14 (0.15)	0.76 (0.15)	1.17 (0.16)	1.06 (0.16)	1.00 (0.13)	0.75 (0.13)	2.04 (0.15)	0.43 (0.15)	1.72 (0.16)	0.37 (0.17)	1.03 (0.13)	0.57 (0.15)	3.21 (0.045)	6.24 (0.014)	11.13 (0.000)	51.55 (0.000)	1.58 (0.212)	12.67 (0.000)
o-Cymene	0.00 (0.11)	0.19 (0.11)	0.05 (0.12)	0.19 (0.12)	0.15 (0.10)	0.24 (0.10)	0.47 (0.11)	0.76 (0.11)	0.34 (0.12)	0.55 (0.12)	0.33 (0.10)	0.09 (0.11)	20.17 (0.000)	20.17 (0.000)	3.21 (0.137)	5.85 (0.004)	0.16 (0.690)	2.59 (0.081)
Limonene	0.77 (0.17)	0.69 (0.17)	1.73 (0.18)	0.64 (0.18)	2.06 (0.14)	1.09 (0.15)	2.32 (0.17)	0.44 (0.17)	2.33 (0.18)	0.76 (0.19)	1.94 (0.15)	1.34 (0.17)	14.18 (0.000)	14.18 (0.000)	113.4 (0.000)	3.28 (0.042)	10.88 (0.001)	11.50 (0.076)
β-Phellandrene	0.49 (0.11)	0.37 (0.11)	1.06 (0.12)	0.36 (0.12)	1.84 (0.10)	0.92 (0.10)	1.41 (0.11)	0.25 (0.11)	1.38 (0.12)	0.21 (0.13)	1.67 (0.10)	1.07 (0.11)	55.23 (0.000)	6.12 (0.015)	3.78 (0.024)	9.63 (0.003)	1.60 (0.207)	10.26 (0.000)

<i>cis</i> -Ocimene	0.51 (0.20)	1.01 (0.20)	1.73 (0.21)	1.20 (0.21)	1.15 (0.17)	0.61 (0.18)	2.73 (0.20)	0.63 (0.20)	2.64 (0.21)	0.37 (0.22)	1.07 (0.18)	0.61 (0.20)	7.21 (0.009)	10.10 (0.000)	60.93 (0.000)	7.30 (0.001)	38.71 (0.000)	5.13 (0.008)	13.03 (0.000)
Terpinolene	0.09 (0.04)	0.14 (0.04)	0.38 (0.04)	0.18 (0.04)	0.46 (0.03)	0.23 (0.03)	0.46 (0.04)	0.09 (0.04)	0.44 (0.04)	0.11 (0.04)	0.35 (0.03)	0.16 (0.04)	0.83 (0.305)	8.82 (0.000)	95.32 (0.000)	11.89 (0.000)	15.70 (0.000)	1.69 (0.019)	9.36 (0.000)
Borneol	1.32 (0.51)	0.77 (0.50)	0.19 (0.53)	0.24 (0.53)	3.94 (0.43)	2.87 (0.46)	2.43 (0.51)	1.21 (0.51)	1.28 (0.53)	0.71 (0.57)	6.19 (0.45)	1.91 (0.50)	6.40 (0.013)	43.17 (0.000)	19.00 (0.000)	0.02 (0.978)	6.62 (0.012)	6.45 (0.002)	2.38 (0.098)
Piperitone	0.03 (0.42)	0.92 (0.42)	1.93 (0.45)	1.33 (0.45)	4.67 (0.36)	4.92 (0.38)	4.45 (0.42)	2.62 (0.42)	5.20 (0.45)	2.14 (0.48)	5.88 (0.38)	5.48 (0.42)	66.91 (0.000)	71.07 (0.000)	10.42 (0.002)	7.28 (0.001)	15.90 (0.000)	4.52 (0.013)	1.96 (0.146)
Isobornylacetate	49.28 (2.49)	21.98 (2.49)	49.53 (2.61)	29.53 (2.61)	46.65 (2.13)	48.52 (2.24)	36.60 (2.49)	53.19 (2.49)	39.43 (2.61)	50.96 (2.79)	40.87 (2.23)	55.84 (2.47)	13.48 (0.000)	11.31 (0.000)	10.7 (0.786)	3.27 (0.043)	106.9 (0.000)	10.31 (0.000)	10.78 (0.000)
β -Caryophyllene	2.60 (0.25)	1.74 (0.25)	1.97 (0.27)	2.19 (0.27)	1.41 (0.22)	3.45 (0.23)	1.60 (0.25)	2.08 (0.25)	1.43 (0.27)	3.16 (0.28)	1.36 (0.23)	2.66 (0.25)	1.48 (0.226)	0.83 (0.438)	31.35 (0.000)	1.80 (0.171)	5.85 (0.018)	14.66 (0.000)	6.69 (0.002)
β -Gurjunene	0.16 (0.07)	0.26 (0.07)	0.08 (0.08)	0.14 (0.07)	0.14 (0.06)	0.33 (0.07)	0.16 (0.07)	0.10 (0.07)	0.07 (0.08)	0.09 (0.08)	0.05 (0.06)	0.30 (0.07)	1.82 (0.180)	0.24 (0.096)	4.78 (0.031)	0.15 (0.860)	0.34 (0.561)	2.60 (0.080)	0.607 (0.547)
α -Humulene	6.22 (0.56)	4.18 (0.56)	4.80 (0.59)	5.80 (0.59)	3.82 (0.48)	9.76 (0.51)	3.74 (0.56)	7.41 (0.56)	3.59 (0.59)	8.36 (0.64)	4.02 (0.64)	9.80 (0.56)	1.46 (0.229)	8.45 (0.000)	95.74 (0.780)	0.025 (0.000)	22.94 (0.000)	21.89 (0.000)	7.80 (0.001)
γ -Muurolene	0.23 (0.34)	0.44 (0.34)	0.18 (0.35)	0.46 (0.35)	0.60 (0.29)	1.11 (0.30)	0.62 (0.34)	1.93 (0.34)	0.42 (0.35)	1.06 (0.38)	0.36 (0.30)	0.71 (0.33)	3.27 (0.074)	0.62 (0.538)	8.07 (0.006)	3.94 (0.023)	1.24 (0.268)	0.29 (0.745)	0.96 (0.386)
Germaacrene D	8.99 (2.44)	21.27 (2.44)	2.51 (2.56)	16.42 (2.56)	2.26 (2.09)	3.20 (2.20)	2.02 (2.44)	4.28 (2.44)	1.16 (2.56)	8.07 (2.75)	1.44 (2.19)	2.81 (2.43)	17.17 (0.000)	8.67 (0.000)	19.82 (0.000)	5.99 (0.004)	3.87 (0.052)	3.86 (0.025)	1.35 (0.264)
Viridiflorene	0.04 (0.03)	0.12 (0.03)	0.08 (0.04)	0.12 (0.03)	0.05 (0.03)	0.21 (0.03)	0.14 (0.03)	0.12 (0.03)	0.15 (0.04)	0.16 (0.04)	0.09 (0.03)	0.07 (0.03)	0.84 (0.361)	0.52 (0.597)	4.68 (0.033)	3.59 (0.032)	8.73 (0.004)	0.60 (0.548)	1.36 (0.261)
γ -Cadinene	0.68 (0.16)	0.82 (0.16)	0.54 (0.17)	0.88 (0.17)	0.52 (0.14)	1.89 (0.15)	0.83 (0.16)	1.62 (0.16)	0.78 (0.17)	1.78 (0.18)	0.68 (0.15)	1.67 (0.16)	12.75 (0.001)	2.07 (0.132)	66.48 (0.000)	4.23 (0.017)	2.71 (0.103)	5.45 (0.006)	3.65 (0.030)
δ -Cadinene	1.24 (0.27)	1.23 (0.27)	0.80 (0.28)	1.46 (0.28)	0.68 (0.23)	2.05 (0.24)	1.27 (0.27)	2.96 (0.27)	1.21 (0.28)	3.16 (0.30)	1.10 (0.24)	2.26 (0.26)	24.09 (0.000)	0.44 (0.644)	54.60 (0.000)	2.24 (0.113)	9.09 (0.003)	0.94 (0.396)	3.91 (0.023)

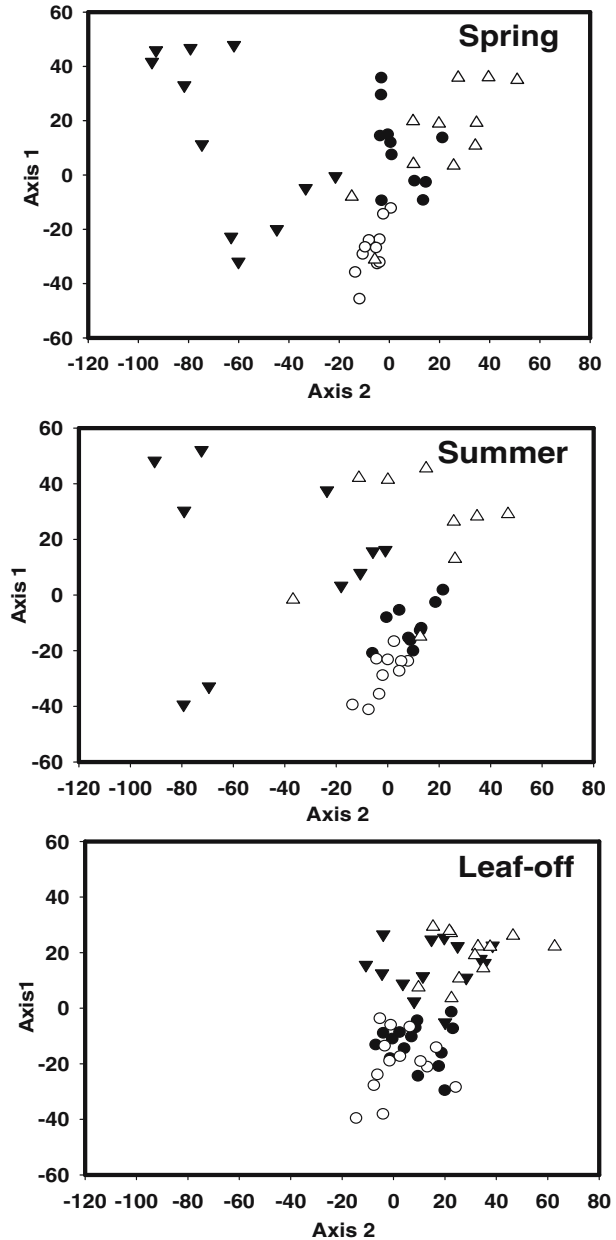
Table 1 summarizes the mean values (with standard error) of the percentages and the statistical tests associated with the variables measured for the terpenoids. Additional terpenoids not listed in Table 1 were present at levels below 0.1% and could not be reliably quantified. There were significant main effects and interactions for most of the measurable terpenoids. The data are complex and patterns are not readily visualized. For instance, isobornyl acetate, the most abundant terpenoid, is at higher levels in the new growth needles and at lower levels in the new growth leaf cushions during the spring and summer periods. During the leaf-off period when the foliage has visually matured, the percentages of isobornyl acetate are similar in both the needles and cushions in both the current and previous year's growth. The statistical tests indicate that the needle and leaf cushion tissues are not different generally, but do differ from each other within age and period. Hence, the isobornyl acetate content in the needles and leaf cushions depends on the age of the foliage and the time of the year. Although inferences can be drawn from Table 1 for other terpenoids by visual inspection, ordination analysis provided a more objective and powerful method to discern the terpenoid inter- and intrarelations.

CMDs was used to assess the relationships among the two tissues and two age growth classes in each of the three time periods, based on the composition of all 23 terpenoids (Fig. 2). This approach demonstrates three key patterns relating host chemistry and adelgid feeding. First, the ordination shows that the chemical composition of both old and new needles is similar, and that the variation in chemical composition within and between both groups is relatively small. In other words, needles vary little spatially or temporally. This is not the case for leaf cushions. For the leaf cushion, there are two patterns. The leaf cushions are widely scattered and in separate groups, and this changes with the time period. The widely scattered points for the leaf cushions in both the spring and summer periods indicate that the adelgid crawlers of both generations encounter a highly unpredictable chemical mixture. In the last period, by leaf-off, the scatter for the leaf cushions is greatly reduced and data points coincide with those for the needles.

PCA was used to identify which individual terpenoids are varying in similar patterns and which may be phytochemically important. The correlation matrix was analyzed by using the 23 terpenoids in the 138 tissue and age samples as variables for the PCA. Next, dummy variables for the age, period, and tissue factors were introduced in order to more easily identify which factors were influencing the component loadings. Introducing the dummy variables had little influence on the loading of the terpenoids. The initial run produced six latent roots (eigenvalues) greater than 1.0 and explained 73% of the total variance. It was difficult to find meaning in so many components, so the components were limited to three (Table 2). First, second, and third components accounted for 30.3%, 14.3%, and 9.7% of the total variance, respectively (54.7% total).

The first component (Table 2) seems to be most associated with the tissues. There are nine terpenoids with high, positive loadings (>0.5). Table 1 indicates that these are generally higher in the needles than in the leaf cushions. There are four terpenoids with negative loading (<-0.5), and these have higher levels in the leaf cushions than in the needles. This pattern among these fourteen terpenoids with positive or negative high loadings is often reversed in the current growth tissues in the spring period. This was identified in the second component, which is interpreted as the interaction between the age and period factors. There are high, positive loadings for isobornyl acetate and piperitone, and high, negative loadings for myrcene and germacrene D. The third component represents a weak relationship that may be represented by the interaction among all three factors in the statistical model (age, period, tissue). The high positive loading terpenoids (*o*-cymene, viridiflorene, δ -cadinene, and γ -cadinene) have higher percentages in leaf

Fig. 2 Classical multidimensional scaling ordination as a function of the terpenoid composition of each sample: 2002 leaf cushion (Δ), 2003 leaf cushion (\blacktriangledown), 2002 needle (\circ), and 2003 needle (\bullet). The positions were simultaneously calculated for all samples and then separated into time periods of June 6–July 14 (spring), July 15–October 14 (summer), and October 15–May 10 (leaf-off)



cushions than needles except during the leaf-off period. This pattern is reversed for the high negative loading terpenoids isobornyl acetate and sabinine. Except for isobornyl acetate, the terpenoids with the higher loadings in this component are generally present in the samples at <1.0%. The importance of these low-level terpenoids is uncertain because in the PCA analysis the mean is subtracted across the data dimensions; hence, the PCA analysis does not consider the overall level of the terpenoid in the eastern hemlock.

Table 2 Values of component loadings for the three factors and 23 terpenoids on the three components of the PCA

	Components		
	1	2	3
<i>Age</i>	0.126	0.444	0.373
<i>Period</i>	0.060	0.624	-0.231
<i>Tissue</i>	-0.783	-0.076	0.268
Tricyclene	0.697	0.009	0.015
α -Pinene	0.745	-0.449	0.133
Camphene	0.809	0.098	-0.022
Sabinene	0.024	0.013	-0.415
β -Pinene	0.839	-0.056	0.222
Myrcene	-0.064	-0.725	0.088
α -Phellandrene	0.705	0.037	0.362
<i>o</i> -Cymene	0.038	0.033	0.579
Limonene	0.824	0.335	0.120
β -Phellandrene	0.713	0.427	-0.021
<i>cis</i> -Ocimene	0.677	-0.068	0.307
Terpinolene	0.784	0.265	0.130
Borneol	0.127	0.342	-0.104
Piperitone	0.327	0.737	0.111
Isobornyl acetate	-0.264	0.592	-0.510
β -Caryophyllene	-0.583	0.314	0.031
β -Gurjunene	-0.312	0.024	0.342
α -Humulene	-0.731	0.434	-0.067
γ -Muuroloene	-0.301	0.069	0.396
Germacrene D	-0.357	-0.520	0.268
Viridiflorene	-0.166	0.198	0.492
γ -Cadinene	-0.612	0.405	0.468
δ -Cadinene	-0.607	0.299	0.524

We identified six terpenoids considering components 1 and 2 together, and plotted their means in Fig. 3. Here, the focus was on comparison of the tissues on which the adelgid feeds (denoted with an \times) with the other tissues. Although the adelgid does not feed on needles, we hypothesized that we could use them as a means to probe host–insect chemical interactions, as the terpenoid levels in needles are more readily analyzed. Although there was not an exact match between terpenoid percentages between the two tissues, levels were similar. Furthermore, all plots in Fig. 3 show that during adelgid feeding periods in the leaf cushion, the tissue that is being fed upon has a stable percentage of the terpenoid.

Myrcene levels in the leaf cushion, but not the needles, were elevated in the immature foliage shortly after shoot extension. Myrcene dramatically increased to become a dominant terpenoid in the leaf cushion during the period over the summer months and decreased by leaf-off to endogenous levels present in previous year's growth tissues. The other dominant terpenoids in the leaf cushion were isobornyl acetate and germacrene D. In November, the needle took on a dark green color and a dramatic hardening of the cushion was evident. Thus, the visual physical maturation of new growth coincided with the chemical maturation of the new growth as well as with the conclusion of estivation and the beginning of the sistens feeding period. Throughout this time period, myrcene levels in the needles remained equivalent to the current and previous year's growth needles.

The period behavior of the sesquiterpene germacrene D in leaf cushions was similar to that of myrcene in leaf cushions. However, unlike myrcene, slightly elevated levels of

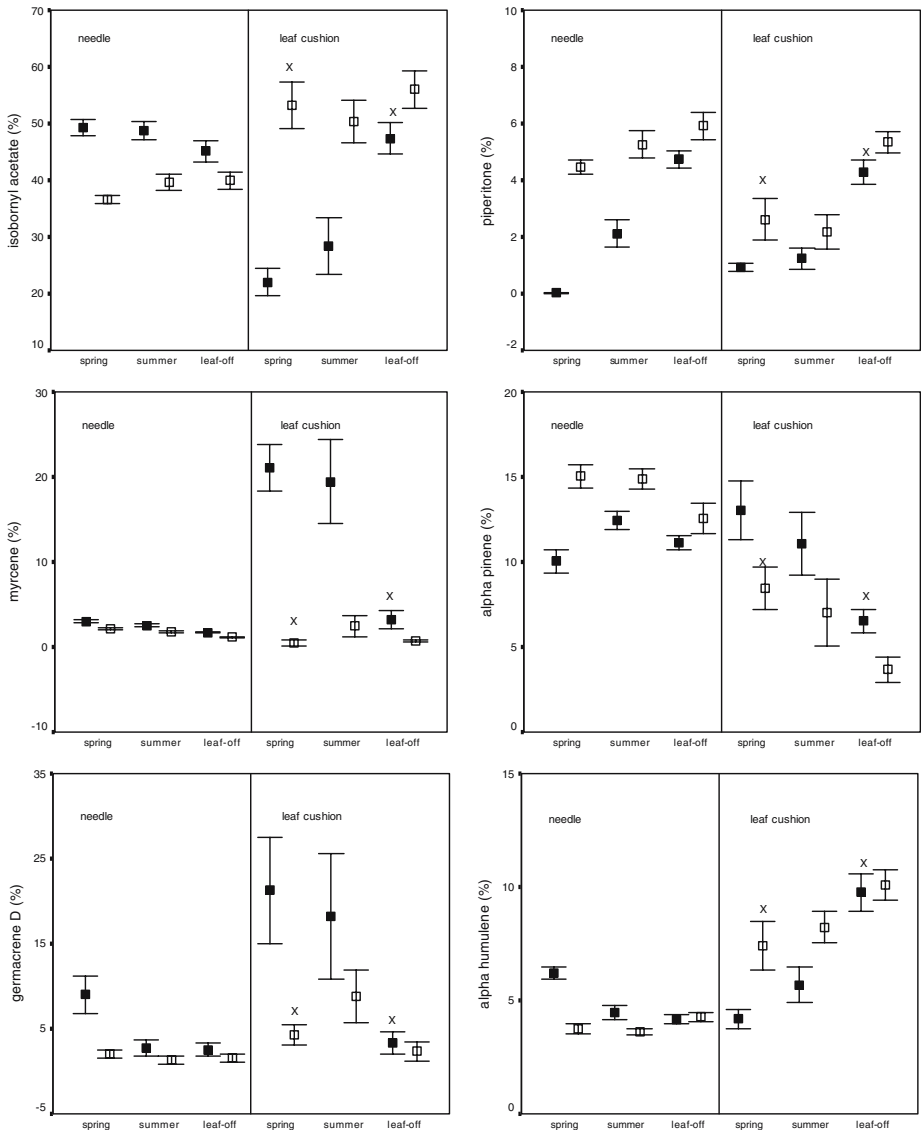


Fig. 3 Select terpenoid percentages (mean±standard deviation) as a function of the period factor grouped according to tissue and clustered by age: current year's (2003) growth (■), previous year's (2002) growth (□). The symbol × indicates a period and tissue fed upon by the hemlock woolly adelgid

germacrene D were also found in needles during the spring period. Once again, by approximately November 2003, the levels of germacrene D decreased to levels that are indistinguishable from the new and previous year's growth in both areas of tissue. The monoterpene, α -pinene behaved similarly to germacrene D, as indicated by its 2nd component PCA loading. As indicated by a positive 1st component loading, α -pinene is slightly more abundant in needles than leaf cushions.

Isobornyl acetate is the predominant volatile chemical constituent of both the needle (40–45%) and leaf cushion (50–55%) in mature tissue. In spring, new growth needle tissue, isobornyl acetate levels are increased relative to old growth needle tissue while the leaf cushion tissue exhibits a depletion in isobornyl acetate compared to previous year leaf cushions. Although at lower relative percentages, α -humulene, and piperitone display depletion in the leaf cushion similar to isobornyl acetate as indicated by their 2nd component PCA loading.

Discussion

The objective of this study was to determine if temporal and spatial variations in percentages of volatile terpenoids in eastern hemlock are linked to the biological life cycle of the HWA. The HWA positions itself distally to the leaf abscission layer but the stylet bundle is inserted proximally to the leaf abscission layer. Electron micrographs reveal that the 1.04- to 1.27-mm stylet bundle penetrates in the proximal direction of the twig and not in the distal direction of the needle tip, terminating in the ray parenchyma cells located within the leaf cushion (Young et al., 1995). A statistically detectable temporal and spatial variation of germacrene D and isobornyl acetate exists within the leaf cushion. In our previous work, PCA identified germacrene D as a compound with a high factor loading in the resistance/susceptibility component of an interspecies comparison among worldwide *Tsuga* (Lagalante and Montgomery, 2003). Comparison of germacrene D levels in the needles among seven species of *Tsuga* revealed that elevated percentages are found in species that are considered resistant to mortality from the HWA as compared to susceptible species (21.65% in *T. mertensiana* and 10.58% in *T. sieboldii*). Also, isobornyl acetate levels in these *Tsuga* species are elevated in the susceptible species. Unlike germacrene D, isobornyl acetate may function as a potential attractant to the HWA.

If these terpenoids have the ability to attract or deter HWA, then settling preferences may be distinguishable between the sistens and progrediens generations of the adelgid. Generally speaking, terpenoids are often toxic or repellant to insects due in part to their ability to inhibit acetylcholinesterase in the neuromuscular junction (Harrewijn et al., 2001). Although monoterpenes do not act on the acetylcholine receptor directly, they can function as competitive inhibitors through binding with acetylcholine. Additionally, HWA, like many herbivorous insects, harbors symbiotic microorganisms that are essential to its survival (Shields and Hirth, 2005). Such symbionts may be susceptible to antibiotic effects of terpenoids.

The crawler phase of the 1st instar of both the sistens and progrediens generations is the only stage of adelgid on hemlock that is capable of selecting feeding sites. Thus, variation in terpenoid levels during each generation's crawler stage may influence settling preferences and survival. For the sistens generation, depending on local climate and elevation, crawler settling generally occurs by mid-July, at the end of the "spring" period. If the sistens crawler settles on new growth, it is likely to encounter a different terpenoid profile in needle and leaf cushion tissue. Depending on the health of the tree, sistens crawlers will settle preferentially on new growth in July (McClure, 1991). However, they do not feed during the summer but instead enter estivation until cooler weather in October when estivation is broken. The adelgid then feeds over the colder winter months and matures to produce up to 300 eggs by April. Following initial high colonization on new growth foliage, the production of the next year's new growth will be severely inhibited, forcing colonization on previous years growth and a subsequent population crash (McClure, 1991).

Most aphids and adelgids avoid mature tissues and feed preferentially on new growth, possibly because older foliage has lower levels of nutrients and higher levels of allelochemicals (Miles, 1990). The primary content of the parenchyma cells of softwoods are steryl esters, fats, and waxes, whereas the canal resin is primarily composed of terpenes and terpenoids (Back, 2002). The salivary sheaths of aphid and adelgid stylet bundles may be able to seal off cell ruptures along the feeding path to avoid plant chemical defenses and wounding responses by absorbing allelochemicals before the undamaged, surrounding cells can be signaled (Miles, 1987, 1990). Since the function of the parenchyma cells is to store metabolites that are mobilized for new growth the following spring, the parenchyma cells of the new growth tissue should not possess high nutrient levels until mid-fall. Thus, the sistens crawlers could not detect high nutrient levels to select an optimal feeding site during stylet probing in July.

Given the general unsuitability of older growth foliage, one might conjecture that a seasonal chemical signal may serve as an indicator of tissue suitability during the period when the adelgid probes leaf cushion tissue with its stylet bundle. The spring emergence of western spruce budworm (*Choristoneura occidentalis* Freeman) larvae has been associated with the annual cycle of select foliar monoterpenes in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) as an indicator, not the cause, of foliage suitability (Nealis and Nault, 2005). Similarly, elevated relative levels of either myrcene or germacrene D detected while probing leaf cushions may be indicators to settle on new growth tissue that will be nutrient-rich during fall. Although myrcene and germacrene D were identified as potential deterrents to HWA in our previous study, the adelgid estives with its stylet inserted, but does not actually feed during summer when these chemicals are elevated; thus, any possible toxic effects are avoided. By the time feeding commences in mid-October, terpenoid levels in new growth tissues have been reduced to background endogenous levels.

Studies linking terpenoid composition to HWA infestation levels are limited to a single reported study in which correlations were made between the volatile emission from eastern hemlock and adelgid population levels between August 20 and August 28, 2001, in a nursery plot in Blacksburg, VA, USA (Broeckling and Salom, 2003). These researchers reported that in respect to the relative percentage of individual terpenoids, there was a stronger correlation with adelgid population levels than with foliage age, although the two variables were not independent of one another. The hemlock trees selected in our study were healthy, displaying no outward signs of even moderate adelgid infestation. Thus, we would not have observed this density-dependent feedback with volatile emissions from the previous year's growth tissues.

Unlike the sistens generation, the progrediens generation 1st instar crawler does not have a choice of settling on new or previous year's growth because all the foliage is mature in March. From April to June, the progrediens generation crawlers will feed in the previous year's growth tissue that has a more stable composition of terpenoids. Interestingly, if the population of overwintering sistens is high on the shoot, the progrediens crawlers appear to survive better on the older growth, more proximal on the shoot. The progrediens crawlers mature to produce approximately 50 eggs that will become the sistens generation. Late hatching progrediens crawlers also have the possibility of settling on new foliage. Unfortunately, we could not sample this new growth (immediately post bud-break in May) due to difficulties in dissecting the pliant, young plant tissue. Therefore, the temporal and spatial variations in terpenoid levels in this study are more open to interpretation for the sistens generation.

Mortality of progrediens crawlers on new growth foliage is in agreement with our previous PCA assertion that identified myrcene and germacrene D as potential chemical deterrents (Lagalante and Montgomery, 2003). Unlike the sistens crawler that settles on

new growth and estivates, a progrediens crawler that settles on new growth immediately begins feeding on the immature leaf cushion and may ingest elevated levels of the potentially toxic myrcene and germacrene D. Although we did not separate enantiomers on a chiral GC column, germacrene D isomers are reported in volatile chemical communications in the aphids *Euceraphis punctipennis* (Zetterstedt) (Francis et al., 2005) and *Therioaphis maculate* (Buckton) (Bowers et al., 1977). (–)-Germacrene D elicited a strong repellent response in *Sitobion avenae* (F.) although (+)-germacrene D isomer elicited no response (Bruce et al., 2005). High concentrations of limonene and myrcene in Douglas-fir deter *A. cooleyi* (Gillette) (Stephan, 1987), and high concentrations of myrcene and piperitone in Sitka spruce [*Picea sitchensis* (Bong.) Carr.] deter several species of aphids (Jackson et al., 1996). Thus, it is reasonable to hypothesize that elevated relative levels of select terpenoids in new growth of eastern hemlock leaf cushion tissue would promote mortality in the progrediens generation of *Adelges tsugae* that settles on new foliage. Likewise, if isobornyl acetate is a chemical attractant for HWA, decreased relative levels in new growth leaf cushions may promote stylet insertion in cushions from the previous year's growth where levels of isobornyl acetate are elevated (Fig. 3). This chemical cue would promote survival of the progrediens generation crawler on previous year's growth, as levels of the all terpenoids are already reduced to endogenous levels during the progrediens crawler stage. The crawler stage of *Adelges piceae* (Ratzeburg) on *Abies balsamea* (L.) continually touch the bark surface with their antennae until settling, suggesting that the crawler is possibly seeking a stimulus from parenchyma cells (Bryant, 1974).

A detailed study of HWA fecundity and population levels as related to settling preferences may further elucidate the role of temporal and spatial chemical variation in levels of terpenoids and their role in resistance/susceptibility to adelgid infestation. Visual maturation of the eastern hemlock foliage was coincident with the levels of all volatile terpenoids reaching the endogenous levels found in the previous year's tissues. We conclude that deviations from endogenous levels for isobornyl acetate, myrcene, and germacrene D have biological implications in relation to the feeding periods of the hemlock woolly adelgid on eastern hemlocks.

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exploring their potential in controlled scientific studies (Lewis et al., 1999). Plant alkaloids are a diverse group of compounds in both structure and function. While there are numerous examples of alkaloids currently utilized in both medicine and industry, considerable potential remains for novel molecules from natural resources.

α -Solasonine and α -solamargine (Fig. 1) are the two dominant steroidal glycoalkaloids (GAs) extracted from plants belonging to the Genus *Solanum* (Ripperger, 1995). Composed of a lipid core and a trisaccharide carbohydrate moiety, they differ only in that α -solamargine terminates in two rhamnose sugars, positioned on the 2' and 4' carbons of the bridging D-glucose sugar, whereas solasonine has a rhamnose and glucose in the 2' and 3' positions of D-galactose, respectively (Fig. 1). α -Solasonine and α -solamargine have shown cytotoxicity against protists, fungi, and various tumor cell lines *in vitro* and *in vivo* (Kupchan et al., 1965; Cham et al., 1987; Fewell et al., 1994; Chataing et al., 1998).

The importance of the carbohydrate moiety in mediating the effects of solasonine and solamargine has been repeatedly confirmed (Roddick et al., 1990, 1992). Studies on cytolytic effects of GAs on Hep3B and Sarcoma 180 tumor cell lines implicated interactions

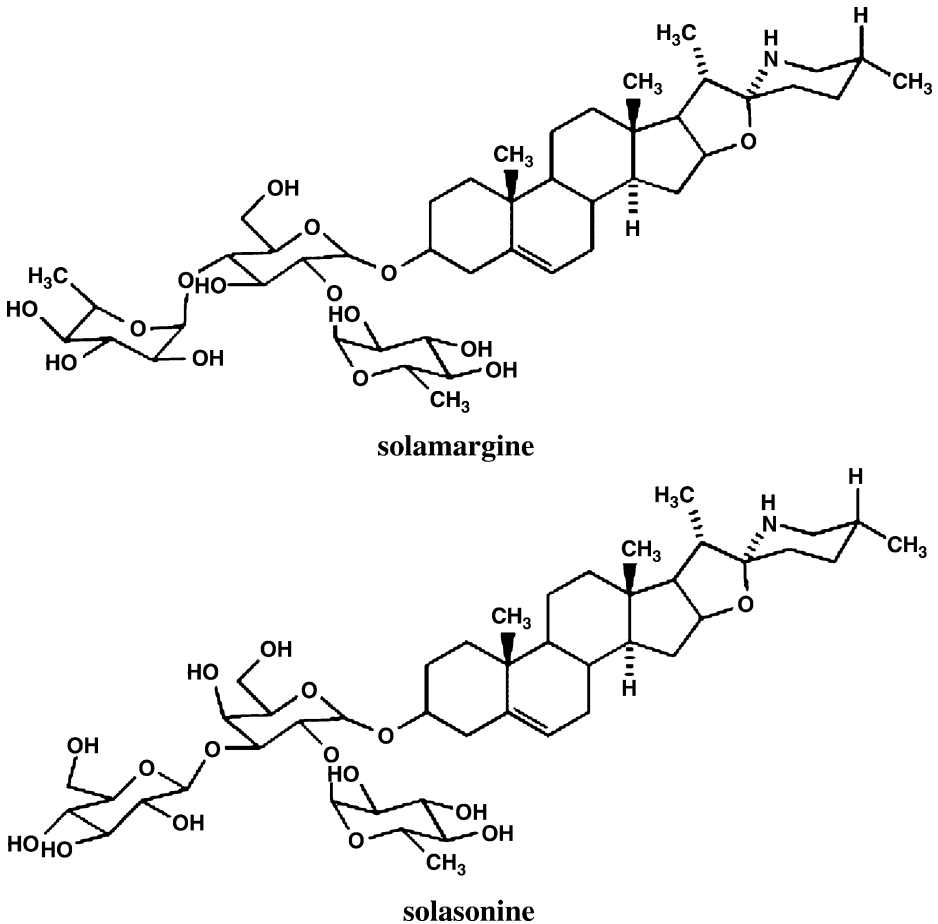


Fig. 1 Structures of solamargine and solasonine

between rhamnose-specific binding proteins (RBPs) on the target cell surface and the terminal rhamnose sugars of the alkaloids (Cham and Daunter, 1990). Separate experiments using the Hep3B cell line found that rhamnose-specific interactions led to the induction of apoptotic mechanisms through tumor necrosis factor (TNF) signaling (Chang et al., 1998). The importance of rhamnose in toxicity against phytoparasitic nematodes has also been confirmed (Udalova et al., 2004).

A 1:1 mixture of α -solasonine and α -solamargine demonstrated synergistic cytotoxic effects on tumor cells (Cham et al., 1987). Similar effects were reported in antifungal studies (Fewell et al., 1994). In the first study, mixtures effectively killed tumor target cells at concentrations significantly lower than with either alkaloid alone. This was taken as an indication of distinct, but cooperative pathways for α -solasonine and α -solamargine function. Experiments testing the efficacy of this 1:1 mixture against S180 sarcoma tumors in mice found an increase in animal survival rate compared to controls deprived of alkaloid (Cham and Daunter, 1990). Subsequent studies reported that the administration of exogenous L-rhamnose significantly reduced survival in S180 challenged mice that received such treatments, suggesting that competitive inhibition of RBPs on the tumor cell surface attenuated the uptake of the alkaloids. A formulation of this compound is currently marketed under the trade name Curaderm as a topical treatment of premalignant and malignant skin cancers in humans (Cham et al., 1987).

Trypanosoma cruzi is a zoonotic hemoflagellate protozoan parasite and the etiologic agent of Chagas disease in humans. Current therapy for *T. cruzi* infection depends on synthetic drugs such as nifurtimox and benznidazole, which are only effective during the acute stage and are frequently associated with severe side effects. One complicating factor in drug development has been that different *T. cruzi* strains and lifecycle stages have not been consistent in their susceptibility to pharmacologic compounds (Chena et al., 2005).

When tested against the EP strain of *T. cruzi* *in vitro*, α -solamargine induced cytolysis of epimastigotes (EMs) (Chataing et al., 1998). In these experiments, α -solasonine was roughly half as effective, suggesting a role for the terminal rhamnose sugars in parasite killing. The same study showed that hydrolysis of the glycosidic residue reduced α -solamargine's anti-parasitic activity, further supporting a role for the carbohydrate moiety in mediating cytolysis. The authors stated that they observed differences in the responses between EMs and bloodstream form trypomastigotes (BSFs) upon exposure to α -solamargine. Although they failed to provide data in support of this statement, they suggested that stage-specific membrane constituents are important in susceptibility to these alkaloids.

This report assesses the efficacy of α -solasonine and α -solamargine against two distinct *T. cruzi* genotypes. We also evaluated lifecycle stage susceptibility over a range of solasonine and solamargine concentrations, as well as the effects of 1:1 compounding on parasite cytolysis. The role of rhamnose in mediating parasite attrition is also investigated through competitive inhibition experiments.

Methods and Material

Alkaloid Purification and Preparation

α -Solasonine and α -solamargine were extracted and purified from the fruits of *Solanum khasianum* by previously described procedures (Cipollini and Levey, 1997a). The purity of each was verified by high-performance liquid chromatography (HPLC) as described by Bushway et al. (1986). Authenticated preparations of α -solasonine and α -solamargine for

quality control monitoring during chromatography and HPLC procedures were obtained from Plantech (Reading, UK). Each alkaloid was separately freeze-dried and stored at -20°C until use. For culture, the purified alkaloids were initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 7 mg/ml. For preparation of 1:1 SM/SL, an equal weight ratio of solasonine and solamargine were combined and dissolved in DMSO as above. Alkaloid solutions were diluted to working concentrations in complete Dulbecco's modified Eagle's medium (cDMEM) for assays involving BSF stages, whereas those requiring EMs were diluted in complete liver infusion tryptose broth (cLIT) (Powell and Kuhn, 1980).

Parasites and Cultures

The type I Brazil strain (BS) and a type II North American isolate (LI) of *T. cruzi* were routinely maintained by serial passage in BALB/c mice or in cLIT. BSF parasites were routinely obtained by coculturing with DH-82 canine monocytes in cDMEM at 37°C , 5% CO_2 . EMs were recovered from log growth phase cLIT cultures.

Cytotoxicity Assay

For cellular toxicity assays, 96-well tissue culture plates were seeded with 100 μl of cDMEM containing 2×10^4 BSF stages. EM stages were prepared in cLIT and diluted to $1 \times 10^6/\text{ml}$. Stock solutions of alkaloids were prepared by dissolving in DMSO at 5–7 mg/ml. The appropriate amount of stock alkaloid solution was added to media to achieve the desired final concentration (0–500 $\mu\text{g}/\text{ml}$) in each well. To account for any potential toxic effects of DMSO, control cultures were incubated with media containing equal concentrations of this reagent. Preliminary experiments showed DMSO to be of negligible toxicity at the concentrations used. DMSO did not synergize with the alkaloids to affect parasite attrition. Cultures in cDMEM were maintained at 37°C , 5% CO_2 ; those in cLIT were kept in an environmental chamber at 27°C . Each well was microscopically examined at 1, 24, 48, or 72 hr, and the mean number of surviving parasites was calculated from counts made throughout three separate microscopic fields of view (400 \times). This value was used to obtain percentage of surviving parasites, expressed as viability, as compared to DMSO-matched controls. One hundred percent viability was set at the lesser value between the initial seeded concentrations or DMSO control culture viability at the appropriate time point. This was done to minimize the exaggeration of alkaloid-dependent parasite attrition due to proliferation in control cultures.

Competitive RBP Inhibition

To test the role of rhamnose-binding proteins in solasonine and solamargine toxicity against *T. cruzi*, cytotoxicity assays were performed as described above with the addition of exogenous L-rhamnose (Sigma, St. Louis, MO, USA) to the media to achieve final concentrations of 50 or 500 mM. Results are expressed as described above.

Statistical Analysis

The Bonferroni all-pairwise comparisons test of cells (Statistix 8.0) following factorial ANOVA was used to assess statistical differences among treatment groups. All experiments were performed at least three times.

Results

Strain Susceptibility

Cytotoxicity assays performed on BSFs and EMs demonstrated that the BS and LI strains were equally susceptible to the effects of α -solamargine and α -solasonine (Table 1, Experiment 1). In BSF cultures at 24 hr, significant attrition was noted for both the BS and LI at titers as low as 10 $\mu\text{g/ml}$ of α -solamargine (Fig. 2a). These titers reduced culture viability to approximately 37% of the DMSO controls lacking α -solamargine. Increasing α -solamargine concentrations to 20 $\mu\text{g/ml}$ reduced parasite survival to approximately 8% for both strains. Concentrations of 40 $\mu\text{g/ml}$ resulted in the clearance of BSFs entirely from culture. Similar results were obtained with α -solasonine except that concentrations approximately twice higher were required to elicit effects equivalent to those of α -solamargine (Fig. 2a).

Similar results were found with EMs cocultured with each alkaloid (Fig. 2b). Significant EM attrition was observed at 10 $\mu\text{g/ml}$ of α -solamargine, where reductions in viability of approximately 23% (BS) and 25% (LI) were measured. Increasing the concentrations of α -solamargine to 30 $\mu\text{g/ml}$ decreased parasite survival for both strains to less than 20%.

As noted for BSF stage parasites, α -solasonine titers had to be increased approximately 1.5–2 times to elicit cytotoxic effects in EM cultures similar to those of α -solamargine.

Table 1 Factorial analyses of variance for the effects of alkaloid type, concentration, parasite strain, and inhibition experiments on parasite cytolysis

Effect	EMs				BSFs			
	<i>df</i>	SS	<i>F</i>	<i>P</i>	<i>df</i>	SS	<i>F</i>	<i>P</i>
Experiment 1: strain specificity								
Alkaloid	1	4187	150.38	< 0.001	1	618.3	43.95	<0.001
Concentration	7	139,046	713.4	< 0.001	5	17,592.2	250.09	<0.001
Strain	1	3	0.12	0.729	1	33.3	2.37	0.1302
Alkaloid \times Strain	1	2	0.07	0.787	1	11.7	0.83	0.367
Alkaloid \times Concentration	7	4542	23.3	<0.001	5	683.6	9.72	<0.001
Strain \times Concentration	7	62	0.32	0.944	5	159.2	2.26	0.063
Alkaloid \times Strain \times Concentration	7	173	0.89	0.521	5	42.2	0.60	0.700
Error	64	1782			48	67.3		
Experiment 3: 1:1 compound effects								
Alkaloid	6	104,441	651.20	<0.001	4	48,784.3	446.56	<0.001
Concentration	2	2625	49.11	<0.001	2	5236.9	95.88	<0.001
Alkaloid \times Concentration	12	3110	9.70	<0.001	8	2204.6	10.09	<0.001
Error	42	1123			30	819.3		
Experiment 4: L-rhamnose inhibition								
Alkaloid	1	5344	223.22	<0.001	1	5922.35	304.36	<0.001
Concentration	6	137,519	957.37	<0.001	5	119,710	1230.42	<0.001
L-Rhamnose	1	1	0.03	0.859	1	4.01389	0.21	0.652
Alkaloid \times Concentration	6	6138	42.73	<0.001	5	7741.40	79.57	<0.001
Alkaloid \times L-Rhamnose	1	3	0.13	0.723	1	0.12500	0.01	0.936
Concentration \times L-Rhamnose	6	75	0.52	.790	5	100.069	1.03	0.412
Alkaloid \times Concentration \times L-Rhamnose	6	53	0.37	.896	5	106.069	1.09	0.377
Error	56	1341			48	934.0		

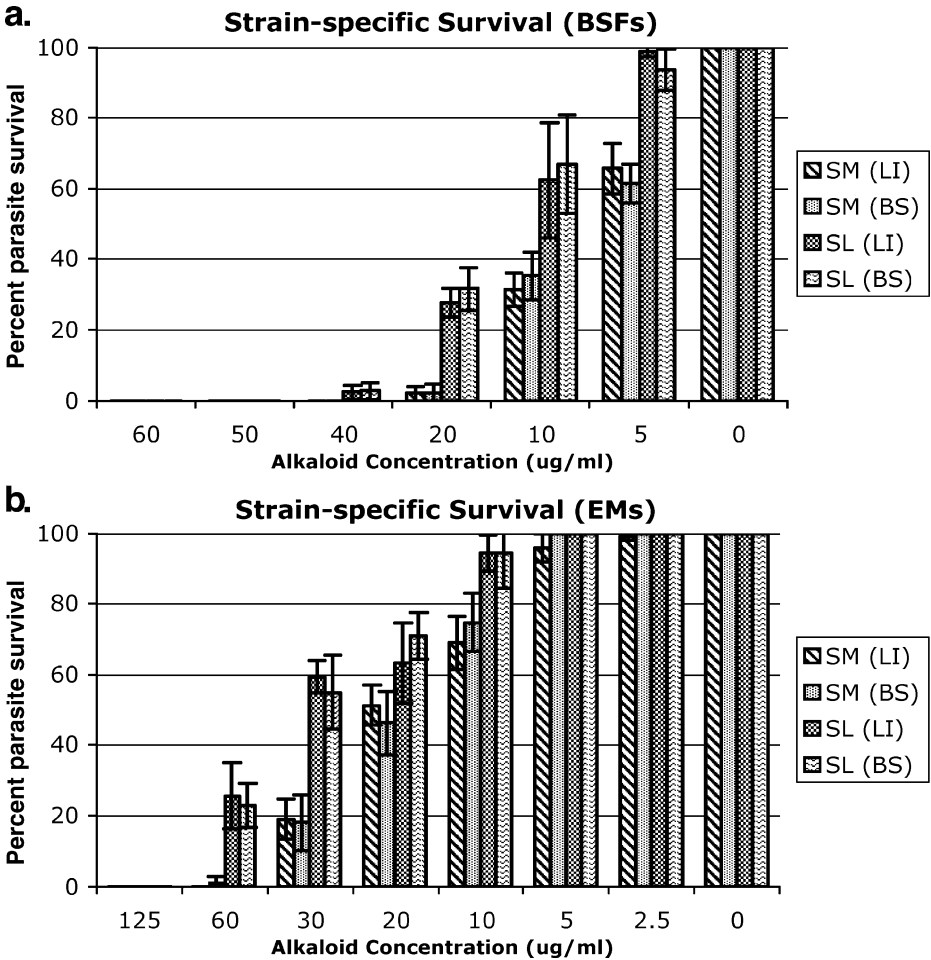


Fig. 2 Susceptibility of the Brazil strain (BS) and Lemur isolate (LI) of *Trypanosoma cruzi* to solasonine (SL) and solamargine (SM). (a) Shows the effects of the alkaloids on blood stream forms (BSFs) and (b) on epimastigotes (EMs). Parasites were cocultured for 24 hr in media containing the appropriate alkaloid at concentrations ranging from 0 to 125 $\mu\text{g/ml}$. For each treatment, parasite survival (mean \pm SD) was calculated as a percentage of the DMSO-matched control cultures. Each value is derived from three independent counts

While statistically significant differences could be attributed to both the concentration and type of alkaloid, no differences in susceptibility could be attributed to parasite strain (Table 1, Experiment 1). These assays suggested higher resistance for the EM stage as compared to BSFs, but given the differences in initial parasite concentrations and media constituents such conclusions cannot be wholly supported.

Since it was determined that the BS and LI strains were equally susceptible to the effects of α -solamargine and α -solasonine, subsequent experiments were limited to the more easily cultured Brazil strain.

Timed Assay

Cytotoxicity assays were performed and culture viability evaluated at 1, 24, and 48 hr to establish the timing of parasite attrition and potential recovery. Assays carried out on BSFs showed that the majority of attrition occurred within the first hour of exposure (Fig. 3a). When cultured at concentrations of α -solamargine $\geq 20 \mu\text{g/ml}$, viability declined rapidly with cultures cleared of parasites within 24 hr. α -Solamargine at 5–10 $\mu\text{g/ml}$ showed an initial decline in BSF viability at 1 hr, followed by a steady increase over 48 hr. Significant decreases were also apparent in EM cultures as early as 1 hr after exposure at all concen-

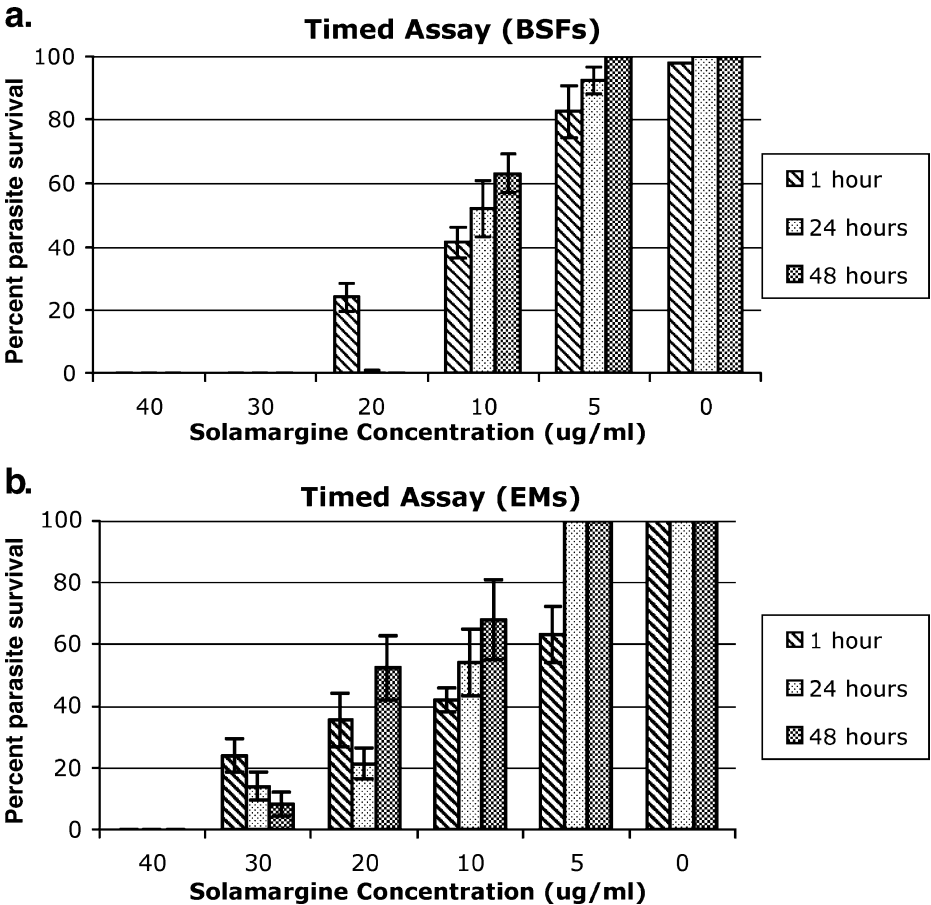


Fig. 3 Timing of attrition induced by different concentrations of solamargine on (a) blood stream forms (BSFs) and (b) epimastigotes (EMs) of the Brazil strain of *Trypanosoma cruzi*. The number of parasites (mean \pm SD) surviving at each concentration after 1, 24, and 48 hr of culture was calculated as a percentage of the DMSO matched controls. Each value represents three independent counts. Pairwise comparisons test of cells identified homogenous groups as follows (Group: concentration/time): (A) 0/1, 0/24, 0/48, 5/24, 5/48; (B) 10/48, 5/1, 10/24, 20/48; (C) 10/24, 20/48, 10/1; (D) 20/48, 10/1, 20/1; (E) 10/1, 20/1, 30/1; (F) 20/1, 30/1, 20/24; (G) 30/1, 20/24, 30/24, 30/48; (H) 30/24, 30/48, 40/1, 40/24, 40/48

trations of α -solamargine tested (Fig. 3b). At concentrations of α -solamargine ≥ 40 $\mu\text{g/ml}$, viability was reduced to zero by 1 hr, whereas those containing 30 $\mu\text{g/ml}$ continued to decline through all time points measured. When alkaloid titers were reduced to 20 $\mu\text{g/ml}$ or lower, cultures initially declined but demonstrated signs of recovery as measured by increases in parasite numbers and activity. Rosettes of replicating EMs were frequently visible by 48 hr in these cultures. Results obtained from EM and BSF cocultured with α -solasonine gave results similar to those with α -solamargine, except for the previously noted requirement for higher alkaloid concentrations to obtain the same effects (data not shown).

Synergistic Effects of 1:1 SM/SL Compound

As tested here, a 1:1 compound of α -solasonine and α -solamargine failed to increase parasite attrition (Fig. 4), although significant differences were noted between each of the three alkaloid preparations tested (Table 1, Experiment 3). The 1:1 mixture was consistently measured as being between that of pure α -solasonine and pure α -solamargine in attrition for both BSFs (Fig. 4a) and EMs (Fig. 4b). α -Solamargine was significantly more effective at eliminating BSFs than the 1:1 mixture at concentrations of 5, 10, and 30 $\mu\text{g/ml}$. α -Solasonine was less effective than either the 1:1 mixture or α -solamargine at 10 and 20 $\mu\text{g/ml}$, with titers of α -solasonine at 10 $\mu\text{g/ml}$ decreasing culture viability by 13% as compared to 36% and 62% for the 1:1 mixture and α -solamargine respectively. Results from coculture of EMs with the 1:1 mixture were consistent with those from BSF assays, eliciting attrition levels between that of α -solasonine and α -solamargine (Fig. 4b).

L-Rhamnose Inhibition Assays

The role of RBPs in mediating cytotoxicity was tested *in vitro* by performing assays in the presence or absence of 50 mM L-rhamnose as a competitive inhibitor. In both the BSF and EM cultures, L-rhamnose failed to significantly increase parasite survival over those cultured with either alkaloid alone (Table 1, Experiment 4). Increasing the final concentration of L-rhamnose to 500 mM generated nearly identical results, with no significant effect on parasite survival (data not shown).

Discussion

T. cruzi strain-specific characteristics are largely associated with the clonal expansion of geographically isolated populations (Revollo et al., 1998). The drug resistance demonstrated in some *T. cruzi* isolates has been correlated largely to such strain specificity (Andrade et al., 1985). α -Solasonine and α -solamargine as tested in this study were effective at inducing cytolysis in both a South American derived type I strain (BS) and a North American type II isolate (LI). This, coupled with previously cited studies, suggests that these compounds are cytolytic against biologically diverse *T. cruzi* strains. This characteristic was also extended to the different lifecycle stages, with both BSFs and EMs rapidly cleared in the presence of either alkaloid, although requiring different titers (Fig. 2). Concentrations as low as 10 $\mu\text{g/ml}$ of α -solamargine drastically reduced culture viability within 1 hr. We routinely observed significant attrition within 5–15 min of the addition of alkaloid, especially at higher concentrations (>40 $\mu\text{g/ml}$). α -Solamargine was significantly more effective at killing all stages tested than solasonine, which required 1.5–2 \times the concentration of alkaloid.

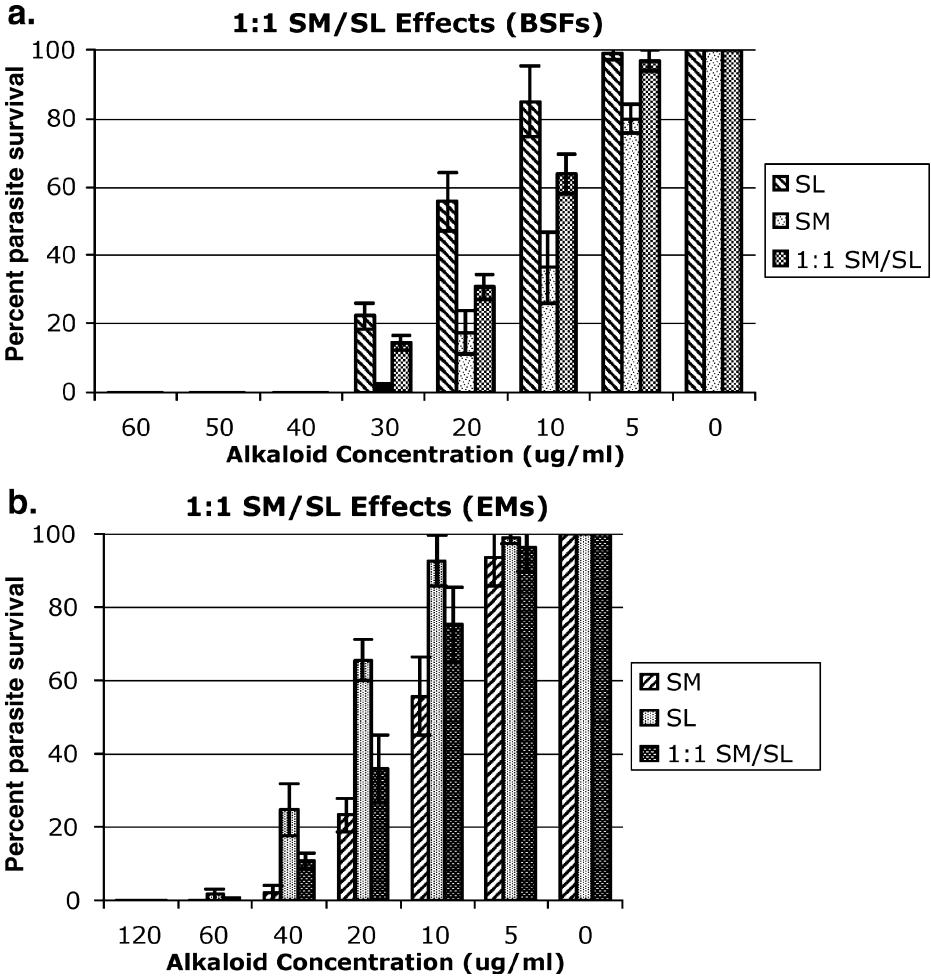


Fig. 4 Cytolytic effects of a 1:1 compound (1:1 SM/SL) of solamargine (SM) and solasonine (SL) on (a) blood stream form trypanosomes (BSFs) and (b) epimastigotes (EMs) of *Trypanosoma cruzi*. Parasites were incubated for 24 hr in the presence of various concentrations of solasonine, solamargine, or the 1:1 compound. Surviving parasites were calculated as a percentage (mean ± SD) of those in DMSO-matched controls. Each value represents three independent counts. Pairwise comparisons test of cells identified homogenous groups as follows (Group: concentration/alkaloid): (A) 0/sm, 0/sl, 0/1:1, 5/sl; (B) 5/1:1, 10/sl; (C) 10/sl, 5/sm; (D) 5/sm, 10/1:1; (E) 10/1:1, 20/sl; (F) 10/sm, 20/1:1; (G) 30/sl, 20/sm, 20/1:1; (H) 30/sl, 20/sm, 30/1:1; (I) 30/1:1, 30/sm; (J) all other comparisons

Although experiments with metacyclic stage parasites found they were cleared from culture at concentrations of alkaloid similar to BSFs, the rapid increase in EMs at lower alkaloid concentrations in these cultures made the data difficult to interpret.

As demonstrated by the reduced titers required by α-solamargine vs. that for α-solasonine, the carbohydrate moiety clearly plays a significant role in the cytolytic properties of these compounds against *T. cruzi*. What is less certain is the mechanism by which the carbohydrates participate. The efficacy of solamargine, with two terminal rhamnose sugars, exceeded both that of 1:1 SM/SL and α-solasonine, in a manner correlated to the relative

amounts of rhamnose in each preparation (Fig. 3). Opposed to the previously cited results in tumor models, the medial efficacy of the 1:1 SM/SL compound as compared to its parent alkaloids discounts the role of separate mechanisms for α -solasonine and α -solamargine for killing. Despite the evidence that rhamnose likely plays a role in antitrypanocidal activity, the inability to competitively inhibit parasite death with exogenous L-rhamnose argues against a direct role for RBPs (Fig. 4). Any suggested requirement for a bivalent rhamnose-dependent receptor would fail to explain the efficacy of solasonine, albeit at somewhat higher concentrations.

We can only speculate that the positioning of the terminal sugars in α -solamargine are sterically more favorable to binding with the parasites mucin-rich cell surface than those of α -solasonine (Ferguson, 1997). Once bound in sufficient concentrations, the alkaloid's lipid moiety would induce cytolysis through hydrophobic interactions with membrane sterols (Roddick et al., 1992, 2001). The difference in the susceptibility noted between EMs and BSFs would be explained by stage-specific differences in membrane constituents, as described by Chataing et al. (1998). The ability to mimic the effects of α -solamargine with higher doses of solasonine likely reflects a dose-dependent increase in bound alkaloid at the cell surface. This would also explain the medial response observed with 1:1 SM/SL and the mechanistic independence from RBPs.

We note that the alkaloids used in this study were derived from ripe fruits of a *Solanum* species that is most likely dispersed by mammals. There have been numerous hypotheses put forth to explain the allocation of secondary metabolites in ripe fleshy fruits (Cipollini and Levey, 1997a; Cipollini, 2000), including the retention of high levels of glycoalkaloids in fruits of some *Solanum* species (Cipollini and Levey, 1997b). The appearance of secondary metabolites in ripe fleshy fruits seems paradoxical in that such fruits are presumably adapted for dispersal by vertebrates yet may contain levels of secondary metabolites that may be toxic or deterrent to vertebrates. Among the hypotheses put forth to explain this phenomenon is the suggestion that high levels of certain secondary metabolites may be necessary to prevent consumption/destruction of fruits by organisms that do not consequently disperse seeds. This "directed toxicity" hypothesis maintains that such chemicals should not have profound effects on vertebrate seed dispersers at normal consumption levels. Considering this hypothesis, we suggest that phytoparasitic organisms may have contributed to selective pressures favoring the retention of high levels of glycoalkaloids in ripe fleshy *Solanum* fruits.

The typical route of transmission for *T. cruzi* and the closely related *T. rangelii* depends on the feeding patterns of the hemophagic reduviid bug. It is likely that, as with most hemophagic arthropods, reduviid bugs are evolutionarily derived from forms that fed upon plant phloem (Grimaldi and Engel, 2005). Other members of the order Hemiptera are phloem feeders and remain important plant pests (Bonjour et al., 1993). Maturing fruits are often targeted by phloem-feeding hemipterans, probably as a consequence of the fruit's role as a carbohydrate sink (M. Cipollini, personal observation). Coincidentally, there are species of trypanosomes, such as *Phytomonas serpens*, that parasitize *Solanum* species. An evolutionary relationship between *P. serpens* and *T. cruzi* has been demonstrated through antigenic cross-reactivity in the mouse model (Bregano et al., 2003). *T. cruzi* is also known to contain a unique pyrophosphatase within their acidocalcisomes that thus far has only been found in plants (Scott et al., 1998). This supports the hypothesis that the trypanosomes that infect animals likely evolved from those that once parasitized plants. Alkaloids such as α -solasonine and α -solamargine may have evolved as a strategy to inhibit parasitism in these plants. High concentrations of these alkaloids in the fruits in particular could: (1) deter feeding by phloem-feeding insects, (2) deter growth of phytoparasitic trypanosomes, and (3) inhibit vertical transmission of phytoparasites to the plant's progeny during seed develop-

ment. One consequence would be that vertebrate seed dispersers could benefit by consuming *Solanum* fruits containing glycoalkaloids.

The hypothesis that animals can self-medicate through strategic feeding upon specific plants or plant parts has been supported by several studies (Hart 1990; Huffman and Caton, 2001; Vitazkova et al., 2001). In the context of our study, it has been suggested that the maned wolf (*Chrysocyon brachyurus*) specifically seeks out *S. lycocarpum* fruits in Brazil and may thus suffer reduced rates of parasitism (Courtenay 1994). Being “medicinal” might be a factor that attracts vertebrates to feed upon certain fruits, including those of *Solanum* species, and thus disperse the seeds. Although there is little experimental evidence that addresses these hypotheses, the potential for coevolution of reduviid feeding strategies with trypanosomes and consequent effects upon vertebrate fruit consumers presents a promising area for investigation. In general, consideration of the evolutionary context of natural products may guide the search for antiparasitic compounds with limited acute vertebrate toxicity and appropriate therapeutic indices. Given their requirement to be eaten by vertebrates, ripe fleshy fruits may be ideal incubators of such compounds in plants.

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major signals contributing to the discrimination were alanine, threonine, glucose, sucrose, feruloyl malate, sinapoyl malate, and gluconapin.

Keywords Herbivory · *Brassica rapa* · *Plutella xylostella* · *Spodoptera exigua* · Metabolic profiling · Two-dimensional nuclear magnetic resonance spectroscopy · Principal component analysis

Introduction

Plants are attacked by a broad array of herbivores and pathogens. Damage to plant tissue may have a profound impact on host plant physiology. In response to herbivore attack, plants have evolved a number of defense mechanisms including physical and chemical barriers, as well as inducible defenses (e.g., Maleck and Dietrich, 1999; Stotz et al., 1999; Ratzka et al., 2002; Rostas et al., 2002).

Many secondary plant metabolites function as repellents or toxins that can reduce feeding by nonadapted herbivores and force adapted herbivores to invest in detoxification (Baldwin et al., 2001). In many cases, however, herbivores evoke a variety of active plant responses, including the induction of defensive secondary metabolites and shifts in plant metabolism. Some responses may be specifically induced by herbivores, while others lack that specificity with regard to the inducing agent. Similarly, the effects of plant responses can be specific depending on the herbivore species (Baldwin and Preston, 1999). Such specificity, in relation to the diversity of abiotic and biotic stress, may have adaptive value.

Plants initiate wound-response pathways when attacked by insects. For instance, feeding by the tobacco hornworm (*Manduca sexta*) stimulates mitogen-activated protein kinase systemically within a few minutes in tomato (Stratmann and Ryan, 1997). Signal transduction pathways in plant defense mechanisms respond differently to different insect species. Therefore, herbivory is thought to cause a profound effect on the plant metabolome. Previous studies have demonstrated alterations of plant metabolism under herbivory. Mauricio and Rausher (1997) as well as Shonle and Bergelson (2000) have shown that total glucosinolate concentration in *Arabidopsis thaliana* and concentration of two individual alkaloids in *Datura stramonium* are under selection pressure by insects. Furthermore, feeding damage of leaf-chewing insect larvae results in the production of a wide variety of volatile organic compounds including mono- and sesquiterpenes, sulfides, (iso)thiocyanates, and others. The ratios among herbivore-induced monoterpenes and sesquiterpenes may vary considerably among plant varieties. The proportion of monoterpenes attains nearly 50% of the total volatile organic compounds emission spectrum in intact white cabbage plants, but remains below 25% in herbivore-damaged plants (Geervliet et al., 1997; Gouinguene et al., 2001). Emission of certain volatiles is enhanced in the plants damaged by their specialist herbivores compared to those damaged by generalists (Vuorinen et al., 2004). Rostas et al. (2002) demonstrated that herbivory by the chrysomelid beetle (*Phaedon cochleariae*) on Chinese cabbage (*Brassica rapa* spp. *pekinensis*) reduces the sucrose concentration of leaves and increases the amounts of indole glucosinolates as well as the total anthocyanins. Yet, these studies have been limited to specific groups of metabolites. Information on the alteration of the whole metabolome of plants under herbivory is necessary.

Examination of the metabolome, however, is a complex task due to the large number of metabolites and different tissues, including special storage organs, with different metabolite complements. It is unlikely that a single analytical method will yield information about all

the metabolites in a plant. Diversity in volatility, polarity, solubility, and chromatographic behavior requires multiple methods. Therefore, a wide spectrum of rapid and reproducible chemical analysis techniques should be used that require only simple sample preparation (Choi et al., 2004a).

^1H NMR can meet these requirements. This method provides metabolic fingerprints with good chemical specificity for compounds containing elements with a nonzero magnetic moment, and can simultaneously detect all proton-bearing compounds such as carbohydrates, amino acids, fatty acids, amines, esters, ethers, and lipids (Sumner et al., 2003). Several studies on plant metabolite profiling utilizing ^1H NMR have been done. For example, Ward et al. (2003) distinguished various ecotypes of *A. thaliana* by using ^1H NMR and multivariate analysis. Choi et al. (2004b) found metabolites that were present in different concentrations in phytoplasma-infected *Catharanthus roseus* leaves vs. healthy leaves, and these could be identified by using one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy followed by principal component analysis (PCA).

Although ^1H NMR is a powerful tool, it has some disadvantages due to low resolution and overlapping signals when applied to mixtures. Thus, when working with complex mixtures, it is essential to reduce the spectral overlap. 2D NMR is an attractive alternative. The use of 2D NMR adds information on the correlation between resonances by dispersing the signals in an extra dimension. The problem of overlap decreases, thereby facilitating the assignment procedure (Nilsson et al., 2004). In particular, 2D J-resolved NMR spectroscopy is helpful because of its relatively short measuring time and reliability for the quantification of metabolites in mixtures.

The present study investigated the metabolomic differentiation of *B. rapa* leaves damaged by two different instars of the crucifer specialist *Plutella xylostella* L. (Lepidoptera: Yponomeutidae), and the generalist *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae), compared to control leaves. For two reasons, we would expect a reaction of the plant specific to larval age. First, young instars are not very mobile and dispersive in comparison to older, and they are, therefore, less easily repelled. Thus, instar-specific differences in induced defense mechanisms could be adaptive. Second, the natural enemies attracted by plants might be different for young and old instars. Therefore, we might expect differences in the induced metabolome.

2D J-resolved NMR spectroscopy coupled with PCA was applied to achieve a more complete interpretation of the ^1H NMR spectra and to discriminate the metabolic differences between the damaged and the healthy leaves.

Methods and Materials

Plant and Insect Materials Turnip (*B. rapa*) plants were grown from seed (commercial seed, Herfstraap Goldana 60773) in a growth chamber (16:8 hr L/D photoperiod, 20°C day/15°C night, RH 70%) in pots (11 cm diam) containing a mixture of 50:50 dune sand/peat. Plants used for the experiments were 2 mo old. Larvae of generalist herbivore, the beet armyworm (*S. exigua*), were reared in a growth chamber on artificial diet. Larvae of the specialist, the diamondback moth (*P. xylostella*) were obtained from the Department of Entomology, Wageningen University, Wageningen, The Netherlands (reared on *Brassica oleracea* L.)

Insect Feeding Experiment Herbivore feeding damage was caused by 48 hr feeding. The following numbers of larvae were transferred to a single leaf: in *P. xylostella*, either 8 2nd- or 8 4th instars; in *S. exigua* either 4 2nd- or 4 4th instars. Five plants were used to analyze

effects of local infestation. For analyzing systemic effects of feeding damage, 2 2nd- or 4th instars of *P. xylostella*, or 2 2nd- or 4th instars of *S. exigua* were placed on 1 leaf of the different plants for 48 hr. Five plants were used.

The particular leaf to which larvae were transferred was covered with a plastic bag. Small holes were made in the bag to provide air circulation. Plants with larvae were placed in transparent plastic cylinders (30 cm diam, 50 cm high). Additional plants were used as controls ($N = 5$) and were kept under the same conditions. Locally damaged and systemic leaves (only the 4th to 7th true leaves) from each treated plant and control plant were harvested. Each leaf sample was separated from the veins, ground into fine powder in liquid nitrogen, and immediately stored at -80°C until needed for analysis.

Extraction of Plant Materials Each sample was freeze-dried. Fifty mg of dry material were transferred to a 10-ml centrifuge tube. After addition of 1.5 ml of 50% methanol- d_4 in buffer (90 mM KH_2PO_4 , pH = 6.0) containing 0.05% trimethyl silyl propionic acid sodium salt (TSP; w/v), the mixture was vortexed at room temperature for 1 min, ultrasonicated for 15 min, and centrifuged at 13,000 rpm at 25°C for 20 min. Each NMR sample consisted of 800 μl of the supernatant.

NMR Measurements ^1H NMR and J-resolved spectra were recorded at 25°C on a 400-MHz Bruker AV-400 spectrometer operating at a proton NMR frequency of 400.13 MHz. The window functions were optimized. The resulting spectra were manually phased and baseline-corrected, and calibrated to TSP at 0.0 ppm, all by using XWIN NMR (version 3.5, Bruker). Two dimensional J-resolved ^1H NMR spectra were acquired by using 16 scans per 32 increments that were collected into 16 K data points, with spectral widths of 5.208 kHz in F2 (chemical shift axis) and 50 Hz in F1 (spin–spin coupling constant axis). A 1.0-sec relaxation delay was employed, giving a total acquisition time of 14.52 min. Data sets were zero-filled to 512 points in F1, and both dimensions were multiplied by sine-bell functions prior to double complex FT. J-resolved spectra tilted by 45° , symmetrized about F1, and then calibrated, all using XWIN NMR (version 3.5, Bruker). Data were exported as the 1D projection (F2 axis) of the 2D J-resolved spectra. ^1H – ^1H COSY, TOCSY, heteronuclear single quantum correlation (HSQC), and heteronuclear bond correlation (HMBC) were measured at 600 MHz Bruker DMX-600 spectrometer operating at a proton NMR frequency of 600.13 MHz. The COSY spectra were acquired with a 1.0 sec relaxation delay, 6361 Hz spectral width in both dimensions. The HSQC spectra were obtained with a 1.0-sec relaxation delay, and 6361 Hz spectral width in F2 and 27,164 Hz in F1. The HMBC spectra were recorded with the same parameters as the HSQC spectrum, except for 30,183 Hz of spectral width in F2. The TOCSY spectra were acquired with a 1.0-sec relaxation delay and 6361 Hz spectral width in both dimensions.

Data Analysis The ^1H NMR and the J-resolved projection spectra were automatically reduced to ASCII files by using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to TSP and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.40–10.00. The region of δ 4.7–5.0 was excluded from the analysis because of the residual signal of water, and the region of citric acid, malic acid, and succinic acid in δ 2.8–2.5 was bucketed by 0.1 ppm to avoid the problems due to chemical shifts of these compounds that depend on concentration. PCAs were performed with the SIMCA-P software (v. 10.0, Umetrics, Umeå, Sweden).

Results

Identification of Metabolites in NMR Spectra The congestion of ^1H NMR spectra due to signal overlapping is to a great extent solved by using 2D J-resolved spectra (Fig. 1). Based on the 2D J-resolved spectra together with other 2D NMR spectra including ^1H – ^1H COSY, HSQC, and HMBC, a number of individual metabolites in *B. rapa* leaves were identified.

The amino acids region in the spectra showed the signals of alanine, threonine, valine, and glutamine. Resonance of alanine at δ 1.48 (d, J = 7.2 Hz, H-3) correlates with δ 3.72 (H-2) in the COSY spectrum and with δ 51.6 (C-2) and δ 176.8 (C-1) in the HMBC spectrum. The threonine signal at δ = 1.33 (d, J = 6.6 Hz, H-5) correlates with δ 4.28 (H-4) and with δ 66.6 (C-4) in the HMBC spectra. Valine signals at δ 1.04 (d, J = 8.0 Hz, H-4) and δ 1.01 (d, J = 8.0 Hz, H-5) show COSY correlation with δ 2.31 (H-3) and HMBC correlation with δ 30.3 (C-3) and δ 61.3 (C-2). Resonance of glutamine at δ 2.13 (m, J = 9.2 Hz, H-3) shows correlations with δ 2.47 (H-4) and δ 3.76 (H-2) in the COSY spectrum, and those with δ 55.8 (C-2), δ 178.7 (C-5), and δ 180.1 (C-1) in the HMBC spectrum. Other compounds detected in this region are α -linoleic acid analogues at δ 0.97 (t, J = 7.5 Hz). A downfield-shifted terminal methyl signal at δ 0.97, when compared with that of general fatty acids at δ 0.94, was identified as a signal of α -linoleic acid analogues including jasmonates by comparison of fatty acid reference compounds. Resonances of malic acid were detected at δ 2.80 (dd, J = 11.2 Hz, 6.6 Hz) and δ 2.63 (dd, J = 11.2 Hz, J = 4.7 Hz), which correlate with H-2 at δ 4.32 in COSY spectra. In the HMBC spectra, those signals show correlations with δ 70.1 (C-2), δ 177.5 (C-1), and δ 179.2 (C-4). Signals found in the sugar region were attributable to sucrose at δ 5.40 (d, J = 3.9 Hz), α -glucose at δ 5.16 (d, J = 3.0 Hz), β -glucose at δ 4.57 (d, J = 7.9 Hz), and the fructose moiety of sucrose at δ 4.22 (d, J = 8.8 Hz). These assignments were confirmed by 2D NMR spectra including J-resolved, COSY, HSQC, and HMBC, and by the comparison of reference compounds.

Apart from those ubiquitous compounds, phenylpropanoids were also detected in *B. rapa* leaves. Recently, we found that major phenolic metabolites of *B. rapa* leaves were malate-conjugated phenylpropanoids including caffeoyl, coumaroyl, feruloyl, and sinapoyl malates (Liang et al., 2006). Each phenylpropanoids malate can be identified by the ^1H NMR signals of H-8 (Liang et al., 2006). Sinapoyl malate (Fig. 2) shows a clear doublet at δ 6.45 (d, J = 16.0 Hz, H-8). The other signals of this compound are detected at δ 6.99 (s, H-2 and H-6) and H-7 at δ 7.66 (d, J = 15.9 Hz, H-7), respectively. Feruloyl malate (Fig. 2) signals are also present in the phenolic region as a major phenolic metabolite: H-7 at δ 6.48 (d, J = 15.9 Hz), H-8 at δ 7.66 (d, J = 15.9 Hz), H-2 at δ 7.25 (d, J = 2.0 Hz), H-6 at δ 7.03 (dd, J = 2.0 Hz, 8.2 Hz), and H-5 at δ 6.89 (d, J = 8.2 Hz). Correlation between H-7–H-8, and H-2–H-3 of the compound is clearly shown in the COSY spectrum. As minor phenolic metabolite, a flavonoid similar to kaempferol was detected. Signals at δ 6.46 (d, J = 2.1 Hz, H-6), δ 6.73 (d, J = 2.1 Hz, H-8), δ 7.79 (d, J = 8.8 Hz H-2' and H-6'), and δ 6.93 (d, J = 8.8 Hz H-3' and H-5') indicate kaempferol analogue.

Signals from an olefinic glucosinolate, gluconapin (Fig. 2), are identified in the 2D J-resolved spectrum (Fig. 3a) in which the anomeric proton from the attached sugar is detected at δ 4.93 (d, J = 9.7 Hz). In the HMBC spectrum, the characteristic correlation is equivocally present. The signal of the anomeric proton of the glucose is correlated with carbon in the ^{13}C NMR spectrum at δ 163.2 (Fig. 3b). Signals from H-2 of gluconapin were observed at δ 2.79 (d, J = 6.4 Hz), H-3 at δ 2.47 (d, J = 6.4 Hz), H-4 at δ 5.14 (ddd, J = 5.0 Hz, J = 4.0 Hz, J = 1.9 Hz), H-5 at δ 5.91 (ddd, J = 10.3 Hz, J = 3.0 Hz, J = 3.1 Hz). In the COSY spectrum, H-2, H-3, H-4, and H-5 are continuously correlated with each other. Those

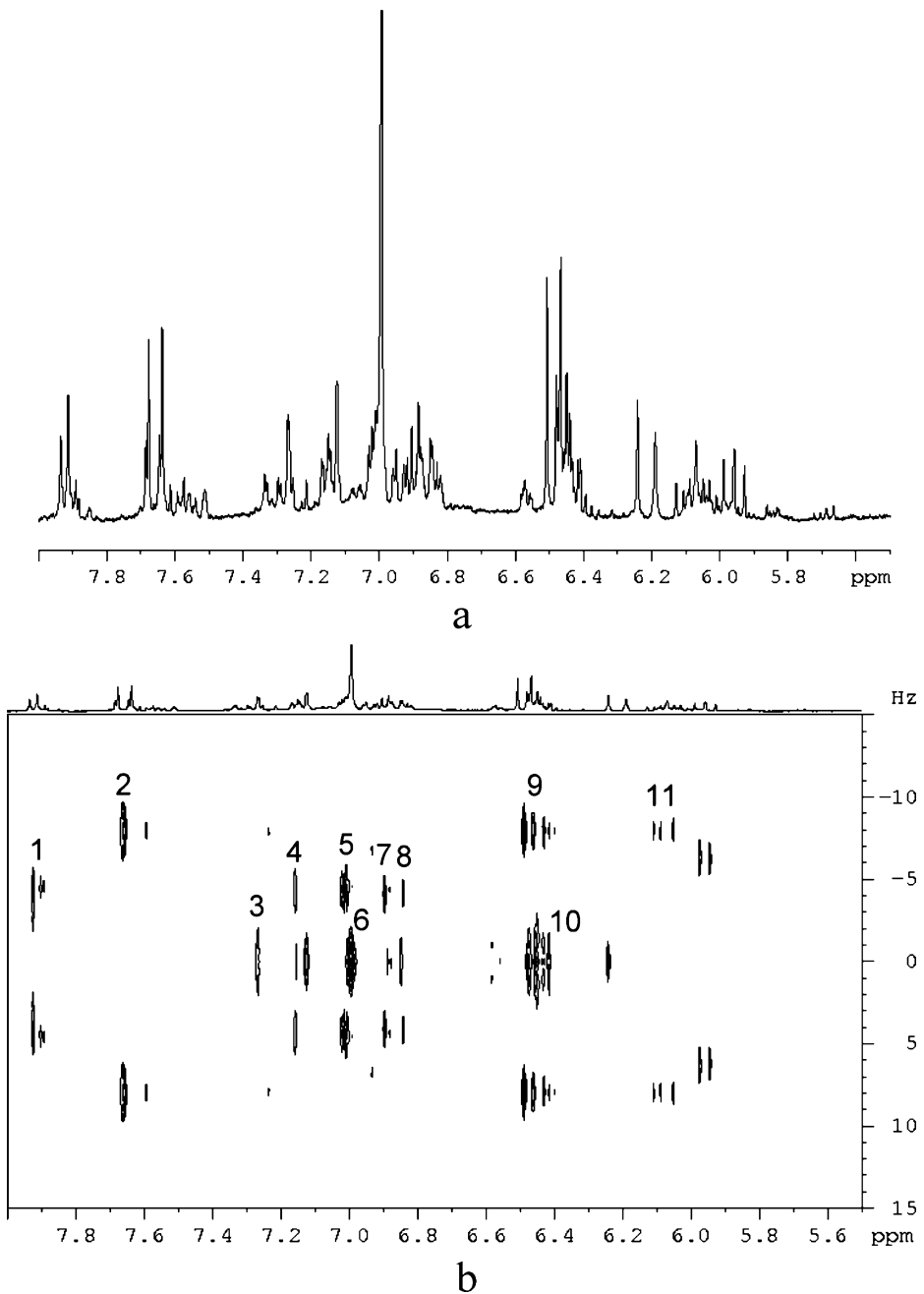


Fig. 1 ^1H (a) and two dimensional J-resolved spectra (b) of *Brassica rapa* control leaves in the range of δ 5.5–8.0. 1: H-2' and H-6' of kaempferol; 2: H-7 of phenylpropanoids; 3: H-2 of feruloyl malate; 4: H-2 and H-6 of tyrosine; 5: H-6 of feruloyl malate; 6: H-2 and H-6 of sinapoyl malate; 7: H-5 of feruloyl malate; 8: H-3 and H-5 of tyrosine; 9: H-8 of phenylpropanoid; 10: H-6 and H-8 of flavonoid; 11: H-8 of minor phenylpropanoid.

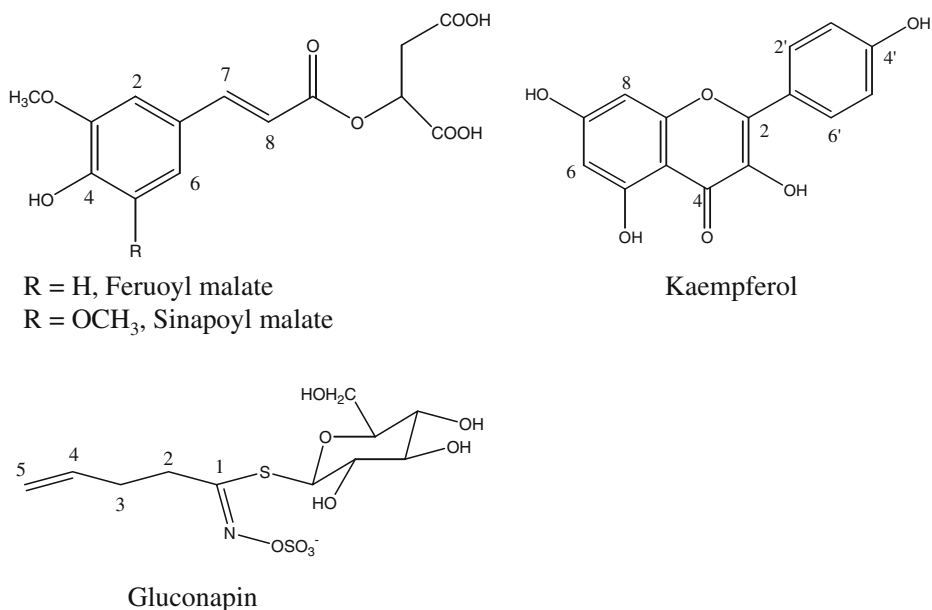


Fig. 2 Chemical structures of secondary metabolites detected in the NMR spectra of *B. rapa* leaves.

signals correlate with carbons at δ 33.9, δ 28.9, δ 137.9, and δ 116.6, respectively, in the HSQC spectrum.

Effect of Herbivory from Different Larval Stages PCA analysis was performed to discriminate between different larval stages of the same species of insect on locally and systemically induced leaves. Projected J-resolved spectra to F2 axis were used for the PCA analysis to increase resolution and overcome the complexity of 1D ¹H NMR spectra. The degree of spectral complexity in the projected J-resolved spectrum is significantly reduced compared to the true 1D data set. We applied this enhanced resolution obtained from the projected J-resolved spectra to monitor metabolic changes in the plants. In spite of the enhanced resolution in J-resolved spectra, a disadvantage is that for strongly coupled spin systems, such as in glucose, glutamine, and glutamic acid, spectral artifacts can arise in J-resolved spectra (Foxall et al., 1993; Nicholson et al., 1995). Such artifacts were included in the PCA as an additional resonance for the metabolites (Viant 2003).

Figure 4a shows that the metabolomes of the local leaves attacked by generalist herbivory are different in young vs. old instars. On locally damaged leaves, the metabolomic profile induced by 2nd generalist instars is separated by PC2 from the control and 4th instar. The loading plot of PC2 and PC3 shows that herbivory of 2nd generalist instars on local leaves increased the level of glucose, ferulic acid, and gluconapin, whereas feeding by the 4th instars caused an increase in levels of alanine and sinapoyl malate.

Figure 4b shows a comparison of the metabolites of systemic leaves attacked by 2nd and 4th generalist instars. The score plot of PC2 and PC3 (Fig. 4b) shows a strong separation. Using a loading plot of PCA, the metabolic difference of the 2nd instar induced *B. rapa* leaves might be ascribed to an increase in glucose, feruloyl and sinapoyl malate when

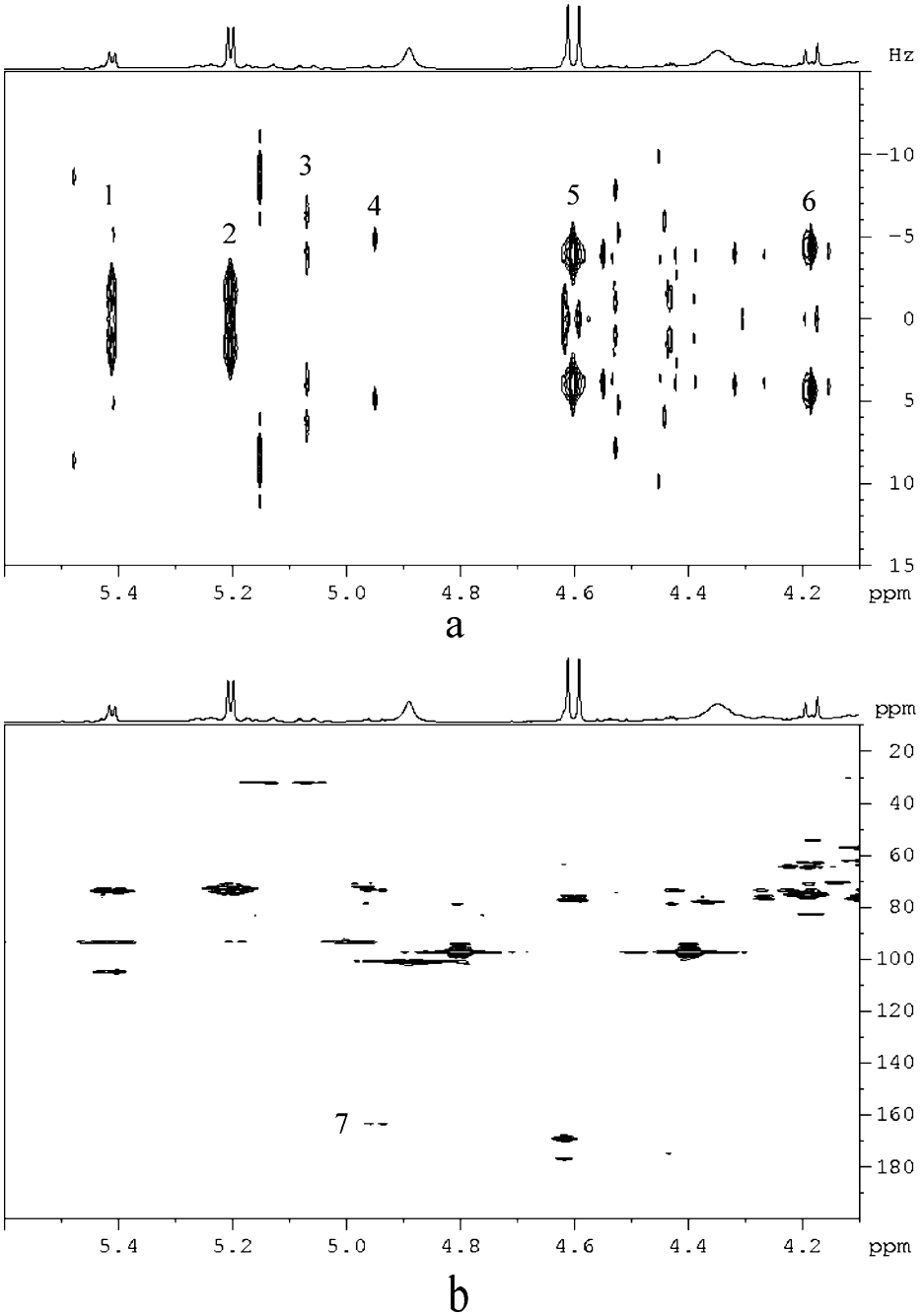


Fig. 3 2D J-resolved (a) and HMBC spectrum (b) of *Brassica rapa* leaves samples in the range of δ 4.0–5.6. 1: H-1 of sucrose; 2: H-1 of α -glucose; 3: H-2' of feruloyl and sinapoyl malate; 4: H-1' of gluconapin; 5: H-1 of β -glucose; 6: H-1' of fructose moiety of sucrose; 7: HMBC correlation between H-1' and C-1.

compared to the control and the 4th instar induced leaves. Feeding by 4th instars was found to induce the highest accumulation of gluconapin and sinapoyl malate in plants.

Figure 4c shows the score plot of PC2 and PC3 of specialist herbivory of different instars on local leaves. Second specialist instars increased levels of glucose, feruloyl and sinapoyl malate, and gluconapin, whereas 4th instars stimulated the plants to accumulate more sucrose.

When comparing the effects of herbivory by 2nd and 4th instar specialists on systemic leaves, PCA analysis showed that when compared with the control, herbivory stimulated plants to synthesize more gluconapin, glucose, feruloyl malate, sinapoyl malate, and threonine (Fig. 4d).

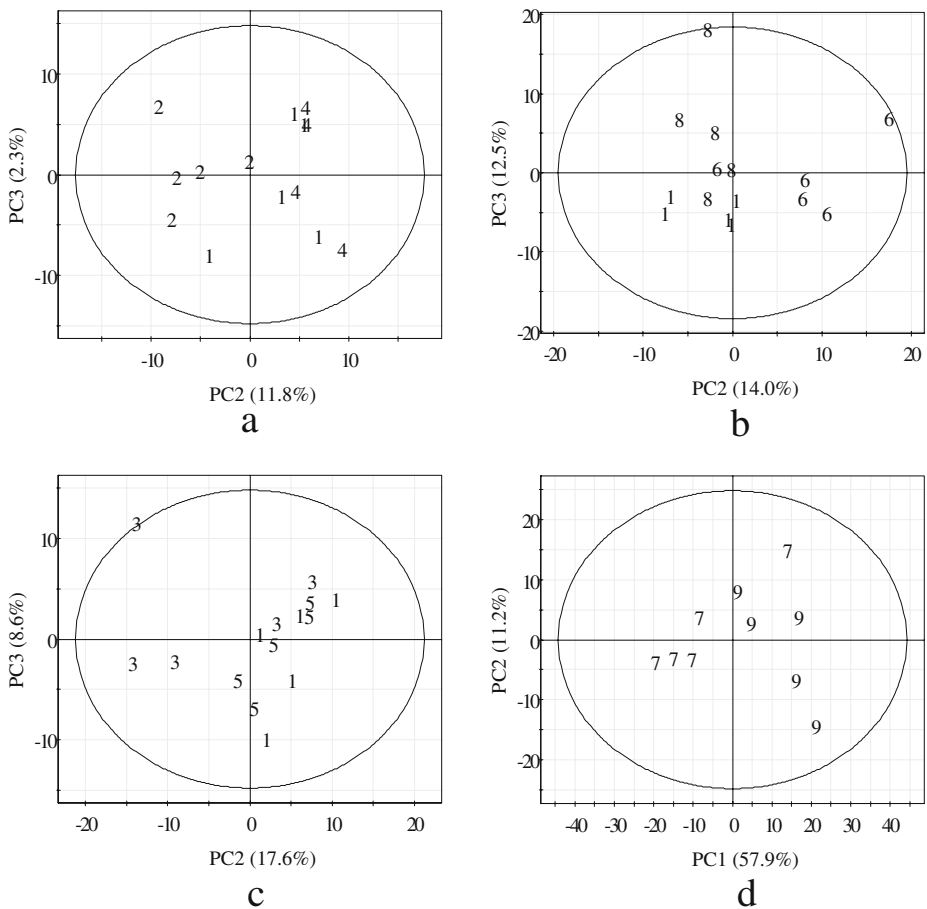


Fig. 4 Score plots of the principal component analysis (PCA) from the projected J-resolved ¹H NMR data sets. (a) 1: Control; 2: local induction of the second instar of the generalist; 4: local induction of the fourth instar of the generalist. (b) 1: Control, 6: systemic induction of the second instar of the generalist; 8: systemic induction of the fourth instar of the generalist. (c) 1: Control; 3: local induction of the second instar of the specialist; 5: local induction of the fourth instar of the specialist. (d) 7: Systemic induction of the second instar of the specialist; 9: systemic induction of the fourth instar of the specialist.

Discussion

We showed that plants respond specifically to herbivory of different instars. Changes in both primary and secondary metabolites occurred in response to different types of herbivory. In particular, *B. rapa* leaves showed different metabolic responses against young (the 2nd) vs. old (the 4th) larvae both of the generalist *S. exigua* and the specialist *P. xylostella*. In reality, young instars of herbivorous insects are not very mobile and dispersive in comparison to old instars. In a certain area of the leaves, the lower mobility of young instars might cause greater damage than that induced by old ones. In both the generalist and specialist larvae, the metabolic alterations induced by younger instars were larger than those of the older. Score values of *B. rapa* leaves attacked by 4th instars are closer to control plants than the those damaged by 2nd instars (Fig. 4a–c). When considering the metabolic pattern of locally damaged *B. rapa* leaves and systemic ones, clear differences were detected, however, in both the local and systemic leaves in the level of phenolics (ferulic acid and sinapic acid) and a glucosinolate (gluconapin).

Although their precise role is not yet understood, phenolics play a role in plant defense. Ferulic and sinapic acid might be involved in guaiacyl and syringyl lignin biosynthesis of *Arabidopsis* (Stout and Chapple, 2004). The modification of cell walls induced by the lignin synthesis may play a role in the defense mechanism. Also, ferulic acid is reported to significantly reduce the incidence of powdery mildew in susceptible (Mustang) and tolerant (Flamingo) cucumber (*Cucumis sativus*) cultivars (Daayf et al., 2000), and it has shown significant antifungal activity against a fungal pathogen, *Sclerotium rolfsii* (Sharma and Singh, 2003). Grant and Langevin (2002) stated that together with sinapic acid, ferulic acid acts as an oviposition deterrent to spruce budworm (*Choristoneura fumiferana*).

Several studies have shown that herbivore feeding damage induces glucosinolate biosynthesis in Brassicaceae. Glucosinolates and their catabolites have wide-ranging biological activities, from feeding deterrence for generalist insects to oviposition stimulation for specialists (Halkier and Du, 1997, Ratzka et al., 2002) and an involvement in the interaction between Brassicaceae with their microbial and fungal pathogens (Doughty et al., 1996) and nematodes (Zasada 2003). We showed in this study that when attacked by insects, *B. rapa* produced an olefin glucosinolate, gluconapin, that is known as one of the most abundant glucosinolates in Brassicaceae. Together with 4-hydroxyglucobrassicin, it accounts for more than 80% of the total glucosinolates in *B. campestris* and *B. rapa* accessions (Peiwu et al., 1999).

The use of 2D NMR spectroscopy is promising for the analysis of complex mixtures of compounds in plant material. Problems of overlap can be decreased, so that the assignments are easier. In particular, J-resolved 2D NMR spectra were helpful because of their relatively short measuring time and the reliability for the assignments of metabolites present in a mixture compared to other 2D NMR methods. When combined with principal component analysis (PCA), the discrimination of peaks can be easily assigned and used to distinguish effects of different types of herbivory.

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detached from the flower and adhered to the fly's dorsum. In this unique mutualistic association, both species receive direct reproductive benefits—the flower's pollinarium is transported for cross pollination, and the fly is offered a bouquet of phenylpropanoids (synomone) that it consumes, converts, and/or sequesters as sex pheromonal components, thus enhancing sexual attraction and mating success.

Keywords *Bulbophyllum vinaceum* · Orchidaceae · Phenylpropanoids · Fruit fly · *Bactrocera dorsalis* · *B. unimaculata* · Tephritidae · Synomone · Sex pheromone · Pollination · Dynamic lip mechanism

Introduction

A number of orchid species in the genus of *Bulbophyllum* (Orchidaceae: subfamily Epidendroideae, subtribe Bulbophyllinae) exhibit an elaborate floral architecture, in addition to characteristic floral fragrances, that attract and bring specific pollinators to the right position for effective pollination. Some of the species, here called “fruit fly orchids,” selectively attract fruit flies of the genus *Bactrocera* (Diptera: Tephritidae) with specific floral volatiles that act as a synomone. It has been reported that the ginger orchid (*Bu. patens* King) flower releases a ginger essence—zingerone, as a floral synomone that attracts fruit flies sensitive to both methyl eugenol (ME) and raspberry ketone (RK)—inclusive of both pestiferous and nonpestiferous species (Tan and Nishida, 2000). In addition, flowers of *Bu. apertum* Schltr. (subspecies *verrucatum*) release RK that attracts RK-sensitive *Bactrocera* species (Tan and Nishida, 2005). Furthermore, the fruit fly orchid flower (*Bu. cheiri* Lindl.) possesses several phenylpropanoids, of which the major component is ME. It attracts male flies of *Bactrocera papayae* Drew and Hancock that assist in its pollination (Tan et al., 2002; Nishida et al., 2004). [It is noted that *B. papayae* is neither a distinct biological nor genetic species different from the Oriental fruit fly, *B. dorsalis* (Hendel) (Naeole and Haymer, 2003; Tan, 2003) and henceforth it is referred to as *B. dorsalis*.] The fruit fly orchid also attracts males of *B. carambolae* Drew and Hancock, *B. umbrosa* (Fabricius), and the hybrid of *B. dorsalis* × *B. carambolae*. None of the above species produces nectar; hence the question of the reward for attracted fruit flies remains unanswered. Flies consume and then either convert the chemical attractant into male sex pheromonal component(s)—in the case for ME-sensitive species, or directly sequester the attractant as part of a male pheromone system in the RK-sensitive species (Nishida et al., 1988, 2004; Tan, 2000; Tan and Nishida, 1995, 1998, 2000, 2005; Tan et al., 2002). Hence, for the three *Bulbophyllum* species—*Bu. patens* King, *Bu. apertum*, and *Bu. cheiri* Lindl.—the respective floral attractants are themselves the rewards for the attracted flies.

The majority of the *Bulbophyllum* species are epiphytes found in the virgin pan-tropical forest (Vermeulen, 1991) in lower montane forest at ca. 1000 m above sea level. The vinaceous orchid, *Bu. vinaceum* Ames & C. Schweinf., is a rare epiphytic plant, that is endemic to the highlands of Borneo Island, such as the Crocker Range and Mt. Kinabalu of Sabah, East Malaysia. Its name is derived from the Latin word “vinaceus” (for dark wine red) (Vermeulen, 1991) due to its deep wine-red colored, single-flowered inflorescence. Its resupinate flower (with lip turned downwards) has a protruding and stiff hamulus (pollinia stalk), whose function is unknown (Rasmussen, 1985), and a “spring loaded” hinged lip that is always held in a closed position (presumably protecting its sexual organs). The flower has a mild sweet scent resembling that of an ester and strongly attracts males of *B. dorsalis*.

The objectives of this paper are to investigate: (1) the behavior of fruit fly visitors and determine species identity of attracted flies; (2) the process of pollinarium removal as well as to determine the role of floral hamulus and the constantly closed lip; (3) the chemical constituents in floral fragrance that attract fruit flies; and (4) the content of phenylpropanoids in various floral organs and rectal gland of fruit fly visitors.

Methods and Materials

Observations of Flies on flowers

Observations of orchid flowers and plants were conducted in Kundasang (ca. 1800 m at the foothill of Mt. Kinabalu), Sabah, East Malaysia, and in Tanjung Bungah (lowland <200 m above sea level), Penang (plants originally obtained from Kundasang). Fly attraction was observed continuously from 07:00 to 17:00 hr on the day a flower bloomed. Investigations of pollinarium removal by a fruit fly were conducted when the first fly visited and fed on a newly opened flower; this process was videotaped whenever possible. Attention was paid to the role of the floral lip (labellum) and hamulus during pollinarium removal. To confirm that fruit flies were attracted to the flower via scent and not visually via floral color or form, certain investigations were performed with the fully developed bud covered with a piece of black mosquito wire netting; the flower was covered at dawn before it bloomed. After observation, flies on the flowers were collected in clear plastic bags, and their respective species identity was confirmed, if necessary, under 40× magnification. To ascertain floral response to lower light intensities especially during the night, newly bloomed flowers were left on the plant for further observation (after exposure to fruit flies). Observations were conducted on at least three flowers.

Instruments

Volatile components were analyzed via gas chromatography-mass spectrometry (GC-MS) using an HP 5989B mass spectrometer coupled with an HP 5890 series II plus gas chromatograph equipped with an HP-5MS capillary column (30 m×0.25 mm, 0.25 μm film thickness) programmed from 60°C (1 min holding) to 280°C at a rate of 10°C/min. Quantification of volatiles was done on an HP 4890A gas chromatograph using an HP-1MS capillary column (20 m×0.25 mm, 0.25 μm film thickness) and programmed from 80°C (1 min holding) to 280°C at a rate of 10°C/min; the GC was equipped with a total ion monitor and flame ionization detection (FID) using internal standards [1-tetradecanol or ethyl 4-(3,4-dimethoxyphenyl)-propanoate].

Headspace Sampling

Volatile components from *Bu. vinaceum* flowers were collected during the day by using a glass jar (250 ml) with a filtered air inlet at the bottom end—connected to an aquarium pump (air flow regulated at ca. 20 ml/min)—and an outlet at the top end—directly connected to a TENAX column (packed with 100 mg in a glass tube). The TENAX column was replaced every 2 hr and washed with 0.5 ml ethanol. The resulting eluate was partially concentrated under reduced pressure (20 mmHg, <20°C) and then subjected to GC-MS and GC-FID analyses, with 1-tetradecanol as an internal standard.

Extraction of Floral Volatiles

Bu. vinaceum plants were originally collected in Kundasang, Sabah, and grown under outdoor conditions in a home garden in Tanjong Bungah, Penang, Malaysia, to obtain the blossoms. Flowers were plucked within 4 hr of blooming (07:00–9:00 hr), weighed, and immersed in sufficient redistilled ethanol in a 20-ml glass vial, and then used for GC quantifications. For individual floral organs (petals, lateral and medial sepals, lip, and column), each part was carefully removed from a freshly bloomed flower (within 3 hr after it bloomed and not exposed to fruit flies), weighed, and soaked in sufficient ethanol in a 5-ml glass vial for quantification of ME.

Extraction of Volatiles from Fruit Flies

Ten wild flies, which were observed feeding on the floral tissues of *Bu. vinaceum* flower, were captured in the orchid habitat in Kundasang, Sabah (October 1, 2002). Flies (captured in the afternoon) were kept alive until the evening (ca. 6 hr—sufficient time to incorporate and sequester the phenylpropanoids into the rectal pheromone glands)—and then dissected. The rectal gland from each fly was removed and individually soaked in ethanol (0.25 ml in 1-ml vial). Each fly body received the same treatment. A laboratory-reared male (18 d old, and not previously exposed to any phenylpropanoids) of *B. dorsalis* was processed in a similar manner after feeding 10–20 min on a flower of a potted *Bu. vinaceum* plant in Penang (October 2003) and kept alive for 24 hr before rectal gland extraction.

Authentic Samples

Eugenol and methyl eugenol were purchased from Tokyo Chemical Industries (Osaka, Japan). 2-Allyl-4,5-dimethoxyphenol, eusarone (5-allyl-1,2,4-trimethoxybenzene), *cis*- and *trans*-coniferyl alcohol, and *trans*-3,4-dimethoxycinnamyl alcohol were synthesized as previously described (Tan and Nishida, 1998; Nishida et al., 2004).

Results

Fruit Fly Species

In the highlands of Kundasang, males of two sibling species belonging to the *B. dorsalis* complex—*B. dorsalis* (most abundant) and *B. unimacula* Drew and Hancock visited flowers of *Bu. vinaceum*. Many males were observed feeding on a single flower and at times, the flower was completely covered with attracted flies (>20). The number of visitors dwindled towards dusk. Of the 10 flies captured from a single flower for rectal chemical analysis, eight were *B. dorsalis* and two were *B. unimacula*; all were male.

A similar phenomenon was observed in the lowland (nonendemic area), but with one or two individuals of *B. umbrosa* and *B. carambolae* occasionally joining the abundant *B. dorsalis* and the wild hybrid (between *B. dorsalis* and *B. carambolae*; Wee and Tan, 2005). Besides being major agricultural pests, all three are ME-sensitive species that are known to respond to ME and have been captured in ME baited traps (Tan and Lee, 1982). No other organism or insect visited the flower. When a flower was covered with fine black wire netting—through which neither the shape nor the color of the flower can be distinguished—flies landed on the netting and were observed to frantically search before settling on the

area of netting nearest to the flower. This indicated that they were attracted by the fragrance and not the floral color or shape. No female flies were attracted to the flower, irrespective of time or location.

Pollinarium Removal

An attracted fly usually landed in the general vicinity of the flower before climbing onto a petal or sepal to begin probing and feeding on the floral surfaces. The fly seemed able to detect a higher concentration of the attractant located on the inner side of the floral lip, and hence eventually headed towards the lip and climbed onto it. This hinged ovately shaped lip is always in its “closed” position (Fig. 1a and b) thus protecting the sexual organs. Should the fly climb onto the lip, particularly the area towards the tip, its weight was enough to pivot the lip into its open position (Fig. 1c). This exposed the bulbous swelling of the hamulus protruding from the gynostemium (Fig. 1d) that is always facing the flower’s reproductive organs. Due to the location of the attractant on the lip and the lip’s architecture—a concave or U-shaped channel—the fly eventually aligned itself along the longitudinal axis on the adaxial side of this ovate lip (Fig. 1d). It continued to lap up chemicals on the lip with its proboscis, and as this was depleted, it moved further in towards the base. After the fly passed the point of instability, at about the midpoint of the lip’s length, the “spring loaded” lip suddenly sprang back to its closed position (in less than 0.04 sec). This pitched the fly headfirst into the column cavity. On the fly’s parabolic flight path into the cavity, it brushed forcefully against the sticky bulb of the hamulus. The relatively long and stiff hamulus acted like a crowbar to force out or dislodge the pollinia from the anther leaving behind just the anther cover. Hence, the whole pollinarium (hamulus and pollinia) was detached from the anther and adhered to the fly’s dorsum.

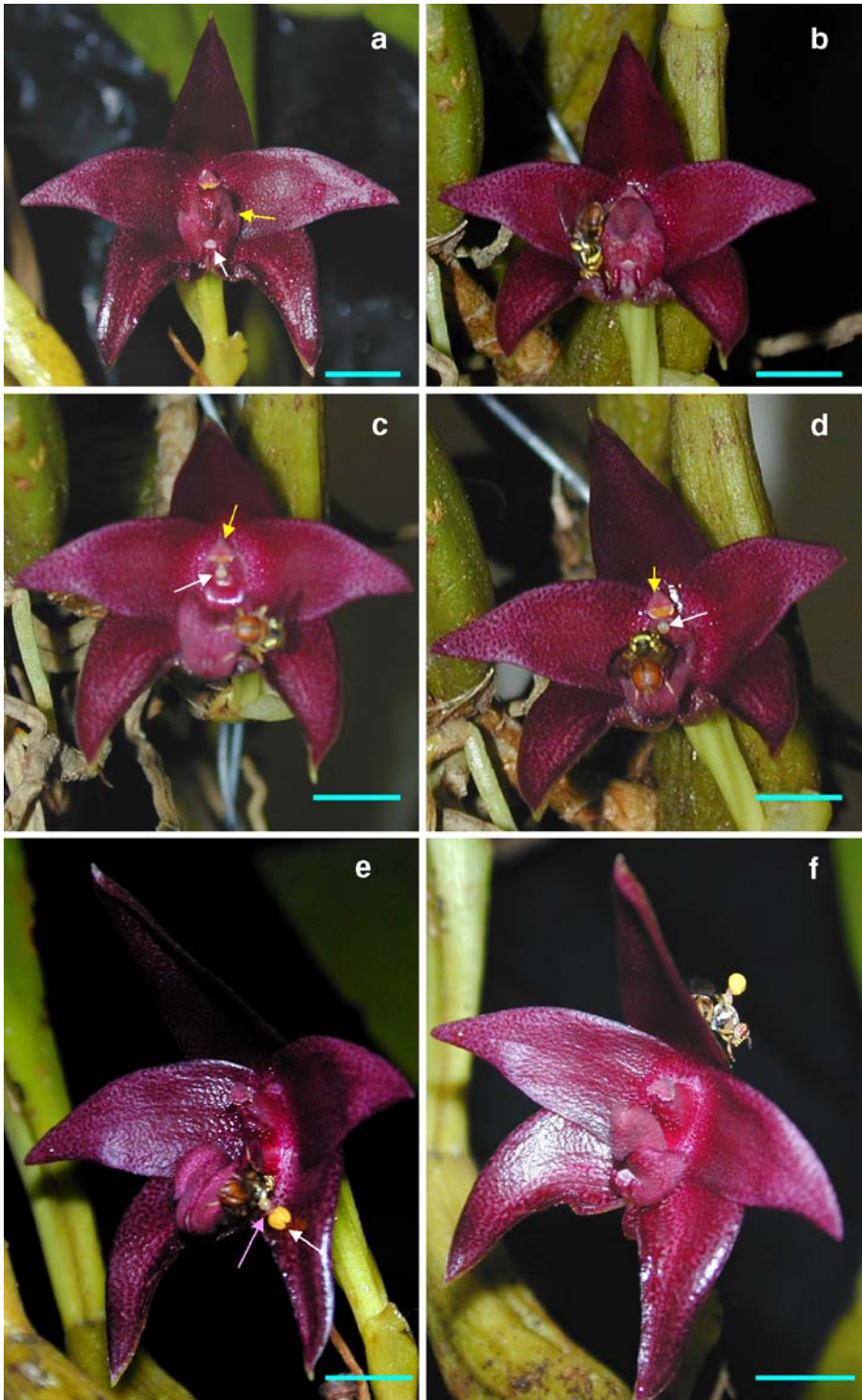
Apparently shocked and shaken, the fly retreated from its temporary confinement between the closed lip and the floral column—by backing out towards the tip of the lip, hence pivoting the lip open again. Figure 1e and f shows the fly exiting the flower with the hamulus and the pollinia (ca. 2 mm from the surface of the fly’s thorax) stuck to it. The whole process of actual pollinarium dislodgement from the anther and its adhesion to the fly was precise, smooth, and nearly instantaneous—occurring in less than 1 sec (as determined from video footage). Once free, the fly either took off (one in five observations) or dismounted from the lip and continued to settle on the flower (Fig. 1f) while intermittently feeding, resting, preening, and cleaning its proboscis for 6–40 min if left undisturbed—until satiation. In the latter situation, no self-pollination was observed.

As the fly dismounted or took off from the opened lip, the lip immediately sprang back to its normal closed position (in less than 0.02 sec—the speed of a single frame of the video clip). In all five cases observed, it was the first fly that landed on the flower that removed the pollinarium; and because there is only one pollinarium, subsequent flies regardless of number were just visitors (>35 fruit flies per flower/d) feeding on floral fragrance on all parts of the flower.

The flower partially closed at night. This process took several hours beginning in late afternoon nearing sunset. The partially closed flower reopened with its petals and sepals spread out again the next day (10:00–12:00 hr).

Identification of Phenylpropanoids

Figure 2 shows a typical gas chromatogram (GC-MS trace) of *Bu. vinaceum* whole-flower extracts, exhibiting eight phenylpropanoid peaks, which were identified as eugenol (**1**),



methyl eugenol (**2**), eusarone (5-allyl-1,2,4-trimethoxybenzene) (**3**), 2-allyl-4,5-dimethoxyphenol (**4**), *cis*-coniferyl alcohol (**5**), *trans*-coniferyl alcohol (**6**), *trans*-3,4-dimethoxycinnamyl alcohol (**7**), and *trans*-3,4-dimethoxycinnamyl acetate (**8**) by direct comparison of GC-MS data with those of authentic samples.

Methyl eugenol (**2**) and *trans*-coniferyl alcohol (**6**) were the most abundant components in all samples of *Bu. vinaceum* flowers (Fig. 3). Besides the phenylpropanoids, 3,4-dimethoxybenzoic acid was also detected in varying quantities (not quantified) in these samples.

1. Compound **1** (eugenol) GC: Rt (min) 11.10; *m/z*(%) 164(100, M⁺), 149(35), 137(17), 131(24), 121(14), 104(14), 103(22), 91(16), 77(19).
2. Compound **2** (methyl eugenol) GC: Rt (min) 11.67; MS: *m/z*(%) 178(100, M⁺), 163(33), 147(18), 135(10), 107(21), 103(20), 91(21), 77(9).
3. Compound **3** (eusarone). GC: Rt (min) 13.77; MS: *m/z*(%) 208(100, M⁺), 193(53), 177(20), 165(14), 133(10), 124(10), 105(7), 91(9), 77(7).
4. Compound **4** (2-allyl-4,5-dimethoxyphenol). GC: Rt (min) 14.33; MS: *m/z*(%) 194(100, M⁺), 179(87), 163(9), 151(12), 123(31), 91(17), 77(12), 69(12).
5. Compound **5** (*cis*-coniferyl alcohol). GC: Rt (min) 14.93; MS: *m/z*(%) 180(70, M⁺), 152(15), 147(10), 137(100), 124(42), 119(23), 103(15), 91(32), 77(19).
6. Compound **6** (*trans*-coniferyl alcohol). GC: Rt (min) 15.67; MS: *m/z*(%) 180(82, M⁺), 152(10), 147(10), 137(100), 124(48), 119(24), 103(12), 91(31), 77(14).
7. Compound **7** (*trans*-3,4-dimethoxycinnamyl alcohol). GC: Rt (min) 16.08; MS: *m/z*(%) 194(97, M⁺), 177(14), 165(17), 161(23), 152(19), 151(100), 138(55), 119(16), 107(12), 91(33), 77(28).
8. Compound **8** (*trans*-3,4-dimethoxycinnamyl acetate). GC: Rt (min) 17.40; MS: *m/z*(%) 236(100, M⁺), 193(41), 177(59), 165(31), 146(59), 138(18), 119(10), 105(10), 103(10), 91(16), 77(9), 43(26).

Headspace Analysis

Emanation of methyl eugenol (**2**) was confirmed by GC-MS analysis of the headspace of a whole flower of *Bu. vinaceum* (diagnostic ion mass chromatogram: *m/z* 178, 163, 147, 107, 103, and 91; Rt = 11.67 min). Although the exact emission rate could not be determined due to low recoveries, methyl eugenol emanation from a flower (1 and 2 d old) was roughly

◀ **Fig. 1** Flowers of *Bulbophyllum vinaceum* showing the role of “spring-loaded” lip before and after pollinarium removal by a fruit fly, *Bactrocera dorsalis*. Bar=1 cm. (a) Flower with closed lip—snapped at an angle to show anther with partially hidden yellow pollinia. Hinged lip (yellow arrow) in a normal closed position, exposing its whitish ligament—“spring loaded” hinge (white arrow). (b) A male fruit fly feeding on floral volatiles near the base of closed lip that hides the floral anther and stigma. (c) Floral lip forced open by the weight of a male fruit fly (on the right edge of lip) to expose the protruding opaque bulbous swelling of hamulus (white arrow) attached to the partially exposed yellow pollinia underneath the anther cover (yellow arrow). (d) A male fruit fly perched on the floral lip before being toppled into the column cavity to remove pollinarium. Only at this instance that the bulbous swelling of the hamulus (white arrow), attached to yellow-orange pollinia partially hidden by anther cover (yellow arrow), is exposed. (e) Lateral view of flower with posterior view of male fruit fly, bearing the freshly removed pollinarium [consisting of a pollinium package (white arrow) supported by hamulus/pollinia stalk (pink arrow)], ready to dismount from the opened lip. A pollinium package has two lobes—each with a pair of pollinia. (f) Lateral view of the male fruit fly, bearing the freshly removed pollinarium, on the back of medial sepal

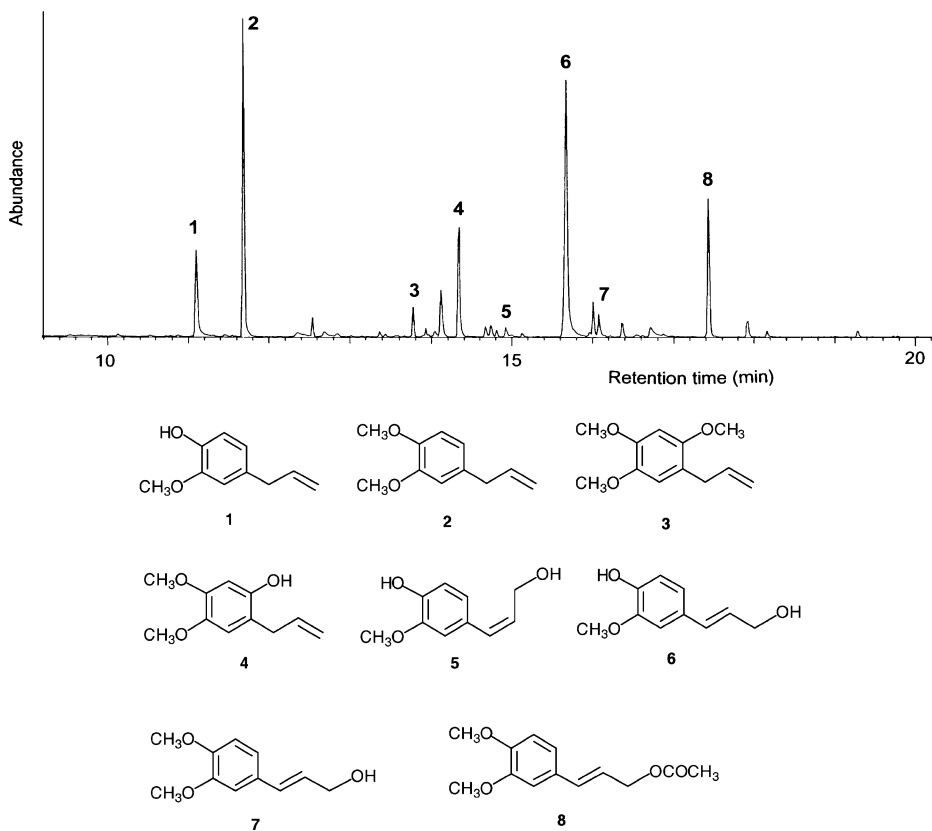


Fig. 2 Top: Gas chromatogram of a floral extract of *Bulbophyllum vinaceum* [MS total ion current: HP-5MS, cross-linked 5% PH ME siloxane, 30 m×0.25 mm, 0.25 μm film thickness, programmed from 60°C (2 min holding) to 240°C at a rate of 10°C/min]. Bottom: Chemical structures of phenylpropanoid volatiles detected in the floral tissues of *B. vinaceum*. 1: Eugenol; 2: methyl eugenol; 3: 5-allyl-1,2,4-trimethoxybenzene (eusarone); 4: 2-allyl-4,5-dimethoxyphenol; 5: *cis*-coniferyl alcohol, 6: *trans*-coniferyl alcohol, 7: *trans*-3,4-dimethoxycinnamyl alcohol; 8: *trans*-3,4-dimethoxycinnamyl acetate

Fig. 3 Contents (μg/flower) of phenylpropanoid volatiles in flowers of *Bulbophyllum vinaceum* (N=11). Compounds 1–8 as in Fig. 2

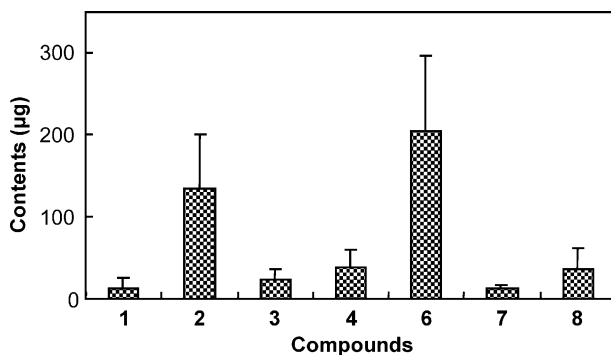
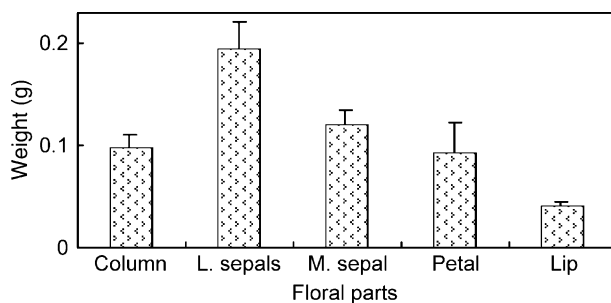


Fig. 4 Weights of various floral parts of *Bulbophyllum vinaceum* ($N=4$)



estimated in a range of 10–100 ng/2 hr. No other phenylpropanoid analogs contained in the flower tissues were detected.

Distribution of Phenylpropanoids in Floral Organs

The average weight for each of the five floral parts, i.e., petals, lateral and medial sepals, lip, and column is shown in Fig. 4; the mean concentration (ppm) of individual components in each organ was obtained to compare the relative quantities among the various floral parts (Fig. 5). Although the lip was the lightest in weight, its relative concentrations of the major compounds (2, 4, 6, and 8) were highest among the five organs. These compounds were detected in lower quantities in the petals. Eugenol (1) was found almost exclusively in the petals.

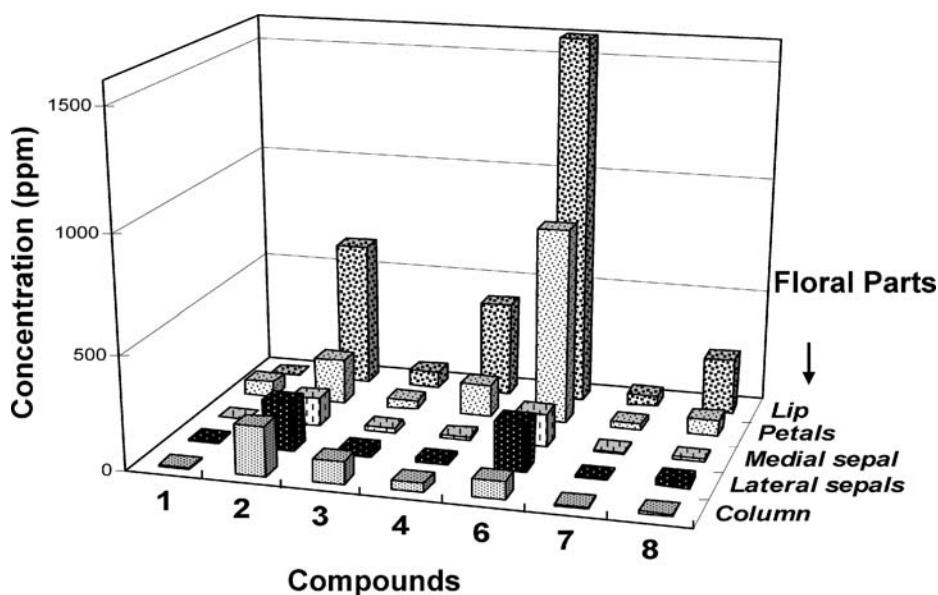


Fig. 5 Concentrations (ppm) of phenylpropanoids in each floral part ($N=3$). Compounds 1–8 as in Fig. 2

Accumulation of Floral Volatiles in Rectal Glands

A laboratory-reared virgin male of *B. dorsalis*, which received the pollinarium on his thorax after voracious feeding on *Bu. vinaceum* flower, sequestered 2.3 and 3.0 μg of compounds **4** and **6**, respectively, in the rectal glands.

Of the 10 males captured on a *Bu. vinaceum* flower under natural conditions in Sabah, eight flies were identified as *B. dorsalis* and two as *B. unimacula*. Among the *B. dorsalis* males, six of them possessed relatively low quantities of compound **4** ($0.51 \pm 0.18 \mu\text{g}/\text{male}$) and a trace amount of **6** (less than $0.10 \mu\text{g}/\text{male}$), whereas the other two possessed 24.5 and 45.7 μg of compound **4**, and 0.5 and 4.9 μg of compound **6**, respectively. Both *B. unimacula* males captured on the same flower possessed a trace amount (approx. $0.03 \mu\text{g}$) of **4**, and 0.99 and 0.49 μg of **6**, respectively, in addition to large quantities of two sesquiterpenic compounds tentatively identified as β -caryophyllene and humulene. These flies appeared to have obtained large quantities of the sesquiterpenes from other plant sources prior to visiting *Bu. vinaceum*.

Discussion

Both *Bu. vinaceum* and *Bu. apertum* flowers have architectures that include prominently protruding hamuli and hinged floral lips. According to Rasmussen (1985), the function of the prominent hamulus for *Bu. ecorutatum* (= *Bu. apertum*) is unknown. However, observations of *Bu. vinaceum* presented here demonstrate the function of the hamulus as a “crowbar” to pry the pollinia out of its protective anther cover.

The morphology of the floral lip in *Bu. apertum*—except for the fact that it is hinged—is like any other petal, i.e., in the open position. Hence, the reproductive organs are always exposed and can be easily or accidentally removed by any part of the fly. Furthermore, the pollinarium once removed can just as easily be deposited anywhere on the petals or medial sepals of the same flower, leading to a total wastage of pollen (Tan and Nishida, 2005). Hence, the function of the “spring loaded” ovately shaped floral lip of *Bu. vinaceum*—always in the closed position—presumably is to protect the pollinarium from accidental removal.

A weight comparison of the portions of the *Bu. vinaceum* lip on either side of the hinge/pivot point demonstrated the presence of a nominal spring restoring force that retains the lip in its normally closed position (Tan, unpublished data). Based on two different methods of calculations performed on preserved flowers, the rotational force required to catapult the fly to the observed velocities was estimated to be between 117 and 122×10^{-9} N m (Tan, unpublished data). Additionally, the fact that the highest concentration of chemical reward occurs on the adaxial side of the lip ensures that an attracted fly will eventually end up here.

Of all the other *Bulbophyllum* species studied so far, this catapulting lip and stiff crowbar-like hamulus is unique to *Bu. vinaceum*. In contrast, other *Bulbophyllum* species, which possess flexible stipes instead of hamuli, such as *Bu. macranthum*, *Bu. cheiri*, and *Bu. patens*, have floral lips that act like seesaws and are normally in an open position. In *Bu. macranthum*, the slippery inner edges of the lateral sepals cause the fly to fall onto the lip, which then tips into the closed position sending the fly—abdomen first—into the column cavity (Ridley, 1890). For *Bu. cheiri* and *Bu. patens*, the floral lip is also usually in an opened position seesawing gently with the breeze. The fly settles on the lip to feed on chemical attractant, and as it moves past the point of imbalance the lip tilts suddenly to a closed position. Thus, for these two species, the fly is sent headfirst into the column cavity

(Tan and Nishida, 2000; Tan et al., 2002). The only other species investigated with a hamulus is *Bu. apertum* (Tan and Nishida, 2005). However, in this flower the hamulus is exposed because of the absence of a spring loaded lip; instead, it has a small triangular seesaw lip that is not large enough to protect the flower's sexual organs.

In *Bu. vinaceum*, the speed of pollinarium detachment and precision of attachment to the fly's dorsum is remarkable. This dynamic process is likely assisted by the stiff hamulus, which allows the momentum of the catapulted fly to be converted into a force that pries out the pollinia from the anther. Conversely, pollinarium removal in *Bu. baileyi* is a much slower process, taking at least 4 orders of magnitude longer (up to 46 min). In this process, the fly adheres to the stipes of the pollinarium, which is attached to the anther, and is hence suspended from the pollinarium. It has to struggle vigorously with wing and leg movements, occasionally clinging onto the small seesaw lip, in order to free itself, and thus, in the process, loosens the pollinia from the anther (Tan, unpublished observation). The adhesion of the *Bu. baileyi* pollinarium to the fly is so strong, that it is believed to be permanent, as reported by Smythe (1969), where a fly—after depositing the pollinia onto the stigma of another flower—remained attached to the pollinarium and suspended from the stigmatic surface until death.

Methyl eugenol (**2**) and *trans*-coniferyl alcohol (**6**) are the two volatile components with the highest concentrations in *Bu. vinaceum* flowers, accompanied by a series of related phenylpropanoids (**1**, **3**, **4**, **5**, **7**, and **8**). However, in another methyl eugenol-producing species, the fruit fly orchid, *Bu. cheiri*, the floral content of **2** varies from 85% to 90% of the total phenylpropanoid volatiles, with some minor ingredients similar to those found in *Bu. vinaceum* (Tan et al., 2002; Nishida et al., 2004). While **2** is known as a potent male attractant for *B. dorsalis*, compounds **4** and **6** and their derivatives **7** and **8** are moderate attractants for male flies and induce compulsive feeding behavior (Tan and Nishida, 1998; Khoo et al., 2000). Compounds **4** and **6** quickly build up in the rectal glands, when the male flies ingest **2** (Nishida et al., 1988). In contrast, if males are fed artificially either with compounds **4** or **6**, these compounds are sequestered unchanged in the rectal glands (Nishida et al., 2004). Compounds **7** and **8** are hydrolyzed by the flies to **6** after ingestion (Nishida et al., 1997). Both rectal compounds **4** and **6** serve as a sex pheromone and attract females during the courtship period at dusk (Tan and Nishida, 1998; Khoo et al., 2000). Thus, the phenylpropanoid cocktail in the *Bu. vinaceum* flower provides both intact pheromone materials (**4** and **6**) and their precursors (**2**, **7**, and **8**) to the male flies. According to the headspace analysis, methyl eugenol (**2**) was the major volatile. The emission rate is sufficient to attract male *Bactrocera* flies to the flower as the flies are extremely sensitive to this compound (ca. 1 ng spotted on a TLC plate was able to attract one or two native *B. dorsalis* males in the field; Tan, unpublished data). It is likely that the flower emits only a small amount of **2** that acts as a long distance signal, and then, provides a variety of less volatile compounds (e.g., **4**, **6**, **7**, and **8**) in its floral tissues that guide the attracted fly towards the lip area where the concentration of phenylpropanoid ingredients is the highest. This demonstrates that the floral volatiles act as synomone in this interaction in which both flies and orchid flowers gain reproductive benefits, i.e., the flower's pollinarium is transported for pollination, and the male fruit fly is rewarded with the chemicals that are used for the production of its sex pheromone (Tan and Nishida, 2000; Tan et al., 2002).

The behavioral and physiological activities (or functions) of phenylpropanoids (**1**–**8**) found in *Bu. vinaceum* flowers—evaluated from previous work on *B. dorsalis* (Nishida et al., 1988, 1997; Tan and Nishida, 1998; Khoo et al., 2000) are as follows: compounds highly attractive to males: **2**; compounds moderately attractive to males: **4**, **6**, **7**, and **8**; compounds marginally attractive to males: **1**, and **3**; compounds with potent phagostimulant activity toward males: **2**,

4, 6, 7, and 8 (5 not tested); compounds sequestered into the male rectal organ after ingestion of methyl eugenol (2): 4 and 6; compounds biotransformed to coniferyl alcohol (6) in the crop after ingestion: 2, 7, and 8; compounds attractive to females during courtship period as a sex pheromone: 4 and 6.

The reason why *Bu. vinaceum* flowers produce such an assortment of phenylpropanoids—in addition to the most attractive compound (2)—is puzzling. The less volatile phenylpropanoids may function as short-range attractants (olfactory) or phagostimulants (gustatory) to arrest male flies, at the same time securing the endowment of the pheromone precursors to the faithful pollinators. Although the flower produces components 4 and 6, which attract and arrest females during courtship and hence would serve as specific “female attractants,” the flower has never been observed to attract female fruit flies—not even during dusk when females are the most sensitive to these chemicals. This absence of attracted females despite the presence of female attractants is an unusual phenomenon—perhaps, it is because floral emission rate of compounds 4 and 6 declines through the day. Furthermore, it is possible that some of the compounds are simply by-products of other processes and, as such, do not have an explicit (or evolved) function in fly attraction. Hence, at this point, we do not have a conclusive answer for the function(s) of all the compounds produced by *Bu. vinaceum*. However, we believe that the original mutualistic interactions between *Bu. vinaceum* and its “true” partner (probably *B. dorsalis*) could shed some light. The hypothesis that the flower and its true partner coevolved, thus allowing the flower to blend the fly’s favorite cocktail in order to charm the male fruit flies is currently being investigated.

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Introduction

Phytoecdysteroids (PE) are plant secondary compounds that include more than 200 molecules (Dinan et al., 2001). One of the most common is 20-hydroxyecdysone (20E). Phytoecdysteroids are found in about 5–6% of the species in most plant genera (Dinan 1998). Since PEs are found in primitive plants such as ferns, it was assumed that these secondary compounds appeared very early in plant evolution. PEs are structural analogs of the molting hormone of insects and are usually considered as defensive compounds against insects. Sensitive species living on plants with low levels of PEs, such as *Bombyx mori* or *Pectinophora gossypiella* (Kubo et al., 1983), are disturbed in their growth and food intake when PEs are incorporated into their diet (Tanaka and Takeda, 1993). *Manduca sexta*, which feeds on *Solanaceae* where some species contain significant level of ecdysteroids, is tolerant to PEs (Dinan 1998). Other polyphagous species are resistant, such as several Noctuidae including *Spodoptera littoralis* (Blackford et al., 1996).

Given the large botanical distribution of PEs, we hypothesize that phytophagous insects have been confronted with these defenses for a long time and that they may have evolved different strategies to cope with them. One way to escape PE intoxication by larvae or juvenile stages is to avoid exposure. This hypothesis is supported by the observation that several larval Lepidoptera are able to avoid feeding on plants or diets treated with PEs (Tanaka et al., 1994; Marion-Poll and Descoins, 2002). This is true even for resistant species such as *S. littoralis* since neonate larvae avoid PE-treated diet (Marion-Poll and Descoins, 2002). This avoidance is mediated by deterrent taste neurons that detect PEs at a low threshold (Ma 1969; Tanaka et al., 1994; Descoins and Marion-Poll, 1999). If PEs exert a selection pressure on phytophagous insects, one would expect females to avoid laying eggs on plants producing them in order to maximize the survival chances of their progeny and, thus, limit exposure of neonate larvae to the toxicant.

How could females achieve such detection? An obvious strategy would be to use the same type of taste receptors as those expressed in larvae, in order to detect PEs on plants' surfaces or within their tissues. To begin testing whether this strategy could be used in polyphagous insects, we chose the European grapevine moth (EGVM) *Lobesia botrana* Denis & Schiffermüller (Tortricidae, Lepidoptera) as a model insect. Although this species is best known as a major pest of grapes, it has been found on more than 20 plant species including rosemary, olive trees (only flowers), privet, or *Daphne gnidium*, which is sometimes considered as its ancestral host plant (Bovey 1966; Maher 2002; Thiéry 2005; Maher and Thiéry, 2006). In this species, first instars can hardly disperse and would not be able to compensate female mistakes (Torres-Vila et al., 1997; Thiéry and Moreau, 2005;). Female decisions to oviposit on different plants or different phenological stages of grapes (Thiéry and Gabel, 2000; Maher et al., 2001) have a strong influence on the fitness of future larvae. Except for grapes, most of the host plants of *L. botrana* are toxic or known for their pharmacological use, but none has been reported to produce PEs in large amounts (Dinan, personal communication). We expect all stages of EGVMs to be sensitive to PEs and possibly to avoid them, a view that is supported by earlier observations on larvae that showed that PEs, and particularly 20E, added to the diet, caused numerous abnormalities in growth, development, and metamorphosis (Mondy et al., 1997). Behavioral studies and electrophysiological observations indicated that larvae do detect PEs from plant extracts, in their diets, by using their taste receptors (Mondy et al., 1999).

In this work, we examined the feeding behavior and oviposition responses of EGVM to 20E and evaluated the sensory responses of taste sensilla of fifth instar and adult females to 20E. In doing so, we described the distribution of the contact chemoreceptors present on the

foreleg of the females of this species, and propose to segregate these taste receptors into three functional groups, based on their responses to a limited set of stimuli.

Methods and Materials

Insects A colony of *L. botrana* issued from insects sampled in Sauternes vineyard in 1998, was reared on a semisynthetic diet (Maher, 2002) at INRA Bordeaux. They were maintained without diapause at $22 \pm 1^\circ\text{C}$ under a 16:8 L/D photoperiod and at 60–65% relative humidity. Electrophysiological and behavioral observations were performed on adults from this strain in 2002–2004 and on larvae in 2005.

Chemicals 20-Hydroxyecdysone was purified from *Leuzea carthamoides* (95% min purity with minor ecdysteroids mixtures of polypodine B, ajugasterone C, and inokosterone; SciTech, Prague, Czech Republic, and R. Lafont, Univ. Paris 6). It was dissolved in ultrapure water or in ethanol according to the different bioassays. For electrophysiology, test solutions were dissolved in 10^{-4} M potassium chloride (KCl, Prolabo) to ensure proper electrical conductivity. 20E was used at concentrations ranging from 10^{-8} to 10^{-3} M for electrophysiological recordings and 10^{-7} to 10^{-2} M for behavioral tests.

Two-Choice Feeding Experiments To determine 20E effects on the feeding behavior of *L. botrana* larvae, two-choice tests were conducted. Two disks of artificial food were placed diametrically opposed on the bottom of a plastic Petri dish. The size of the food disk and of the feeding arenas (Petri dish) varied according to the stage of the larvae (L2=8 mm diam \times 2 mm high food disks into a 3-cm-diam Petri dish; L3=12 mm diam \times 2 mm high food disks into a 5.5-cm-diam Petri dish). One disk was treated with 20E 10^{-3} M in ethanol (12 μl for L2 and 22 μl for L3) and the other with ethanol only (control disk). Given the volume of the diet offered to the larvae, this corresponds to 49 $\mu\text{g}/\text{cm}^3$. A control test was conducted each time in parallel using disks treated with solvent only. Assuming that the solvent had evaporated after 30 min, four larvae starved for 4 hr were introduced into the center of each arena. We measured the number of larvae located on the disks or within a radius of 1 cm around the disks, after 2, 24, 48, and 72 hr. These experiments were repeated 15 times (240 larvae tested). The results were analyzed with nonparametric statistics. Preferences between treated and nontreated disks were compared by using the Wilcoxon test (WX). To determine if there was an increase or a decrease in the deterrency, the number of larvae placed onto or close to the treated disk at different times was analyzed with the Mann–Whitney (MW) test.

Oviposition Tests Oviposition responses of gravid females of *L. botrana* were examined by using a two-choice bioassay (Maher and Thiéry, 2004a). Test solutions were sprayed onto rows of four glass spheres (diam: 1.6 cm, spaced by 2 cm), placed on a glass plate (75 \times 25 mm). Each row was treated either with 0.5 ml of diluted 20E (ranging from 10^{-7} to 10^{-2} M) or with 0.5 ml of pure water (control). These spheres were dried in an incubator at 40°C for 90 min. Given the size of the spheres, the resulting concentration of 20E ranged from 0.8 ng to 80 $\mu\text{g}/\text{cm}^2$. Two rows of spheres, spaced by 6 cm, were placed into the bottom of a plastic box. This box was covered with baize, which prevents oviposition on the experimental area except on the spheres. After introducing a single female per box, each box was closed with a veil and placed into an environmental chamber maintained at 16:8 L/D photoperiod

and 23°C. Females were removed 16 hr later, and the number of eggs deposited on the spheres was counted.

Experiments were done on 3–4 day old females. Recently emerged females and males (less than 12 hr) were grouped one night in a cage. Females were isolated the following night in glass tubes and checked for oviposition in the morning. We used only females that laid eggs during this first night. By using this procedure, more than 90% of females were ready to oviposit during the experiments. Experiments that started at 4 P.M. were run on individual females with a minimum of 20 females per dose.

Oviposition scores were compared with nonparametric statistics. The total number of eggs laid during one experiment was compared by using the MW test. The preferences between treated and nontreated items in each experiment were compared by the WX test. For control experiments, the spheres were arbitrarily assigned a label A or B before experiments. Pilot experiments showed that the females ignored such labels. For convenience, data are presented in the text as mean±SE of the mean.

Electrophysiology Recordings from fifth instars were obtained from isolated heads, mounted on a reference silver electrode. The preparation was oriented in such a way that the galea was extruded, thus giving access to the lateral and medial sensilla styloconica.

Adult females were briefly narcotized with carbon dioxide, decapitated by using fine scissors, and then fixed onto a polystyrene block. A reference electrode, filled with Ringer's solution, was inserted into the abdomen. One of the prothoracic legs was taped to the support in order to expose the sensilla from the ventral side of the fifth tarsomere. Recordings were performed on sensilla chaetica type b (Maher and Thiéry, 2004b) by capping them with a capillary tube (10 µm tip diam) containing the test solution, filled just before recording.

Electrical signals were recorded with an amplifier (TastePROBE DLP-02; Syntech, Hilversum, the Netherlands) with automatic offset compensation (Marion-Poll and Van der Pers, 1996) and further amplified and filtered (CyberAmp, 320, Axon Instruments; gain: 1000; eight poles Bessel band-pass filter: 0.1–30 to 2800 Hz). Data were recorded and stored on a computer with a 16-bit A/D conversion card (DT9803; Data Translation, USA) under the control of a custom software, dbWave (Marion-Poll 1996). Each recording lasted 2 sec and was triggered by a pulse delivered by the amplifier on the initial contact of the electrode with the sensillum. Recordings were analyzed by using dbWave, in order to detect and sort spikes according to their amplitude and shape using interactive procedures. Responses to the different stimuli were evaluated by counting the total number of spikes during the first sec of the recording.

Series of recordings were done with 10^{-4} M KCl (also used as electrolyte of nonconductive solutions) and followed by an ascending series dilutions of 20E (10^{-8} to 10^{-3} M step 10). Consecutive stimuli on a sensillum were applied with an interval of at least 2 min in order to avoid adaptation. For adults, the position of each recorded sensillum was carefully noted in order to compare our results with earlier observations (Maher 2002; Maher and Thiéry, 2004b; Maher et al., 2006).

Results

Two-Choice Feeding Experiment Larvae preferentially fed on nontreated disks rather than on disks treated with 20E (Fig. 1). The difference was highly significant for all observation times for L2 and L3 larvae. Both instars spent significantly more time on or close to the food disk (treated or not) than wandering elsewhere, except during the first 24 hr. Mann–

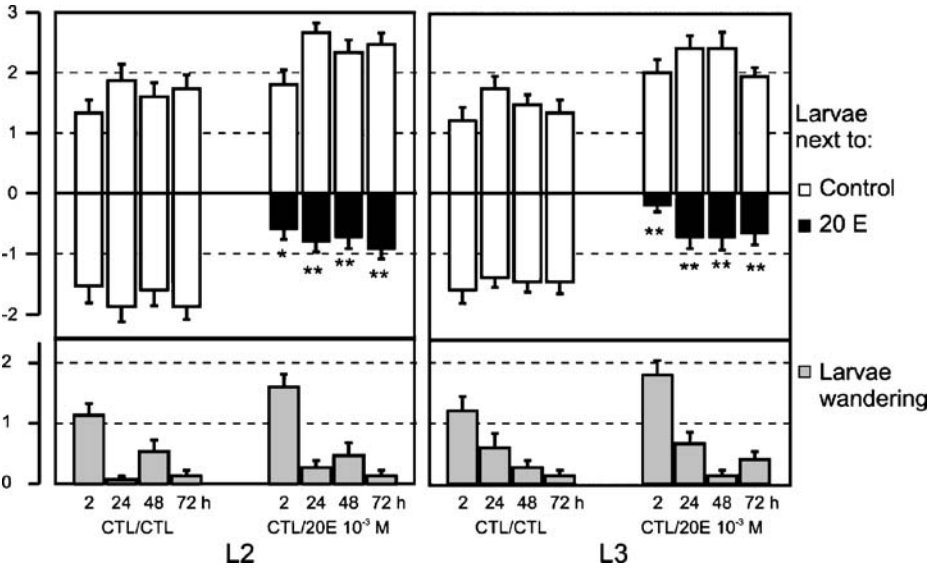


Fig. 1 Behavioral choices of second and third instars of *Lobesia botrana* between two food disks treated or not with 20E. White bars: control diet (solvent alone); black bars: treated diet (20E 10^{-3} M). Bars indicate the number of larvae (mean±SEM) found on or close to each disk at 2, 24, 48, and 72 hr after the start of the experiments. $N=15$ (with four larvae each). Wilcoxon test, 20E vs. control * $P<0.05$, ** $P<0.01$

Whitney test showed that there was no increase or decrease in deterrence according to time. In the control tests (using disks with only solvent), L2 and L3 larvae fed equally on both disks.

Sensitivity of Larvae to 20E In fifth instars, a strong activity in response to 20E was found in the medial and lateral sensilla (Fig. 3). Responses originated from one cell that increased its activity from 10 to 80 spikes sec^{-1} , respectively, for 10^{-5} to 10^{-3} M of 20E (Figs. 2 and 3). At lower concentrations (10^{-6} and 10^{-5} M), the response was initiated after a latency of 50–100 ms as in the adults.

Fig. 2 Response samples of fifth instar lateral sensilla to 20E 10^{-4} M and 10^{-3} M. Trace duration = 1 sec. Vertical bar = 1 mV, horizontal bar = 100 ms

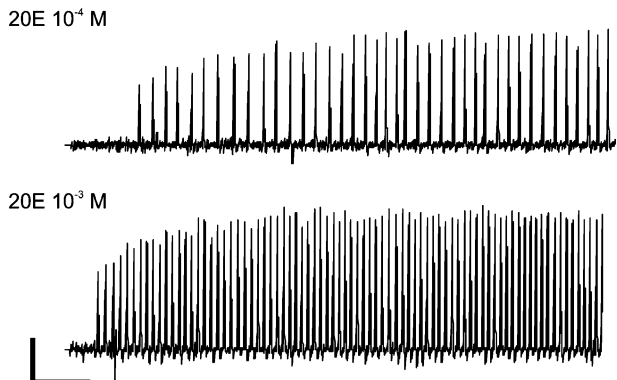
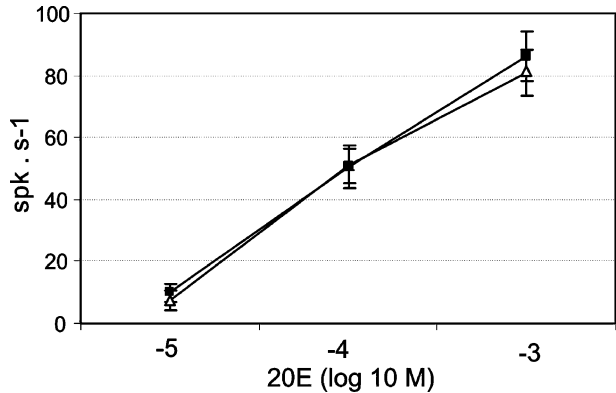


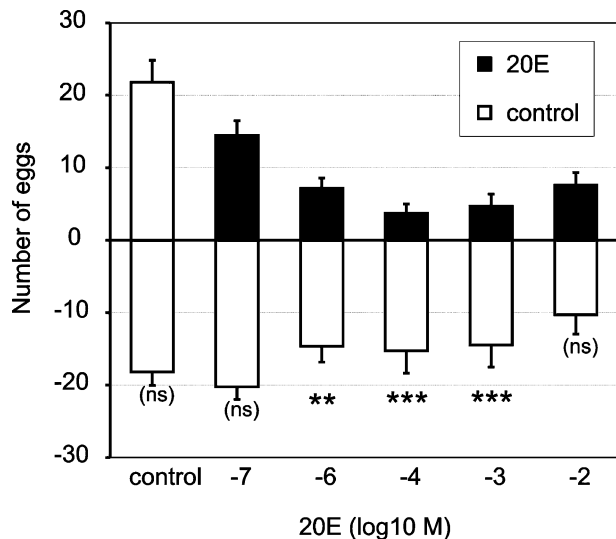
Fig. 3 Electrophysiological responses of fifth instars to ascending concentrations of 20E. Black squares: responses from medial sensilla styloconica; Open triangles: responses from the lateral sensilla styloconica. Each data point represents the average number of action potentials during the first second of the recordings (mean \pm SEM; $N=10$ stimulations)



Oviposition Tests Females laid fewer eggs on treated glass spheres (T) than on nontreated ones (NT) at intermediary doses of 20E, namely, 10^{-6} M (T: 8.1 ± 6.9 vs. NT: 16.7 ± 9.8 ; WX, $P=0.003$), 10^{-4} M (T: 4.7 ± 6.9 eggs vs. NT: 19.1 ± 14.9 eggs; WX, $P<0.001$) and 10^{-3} M (T: 5.2 ± 8.6 eggs vs. NT: 16.5 ± 15.1 eggs, WX, $P<0.001$). This was not the case for low 20E concentrations (10^{-7} M) nor for the highest (10^{-2} M), where the scores were not statistically different between the treated and untreated spheres (10^{-7} M: T: 14.5 ± 10 vs. NT: 20.2 ± 8.6 ; 10^{-2} M: T: 11.2 ± 8.2 vs. NT: 15.1 ± 14 (Fig. 4). The behavioral threshold to 20E is close to 10^{-6} M, a concentration that corresponds to 30 ng of 20E per sphere or 0.037 ng per mm^2 . In addition, the total number of eggs deposited on both glass spheres is significantly reduced at concentrations higher than 10^{-6} M (Mann Whitney: NT vs. 10^{-6} M: $P=0.002$; NT vs. 10^{-4} , $3 \cdot 10^{-4}$ M: $P=0.008$).

Tarsal Taste Sensilla On the ventral side of the fifth tarsomere, adults of both sexes bear a ring of 10 sensilla and four medial sensilla that were described as sensilla chaetica type I, or “sensilla b” (Maher, 2002; Maher and Thiéry 2004b). Although they look morphologically

Fig. 4 Behavioral choices of females *L. botrana* given a choice between glass surrogates treated or not with 20E (from 10^{-7} to 10^{-2} M). Number of eggs laid by one female according to the substrate ($N>20$). Wilcoxon test: 20E vs. control $**P<0.01$, $***P<0.001$



identical, they diverge in their responses to KCl. We propose to distinguish three groups among them (Fig. 5):

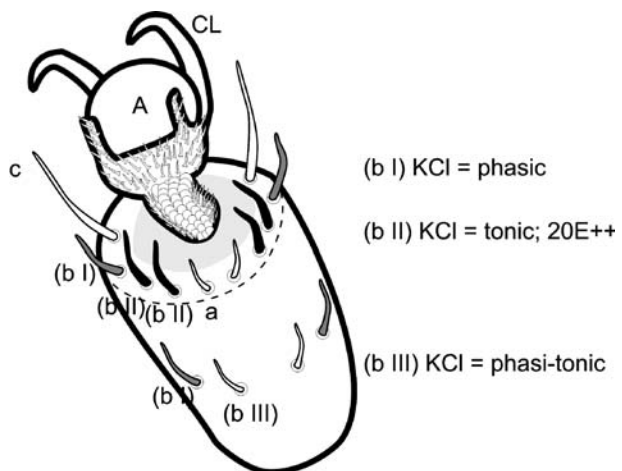
- (a) Group I sensilla (Fig. 6a) that fires a few spikes of large amplitude at the beginning of the stimulation (amplitude = 1.5–2 mV; 1.7 ± 0.2 spikes sec^{-1} (average \pm SEM), $N=32$).
- (b) Group II sensilla responds tonically with a single class of spikes (0.3 mV; 57.2 ± 2.7 spikes sec^{-1} , $N=27$).
- (c) Group III sensilla (Fig. 6b) that responds phasitonicly with two classes of spikes: one of large amplitude (1–2 mV; 2.6 ± 0.3 spikes sec^{-1} , $N=6$ recordings) and a second one of smaller amplitude (0.2 mV; 48.4 ± 6.4 spikes sec^{-1}). On some recordings, this second cell started to fire after a variable delay.

The large amplitude spikes observed at the beginning of the recordings (Fig. 6a, b) differ from the activity of mechanoreceptors. When a deflection was imposed on the hair by moving the capping electrode laterally, these mechanoreceptors elicited spikes of much smaller amplitude (1–2 mV) superimposed on a downward deflection of the baseline during the bending (data not shown). Only sensilla of group II responded to 20E; therefore, all subsequent recordings were performed on them.

Sensitivity of Adults to 20E Although two spike amplitudes were observed in the responses of group II sensilla to 20E, we consider that only one neuron was activated (Fig. 7a, b). One neuron consistently fired spikes of medium amplitude (0.3–0.4 mV), but its activity did not vary with changes of 20E concentration (Fig. 8a), except by a small decrease of activity at 10^{-3} M (46.5 ± 3.02 spikes sec^{-1}). This cell fired also in the presence of NaCl or of sugars (data not shown).

A second cell fired spikes of smaller amplitude (0.2–0.3 mV) in the presence of 20E, starting at concentrations of 10^{-8} or 10^{-7} M, depending on the preparation. Its activity increased with 20E concentration, ranging from 8.25 ± 1.61 spikes sec^{-1} at 10^{-7} M to a maximum of 22.5 ± 2.79 spikes sec^{-1} at 10^{-5} M (Fig. 8b). Its response decreased from 10^{-4}

Fig. 5 Schematic diagram of the fifth tarsomere of *L. botrana* viewed from the ventral side and sensilla types according to their shape and their electrophysiological responses. CL: claw; A: arolium; a, c: sensilla chaetica type II; bI, II, III: sensilla chaetica type I



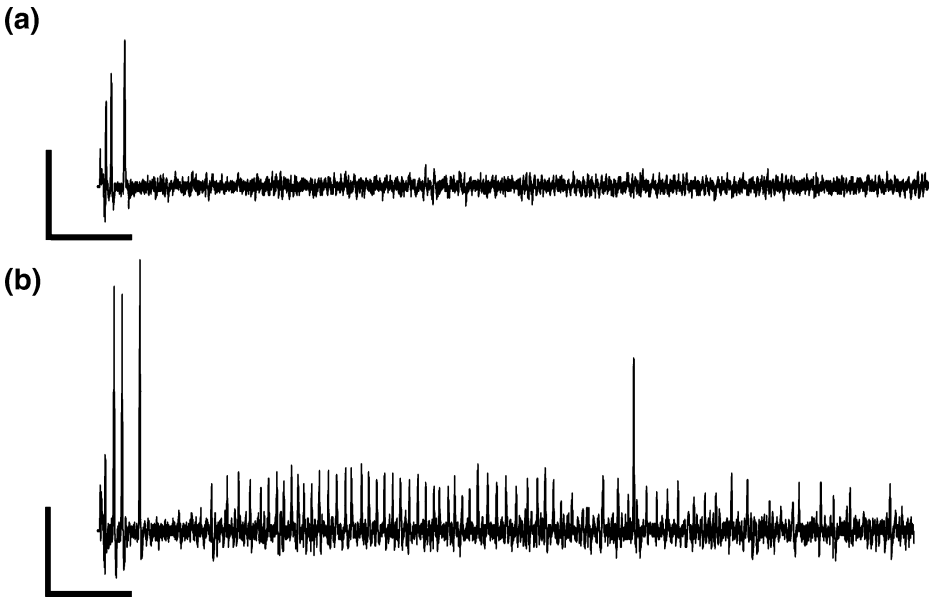


Fig. 6 Response samples of “b” sensilla to 10^{-4} M KCl: (a) sensilla from group I; (b) sensilla from group III. Trace duration=1 sec. Vertical bar=400 μ V; horizontal bar=60 msec

to 10^{-3} M. The responses were tonic and regular over the observation period (2 sec). At low concentrations of 20E, it started to fire with a delay of 200–300 ms. This delay shortened at higher concentrations of 20E.

Discussion

In this work, we demonstrate that *L. botrana* adults and larvae can detect 20E. Electrophysiological recordings indicate that taste receptors responding to 20E are present

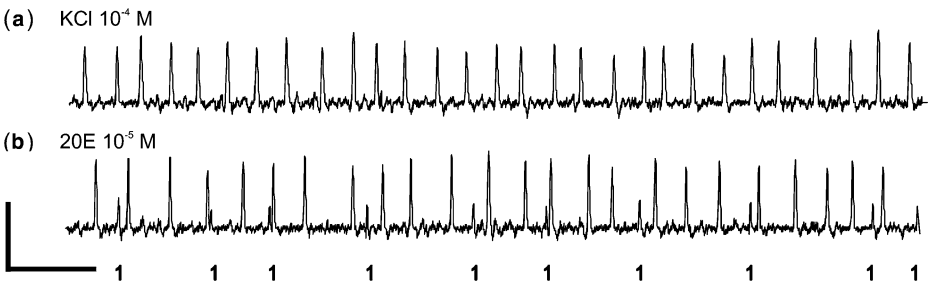
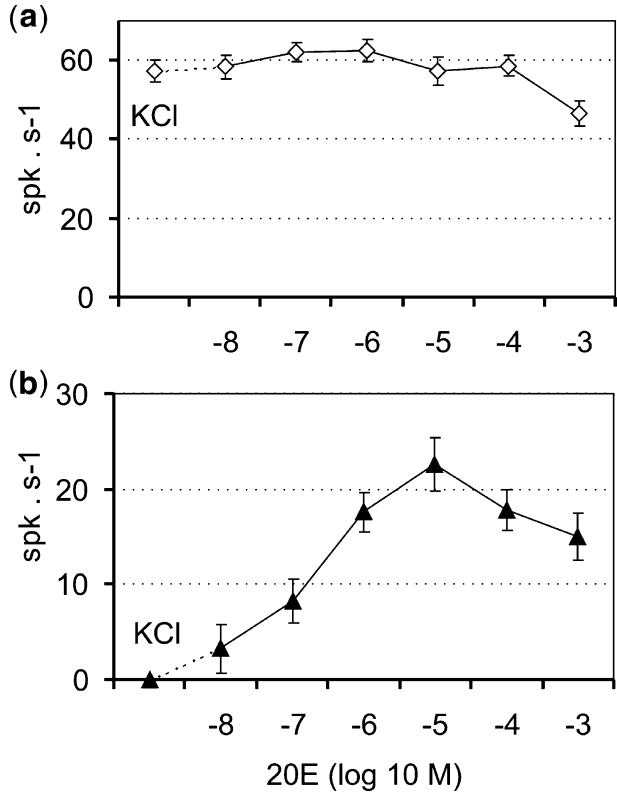


Fig. 7 Response samples of sensilla chaetica b to (a) 10^{-4} M KCl and to (b) 10^{-7} M 20E. Trace duration = 500 msec; vertical bar=400 μ V; horizontal bar=50 msec. Spikes amplitudes fall into two classes: medium-amplitude spikes (class 0) are observed in both types of recordings; small-amplitude spikes (class 1) are observed only in the presence of 20E

Fig. 8 Electrophysiological responses of sensilla chaetica type I (sensilla b—group II) to 10^{-4} M KCl and to ascending concentrations of 20E. (a) White diamonds: responses from class 0 spikes (of medium amplitude). (b) Black triangles: responses from class 1 spikes (of small amplitude). Each data point represents the average number of action potentials during the first second of the recording (mean \pm SEM; $N=12-25$ recordings)



in both stages, and our behavioral studies show that 20E has a deterrent effect on feeding and oviposition. These observations are new because the impact of phytoecdysteroids has been mostly evaluated on larval insects. Using behavioral and toxicity tests on larvae, Blackford and Dinan proposed that species can be rated as sensitive, semitolerant, or tolerant to PEs (Blackford et al., 1996, 1997; Blackford and Dinan, 1997a, b, c). Our own observations on second and third instars as well as former studies on *L. botrana* (Mondy et al., 1997) suggest that this species is sensitive to 20E. These behavioral responses are probably mediated by taste neurons located in the lateral and the medial sensilla of the maxillary palps in which we recorded vigorous and sustained monocellular responses to 20E, at a threshold of 10^{-5} M.

Because larvae are sensitive to 20E, we expected adults to avoid ovipositing on a substrate in the presence of 20E. This hypothesis was tested by monitoring eggs laid on berry surrogates treated with 20E. We observed that 20E-treated berries were avoided and that females laid fewer eggs in the presence of 20E. The most effective concentration of 20E was 10^{-4} M, which is equivalent to 6.3 μg of 20E per sphere, i.e., 0.8 μg of 20E per cm^2 . At higher concentrations of 20E, discrimination between treated and nontreated spheres was less effective. The total number of eggs deposited during the observation period decreased with 20E in a dose-dependent manner, even at higher doses when females did not seem to discriminate between treated and nontreated surfaces.

As in larvae, detection of 20E in adults is certainly mediated by taste sensilla. We studied taste sensilla located on the distal tarsi. From the 16 sensilla present on the ventral

side of the last tarsus of the prothoracic legs, only four were found to house a neuron stimulated by 20E. This neuron fired spikes of small amplitude and responded with a detection threshold of 10^{-7} M. Its response was maximal at 10^{-5} M and decreased slightly at higher concentrations. This decrease is probably attributable to adaptation and to our experimental protocol, because we presented 20E as a series of increasing concentrations. It is likely that we would need to respect resting intervals longer than 2–3 min at these high doses. While 20E stimulated this cell firing small amplitude spikes, a second cell was present in the recordings, characterized by spikes of medium amplitude. Its firing activity did not change in the presence of 20E, except at high concentrations where its activity decreased. This cell could correspond to the ubiquitous water cell found in many taste sensilla of other insects (Inoshita and Tanimura, 2006).

Our anatomical and electrophysiological observations complete earlier descriptions of taste tarsal sensilla in *L. botrana* (Maher, 2002; Maher and Thiéry, 2004b; Maher et al., 2006). By using KCl and 20E as test stimuli, the responses of these taste receptors could be classified into three groups, with only group II sensilla responding to 20E. It remains to be determined if group I–III sensilla respond to other deterrent compounds or if group II sensilla are specialized in triggering an avoidance behavior. The functional arrangement of these tarsal sensilla is reminiscent of what we found with *Ostrinia nubilalis* (Marion-Poll et al., 1992; Marion-Poll and Calas, unpublished data) and with *Drosophila melanogaster* (Meunier et al., 2003), where only a subset of the tarsal sensilla are sensitive to a given deterrent molecule. This means that behavioral decisions taken in the presence of deterrent compounds is taken from integration of responses from several types of sensilla from one or more appendages.

Although similar taste cells responding to 20E have been found in *L. botrana* larvae, as well as in larvae of several other species of Lepidoptera (Ma, 1969; Tanaka et al., 1994; Descoins and Marion-Poll, 1999; Marion-Poll and Descoins, 2002), it is the first time that responses to this compound have been demonstrated in adult Lepidoptera. We postulated that if 20E presented a negative effect on growth and development of the larvae of *L. botrana* (Mondy et al., 1997), this ecdysteroid could be perceived by adults and particularly females as a deterrent compound for oviposition. That females could detect deterrents avoided by larvae is ecologically relevant, since females are much more mobile than larvae. A similar situation was observed in *Trichoplusia ni*, where oviposition of adult females was deterred by toosendanin, a limonoid from the bark of *Melia azedarach*, which inhibits feeding of larvae of this species (Akhtar and Isman, 2003).

Although we have examined the effect of 20E on adults only in regard to oviposition, and have speculated that detection of 20E is relevant in avoiding plants that would lessen the chance of larvae survival, it is still possible that 20E could impact directly on adults, a hypothesis that was not tested in this work. Adults may need to avoid ingesting phytoecdysteroids present in the nectar of flower or from guttation liquids, especially considering that the maturation of the oocytes requires 20E. Avoiding 20E has been noted in Heteroptera, where 20E was found to deter drinking in three species among four tested (Schoonhoven and Derksen-Koepfers, 1973).

The ecological significance of 20E avoidance in the European grapevine moth may have to be considered in a broader frame than the relations between the EGVM and its host plants. Given the number of plants producing PEs in different genera and scattered observations in the literature concerning the detection of PEs, it is possible that PEs have been conserved in many phytophagous insect species, not only in larvae as discussed by Blackford and Dinan (1997a), but also in adult insects.

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Introduction

Low levels of nutrients and high concentrations of plant secondary metabolites (PSMs) often make it difficult for herbivores to meet their daily nutritional requirements. Therefore, most herbivores have adapted a generalist strategy to foraging, eating many different types of plants to meet their nutritional requirements and reduce toxic effects of PSMs (Freeland, 1991; Dearing et al., 2000; Marsh et al., 2003; Sorensen and Dearing, 2003). Out of more than 1000 mammalian herbivores known, only about 1% are considered dietary specialists, obtaining most or all of their nutritional requirements from one plant source (Freeland 1991; Dearing et al., 2000). Specialist herbivores have likely coevolved with their forage plant, developing detoxification pathways specific to its PSMs (McArthur et al., 1991; Marsh et al., 2003; Sorensen and Dearing, 2003; Sorensen et al., 2004).

Monoterpenes are bitter-tasting volatile oils, many of which are responsible for the characteristic aromatic odors of plants such as sagebrush, *Eucalyptus* spp., and conifers (Harborne, 1991). They act as feeding deterrents (Sinclair et al., 1988; Gershenson and Croteau, 1991; Meyer and Karasov, 1991), and at higher concentrations can cause acidosis (Cork and Foley, 1991), destroy or inhibit growth of rumen flora (Nagy et al., 1964; Burritt et al., 2000), irritate mucous membranes, cause neurotoxicity, diuresis and nephritis (Dearing et al., 2000), and require animals to spend additional energy in detoxification (Sorensen et al., 2005). In some instances, terpenes themselves may not be toxic, but as volatile compounds act as an easy-to-detect cue for more potent PSMs they are associated with, such as formylated phloroglucinol compounds (FPCs), lactones, and phenolics (Radwan et al., 1982; Bray et al., 1991; Lawler et al., 1999).

Although most animals avoid eating large amounts of plants that contain toxic PSMs, some animals specialize on them. Koalas (*Phascolarctos cinereus*) and the greater glider (*Petauroides volans*) feed almost exclusively on *Eucalyptus* spp., known to have high levels of terpenes, tannins, and FPCs (Lawler et al., 1998; Moore and Foley, 2000; Marsh et al., 2003). Other herbivores also rely on terpene-containing plants, but to a lesser extent. For example, the terpene-containing juniper (*Juniperus monosperma*) comprises 80–95% of the diet of the Stephen's woodrat (*Neotoma stephensi*; Dial, 1988). Likewise, pygmy rabbits (*Brachylagus idahoensis*) are restricted to deep-soil sagebrush-steppe habitat, consuming up to 99% of their diet in big sagebrush (*Artemisia tridentata*) in winter and up to 50% in summer (Green and Flinders, 1980b; Thines et al., 2004), despite high concentrations of terpenoids (6–23%), and monoterpenes (1–4%) in particular (White et al., 1982b; Meyer and Karasov, 1991). Although little is known about PSMs other than terpenes and sesquiterpenes in sagebrush, 10 coumarins (Shafizadeh and Melnikoff, 1970; Brown et al., 1975) and 13 flavonoids (Brown et al., 1975; Thines, 2006) have been identified.

Several wildlife species, such as mule deer (*Odocoileus hemionus*; Cluff et al., 1982), pronghorns (*Antilocapra americana*; Ngugi et al., 1992), and black-tailed jackrabbits (*Lepus californicus*; Uresk, 1978), consume sagebrush as a portion of their seasonal diet, but no other known mammal depends on sagebrush as extensively for food as does the pygmy rabbit. Consuming moderate amounts of sagebrush (15–50% of the diet) causes digestive upset, reduced rumen motility, rumen lesions, rumenitis, and even death in mice, mule deer, sheep, and cattle (Johnson et al., 1976; White et al., 1982b; Harborne, 1991). Therefore, sagebrush is generally considered toxic to mammals, especially livestock (Burritt et al., 2000).

Although sagebrush dominates 1.1 million km² of the western United States and Canada (Meyer and Karasov, 1991), pygmy rabbits only occupy patchily distributed deep soil habitats dominated by tall, dense big sagebrush (Green and Flinders, 1980a). However,

much of this small (400–500 g), burrowing rabbit's habitat within the Great Basin of the western United States has been degraded, fragmented, and converted to other uses such as agriculture for over a century (McAllister, 1995; Federal Register, 2003). An evolutionarily distinct population of pygmy rabbits in the Columbia Basin of Washington has been listed as endangered by the U.S. Fish and Wildlife Service (USFWS; Federal Register, 2003), and USFWS has received petitions for listing the species range-wide (Federal Register, 2005). However, little is known about the extent to which pygmy rabbits depend on sagebrush for food, how they cope with a diet high in terpenes, and their requirements for energy and nitrogen (N).

In this study, we examined the nutrient requirements of pygmy rabbits and their propensity and ability to select and consume sagebrush. As a comparison, we also examined nutritional requirements and sagebrush consumption by eastern cottontail rabbits (*S. floridanus*), a larger leporid that is considered a dietary generalist, but is also known to occasionally inhabit the sagebrush-steppe region with pygmy rabbits (McAllister, 1995). We expected pygmy rabbits to eat more sagebrush relative to their size than cottontails when sagebrush was offered alone and as part of a mixed diet. We expected them to voluntarily eat enough sagebrush to meet their energy requirements and maintain body mass, and to obtain more digestible nutrients from sagebrush, but less from nonsagebrush foods, than cottontails. Finally, we expected both pygmy rabbits and cottontails to eat more sagebrush as the quality and quantity of other diet items declined.

Methods and Materials

To examine energy and protein requirements and fiber tolerances of pygmy rabbits, we conducted digestion trials with 2–5 adult pygmy rabbits (451 ± 15 g) from populations found in Idaho. All had been born in captivity as part of a captive breeding program at the E.H. Steffen Center at Washington State University (WSU). As a comparison, we also used 3–4 adult eastern cottontails (1209 ± 70 g), captured on the WSU campus. Standard husbandry practices and the experimental protocol were approved by the WSU Institutional Animal Use and Care Committee (#3097). When not used in experiments, animals were housed on soil in large outdoor pens or in stainless steel rabbit cages indoors, and fed a maintenance diet consisting of one or more completely balanced pelleted rabbit diets (e.g., LabDiet[®] 5326 Hi Fiber Rabbit, PMI Nutrition International, Brentwood, MO, USA; Bunny Basics/T[®], Oxbow Pet Products, Murdock, NE, USA). They were also fed a mix of fresh forbs and sagebrush grown in our greenhouse without pesticides, and only supplemented with N for fertilizer. Tap water, trace mineral block, and the pelleted rations were provided *ad libitum*.

In January–March 2003–2005, we conducted total-collection, single-diet digestion trials using a series of seven pelleted diets and sagebrush grown in the greenhouse and outside (Table 1). Cottontails participated in three of the pelleted trials and the greenhouse sagebrush trial. The diets ranged in fiber [24–55% neutral detergent fiber (NDF)] and N content (0.5–4.5%) (Table 1). During the trials, animals were housed individually in stainless steel digestion crates with Dri-Dek[®] flooring (Kendall Products, Naples, FL, USA) and plastic nest boxes in a covered barn. Electric heaters maintained the air temperature outside the crates at an average daily temperature that ranged from 0 to 5°C. The digestion trials lasted from 3 d (sagebrush) to 5 d (pellets) following a 10-d pretrial during which the animals were gradually introduced to the trial food by increasing its contribution to the diet, and acclimated to stainless steel digestion crates. At the end of each trial,

Table 1 Composition of pelleted rations and sagebrush (*Artemisia tridentata*) fed to captive pygmy rabbits (*Brachylagus idahoensis*) and eastern cottontails (*Sylvilagus floridanus*)

Diets	Date	Nitrogen content (% DM)	NDF (% DM)	ADL (% DM)	Gross energy content (kJ/g)
Pellet 1: WSU custom high-quality diet	3/4/05	3.1	28.5	2.6	17.8
Pellet 2: WSU custom low-quality diet	2/17/05	2.3	55.0	8.3	16.8
Pellet 3: WSU custom deer diet	2/22/03	2.8	32.3	4.5	18.2
Pellet 4: Purina [®] Advanced Nutrition Breeders Formula	1/15/04	3.4	41.5	6.7	18.3
Pellet 5: Mazuri [®] Moose Diet	1/30/03	3.2	37.7	6.0	19.3
Pellet 6: Lab Diet [®] 5326 Hi Fiber Rabbit	1/14/03	2.5	49.3	6.5	18.7
Pellet 7: WSU custom low-protein diet	3/14/03	0.5	24.2	1.4	18.3
Greenhouse sagebrush	2/8/05	4.5	41.7	10.3	18.8
Outside sagebrush	3/17/05	2.5	32.1	8.9	20.2

NDF: neutral detergent fiber; ADL: acid detergent lignin; WSU: Washington State University.

animals were moved back to their original pens and weaned back onto their maintenance diets during a 10-d post trial. Animals were weighed before, at least once during, and after each digestion trial. Food and water were offered *ad lib*, and a mineral block was provided. If an animal lost more than 20% of its pretrial body mass, it was removed from the trial.

During the sagebrush trials, fresh sagebrush was offered in the morning and augmented twice during the day as needed. To ensure that sagebrush diets were homogenous, sagebrush was grown in a dedicated greenhouse or food plot outside. Greenhouse sagebrush was grown from seed in individual containers, and watered daily. Greenhouse seedlings used in trials were 2–3 mo old and about 20 cm tall. Sagebrush grown outside in food plots were 3-yr-old shrubs that had originated from greenhouse seedlings. Only current annual growth was collected, the woody component of sagebrush was minimized, no flowers or inflorescences were included, and all leaves were consistent in size and age. Sagebrush was harvested each day immediately before feeding from several flats in the greenhouse or at least six shrubs outside. Orts were collected, weighed, and corrected for dry matter (DM), then subtracted from the forage given the previous day to determine daily DM intake (DMI) of each animal.

Feces fell to mesh screens placed below each digestion crate, urine was funneled into bottles containing ≈ 5 ml of HCl to maintain a $\text{pH} < 7$ and reduce loss of N as ammonia. Each day, the pH of urine (before it reached the HCl) was measured with a pH meter or pH paper between 0700 and 0800 hr. The remainder of the urine was collected daily and stored at -20°C . Samples of the food, feces, and orts were dried daily at 100°C for 24 hr to determine DM content. Additional samples of food and feces were dried at 60°C for 3 d and ground to pass a 1-mm screen and pooled at the end of the 3- to 5-d trial for later nutritional analysis. Samples of sagebrush intended for terpene analyses were harvested from at least six plants between 1000 and 1130 hr to avoid diurnal and interplant variation in terpene concentration (Nicholas, 1973) and stored at -40°C . We made composites of samples intended for tannin analysis from at least six plants, froze them at 20°C , and freeze-dried them before grinding.

We measured the gross energy content of food, feces, and urine by using bomb calorimetry (Table 1). Fiber composition of food and feces was determined from sequential detergent analysis (Goering and Van Soest, 1970) with filter bags, sodium sulfite, and alpha

amylase (Ankom Fiber Analyzer 200/220®; Ankom Technology, Fairport, NY, USA) to determine NDF, acid detergent fiber (ADF), and acid detergent lignin (ADL; Table 1). Kjeldahl analysis and a carbon–nitrogen analyzer (TruSpec®; LECO Corporation, St. Joseph, MI, USA) was used to determine N content of feces, food, and urine. Crude protein (CP) content (%) was estimated as 6.25 times the N content (Robbins, 1993). The capacity of condensed and hydrolyzable tannins to bind proteins in food was determined by the bovine serum albumin (BSA) method (Martin and Martin, 1982).

To extract volatile terpenes from whole plant tissue of greenhouse and outside sagebrush, we used direct simultaneous steam distillation pentane extraction of the frozen (−20°C) plant material with a Likens–Nickerson apparatus (J&W Scientific, Folsom, CA, USA). In this process, we combined plant material with 100 ml deionized water, boiled it for 20 min, and collected distillate in 25 ml of pentane with a standard added to the pentane. After drying and weighing the distilled plant material, we diluted the distillate with pentane (200 µl of sample with 800 µl of pentane) and quantified the components by comparing detector response with a standard of known quantity using capillary gas chromatography (GC) on a Hewlett-Packard 5890 Series II GC equipped with a flame ionization detector (at 230°C), 7673 A autosampler, and a ZB5 column (30 m × 0.25 mm i.d.; Phenomenex, Torrance, CA, USA) using hydrogen (13 psi head pressure) as the carrier gas. Cool on column injection (40°C) was used with a temperature program starting at 40°C, ramped at 10°C/min to 250°C, and then programmed at 40°C/min to 300°C. Data were analyzed by using Chrom Perfect Spirit v. 5.5.2 (Justice Laboratory Software, Denville, NJ, USA). We identified components of the distillate by comparing their mass spectra with spectra from the NIST02 Mass Spectral Library with Windows Search Program v.2.0 (National Institute of Standards and Technology, Gaithersburg, MD, USA). GC-mass spectrophotometry (GC-MS) was performed on a Hewlett-Packard 5840A-5985B MSD system (at 70 eV) using a ZB5 column (30 m × 0.25 mm i.d.; Phenomenex, Torrance, CA, USA) with the same temperature program as described for GC analysis.

Metabolic fecal nitrogen (MFN, g N/100 g feed) for pygmy rabbits was estimated as the negative y -intercept of the line of regression of digestible N (g N/100 g feed) against dietary N (%) for all foods. True N (%) was determined by the slope of this regression line. Similarly, the y -intercept of the regression line of urinary N (mg N/kg^{0.75}/d) against dietary N intake (mg N/kg^{0.75}/d) was used to estimate the endogenous urinary N (EUN, mg N/kg^{0.75}/d). The x -intercept of the regression line for N balance (N ingested – N excreted, mg N/kg^{0.75}/d) against dietary N intake (mg N/kg^{0.75}/d) estimated maintenance N requirements (the amount of N an animal must consume to counteract the minimum constant losses from feces and urine) of pygmy rabbits and cottontails. Minimum dietary protein requirements were derived from the equation [(EUN + MFN (DMI) – 6.25)/DMI/0.74] (Robbins, 1993). Because none of the diets had enough tannins to precipitate proteins in BSA analysis, all diets were included, but only animals gaining mass or losing <1% of their pretrial body mass per d of the trial were included in these analyses. We compared digestible N, urinary N, and N balance among pygmy rabbits and cottontails with analysis of covariance.

Maintenance energy requirements for both pygmy and cottontail rabbits in small cages were estimated from the x -intercept of the regression of average daily change in body mass as a function of digestible energy intake (DEI; kJ/kg^{0.75}/d), where DEI was the product of DMI (g/kg^{0.75}/d), gross energy content of the food (kJ/g), and apparent energy digestibility (AED, %) for each diet. The slope of this line represented body mass change per unit of additional energy intake. We compared intake and digestibility among foods between pygmy rabbits and cottontails by using a two-way analysis of variance with interactions, with Tukey's multiple comparison test at $\alpha = 0.05$.

To compare intake of sagebrush by pygmy rabbits and cottontails as a function of the amount and nutritional quality of supplemental foods, we ran a series of single- and double-choice experiments. In these experiments, we measured the rabbits' voluntary consumption of sagebrush grown in the greenhouse and outside as we varied the amount of high-quality (pellet 1: high N, low fiber) or low-quality (pellet 2: low N, high fiber; Table 1) pellets offered. We used two to three pygmy rabbits and three cottontails for these experiments. Our first experiments determined the amount of sagebrush and high- or low-quality pellets consumed when each was offered *ad lib*. In subsequent experiments, we offered rabbits pellets at 50% and 25% of their *ad lib* intake of pellets and measured sagebrush consumption. We also measured sagebrush consumption when no supplementary pellets were offered, and consumption of greenhouse and outside sagebrush when offered together with 15% of their *ad lib* consumption high-quality pellets. Because of its limited availability, we only conducted experiments with outside sagebrush with 50% *ad lib* intake of both high- and low-quality pellets for both rabbit species, and with greenhouse sage/15% pellets and no pellets for pygmy rabbits.

Food offered was weighed and corrected for DM, and orts were collected, weighed, and subtracted to determine the dry mass of pellets and sagebrush consumed. For trials with both sagebrush types, we separated the food offered and refused into stems and leaves to account for any possible differences in preference for plant parts. We compared DMI and DEI of greenhouse sagebrush consumed by pygmy rabbits and cottontails, and the relationship between urine pH and sagebrush consumption, by using analysis of covariance, with species and pellet quality as class variables and dry mass of pellets consumed as a covariate. Additionally, we compared voluntary intake of greenhouse and outside sagebrush by pygmy rabbits and cottontails when fed 50% of their *ad lib* intake of low- and high-quality pellets by using analysis of variance, and paired *t*-tests.

Results

Energy and Protein Requirements

Pygmy rabbits required 750.8 kJ DE/kg^{0.75}/d and cottontails required 549.2 kJ DE/kg^{0.75}/d to maintain their body mass when residing in small cages between 0°C and 5°C (Fig. 1). Pygmy rabbits had an MFN of 7.5 g N/kg feed (Fig. 2), and an EUN of 22.9 mg/kg^{0.75}/d (Fig. 3). True N digestibility was 88%. They required at least 306.4 mg N/kg^{0.75}/d for

Fig. 1 Body mass gain as a function of digestible energy intake of captive pygmy rabbits (PR, solid line) and cottontail rabbits (CT, dashed line) when consuming pelleted rations and sagebrush (*Artemisia tridentata*, circled points). Daily digestible energy requirements are found at the intersection of the regression lines with the *x*-axis

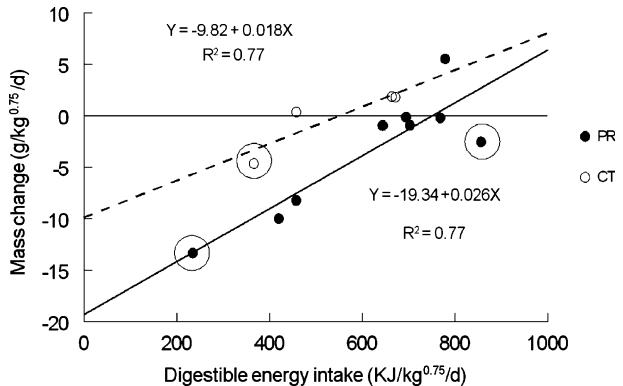
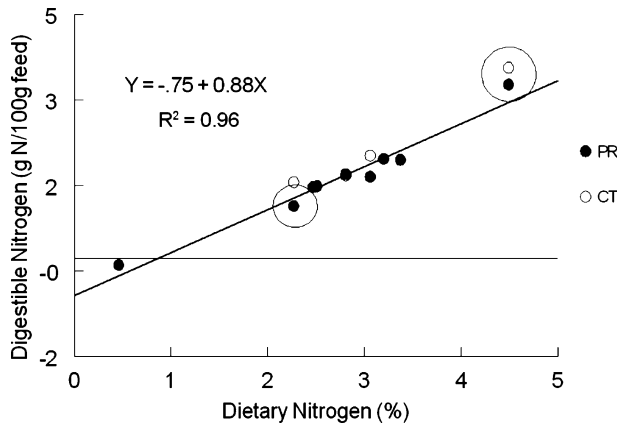


Fig. 2 Mean apparent digestible nitrogen of captive pygmy rabbits (PR) and cottontail rabbits (CT) as a function of dietary nitrogen for pelleted rations and sagebrush (*A. tridentata*, circled points). Metabolic fecal nitrogen is the negative y -intercept



maintenance (Fig. 4). Cottontails had a similar digestible N ($F = 1.13, P = 0.32$), N balance ($F = 0.80, P = 0.39$), and urinary N ($F = 0.02, P = 0.90$) relative to dietary N or N intake to pygmy rabbits (Figs. 2, 3, and 4). Based on their EUN and MFN, when pygmy rabbits eat >9 g DM/d, they require a minimum of 7% CP in their diet.

Digestibility of Foods

Pygmy rabbits digested DM, apparent energy (AE), neutral detergent solubles (NDS) and NDF of the high-quality diet (pellet 1), and NDS and NDF of the low-quality diet (pellet 2), to a lesser extent than the larger cottontails (all $P < 0.05$, Table 2). However, the two rabbit species digested high-quality pellets and greenhouse sagebrush equally (all $P > 0.05$, Table 2). Both pygmy and cottontail rabbits digested DM, AE, and NDF to a greater extent when eating high-quality pellets and greenhouse sagebrush than when eating low-quality pellets (all $P < 0.05$, Table 2). Pygmy rabbits achieved a lower DM digestibility (DMD) and AED on outside sagebrush than on high-quality pellets and greenhouse sage, but greater than that on low-quality pellets ($P < 0.05$, Table 2). Both pygmy and cottontail rabbits digested NDF as a function of the proportion of indigestible of the cell wall (i.e., ADL) similarly to other small hindgut fermenters, and to a lesser extent than ruminants and large

Fig. 3 Mean urinary nitrogen as a function of nitrogen intake of captive pygmy rabbits (PR) and cottontail rabbits (CT) consuming pelleted rations and sagebrush (*A. tridentata*, circled points). Endogenous urinary nitrogen is the y -intercept of the regression line

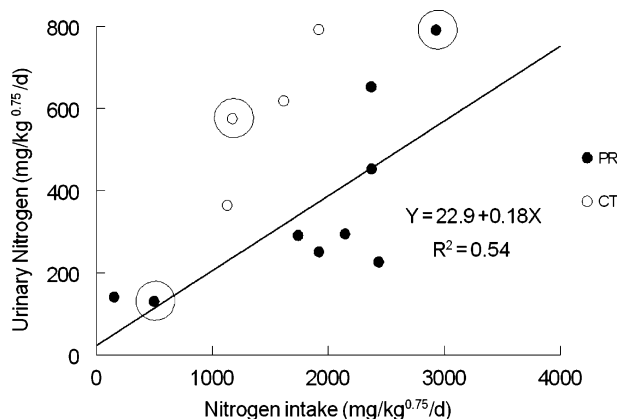
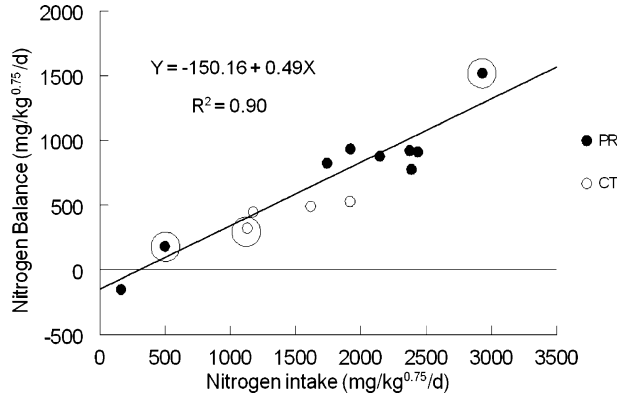


Fig. 4 Mean nitrogen balance as a function of total nitrogen intake of captive pygmy rabbits (PR) and cottontail rabbits (CT) consuming pelleted rations and sagebrush (*A. tridentata*, circled points). Minimum nitrogen requirements are found at the x -intercept



hindgut fermenters (Fig. 5). However, they digested NDF of greenhouse sagebrush better than expected from its ADL content, and better than the other diets (all $P < 0.05$; Table 2, Fig. 5).

A greater proportion of energy consumed was excreted in the urine of both pygmy rabbits ($\bar{X} = 9.5 \pm 2.4\%$) and cottontails ($\bar{X} = 14.1 \pm 2.7\%$) when eating sagebrush than when eating high-quality or low-quality pellets ($\bar{X}_{\text{pygmy rabbits}} = 0.7 \pm 0.2\%$, $\bar{X}_{\text{cottontails}} = 2.8 \pm 0.2\%$, $F = 9.87$, $P < 0.001$). Pygmy rabbits and cottontails excreted in their urine the same proportion of energy consumed when eating greenhouse sagebrush ($F = 0.53$, $P = 0.52$). In contrast, the proportion of ingested energy excreted in the feces when consuming sagebrush was lower than the amount excreted when eating low-quality pellets (all $P < 0.05$), and lower than (i.e., pygmy rabbits eating greenhouse sagebrush, $P < 0.05$) or equivalent to (i.e., pygmy rabbits eating outside sagebrush and cottontails eating greenhouse sagebrush, both $P > 0.05$) the energy excreted when eating high-quality pellets. Pygmy rabbits and cottontails excreted in feces the same proportion of ingested energy when eating greenhouse sagebrush ($F = 4.12$, $P = 0.14$).

Terpene Content of Sagebrush

Using GC and GC-MS, we identified six major peaks (mostly monoterpenes) present in the greenhouse and outside sagebrush (Table 3). Greenhouse sagebrush had over twice the total concentration (% DM) of terpenes of the outside sage, and among the major peaks, had more than twice the amount of artemiseole, 1,8-cineole/eucalyptol, and methyl santolinate, and 10 times more santolina epoxide than did the outside sagebrush. However, greenhouse sagebrush had only half the camphor of outside sagebrush (Table 3). Camphor made up nearly 50% of the major distilled volatile oils of the outside, but only 12% of the greenhouse, sagebrush.

Voluntary intake of sagebrush and pellets

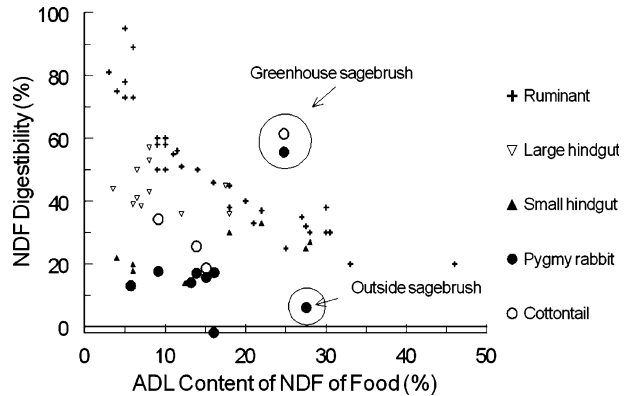
When only pellets were offered, pygmy rabbits ate the same dry mass and digestible energy (DE) relative to their metabolic body mass ($\text{kg}^{0.75}$) as did cottontails (both $P > 0.05$, Table 2). Both pygmy ate more mass of low- than high-quality pellets ($F = 24.4$, $P = 0.001$), but consumed equivalent DE ($F = 0.19$, $F = 0.67$, Table 2).

Table 2 Mean body mass change, digestibility, and intake of pelleted diets and sagebrush (*Artemisia tridentata*) fed captive pygmy rabbits (PR, *Brachylagus idahoensis*) and eastern cottontails (CT, *Sylvilagus floridanus*)

Diet	Body mass change (%/d)		Dry matter digestibility (%)		Apparent energy digestibility (%)		Neutral detergent soluble digestibility (%)		Neutral detergent fiber digestibility (%)		Dry matter intake (g/kg ^{0.75} /d)		Digestible energy intake (kJ/kg ^{0.75} /d)	
	PR	CT	PR	CT	PR	CT	PR	CT	PR	CT	PR	CT	PR	CT
Pellet 1: WSU high quality	0.0	0.2	60.3 ^{*bc}	69.7 ^{ab}	61.5 ^{*bc}	71.4 ^{ab}	72.2 ^{*b}	83.8 ^a	17.7 ^{*ab}	34.3 ^b	70.1 ^{ab}	52.8 ^b	769 ^{abc}	672 ^a
Pellet 2: WSU low quality	0.7	0.2	40.2 ^f	44.1 ^c	43.3 ^{ef}	46.7 ^c	70.0 ^{*b}	75.1 ^b	15.7 ^{*b}	18.7 ^c	107.3 ^a	84.4 ^a	779 ^{ab}	664 ^a
Pellet 3: WSU deer	-0.1	0.0	56.7 ^{cd}	62.5 ^b	60.2 ^{cd}	65.9 ^b	75.5 ^b	80.0 ^{ab}	17.1 ^b	25.6 ^{bc}	68.4 ^{*ab}	40.3 ^{bc}	703 ^{*abc}	458 ^b
Pellet 4: Purina [®] Breeders	-0.1	-	50.0 ^{de}	-	51.3 ^{de}	-	73.2 ^b	-	17.3 ^b	-	70.3 ^{ab}	-	644 ^{abc}	-
Pellet 5: Mazuri [®] Moose	-1.0	-	43.7 ^{ef}	-	46.7 ^{de}	-	71.3 ^b	-	-1.8 ^c	-	54.4 ^{bc}	-	458 ^{bcd}	-
Pellet 6: Lab Diet [®] Rabbit	0.0	-	39.5 ^f	-	42.6 ^f	-	64.2 ^c	-	14.1 ^b	-	94.5 ^a	-	695 ^{abc}	-
Pellet 7: WSU low protein	-3.6	-	69.6 ^a	-	72.2 ^a	-	87.7 ^a	-	13.1 ^b	-	33.6 ^{bc}	-	429 ^{cd}	-
Greenhouse sagebrush	-0.3	-0.4	68.3 ^{ab}	73.7 ^a	70.3 ^{ab}	74.6 ^a	77.4 ^b	82.4 ^a	55.6 ^a	61.4 ^a	65.3 ^{*abc}	26.2 ^c	857 ^{*a}	366 ^b
Outside sagebrush	-1.6	-	54.8 ^{cd}	-	57.2 ^{cd}	-	77.8 ^b	-	6.2 ^b	-	20.2 ^c	-	234 ^d	-

An asterisk denotes significant difference between PR and CT on each diet, and different letters denote significant differences among diets within a column ($\alpha=0.05$). WSU: Washington State University.

Fig. 5 Mean digestibility of neutral detergent fiber (NDF) of pygmy rabbits and cottontail rabbits as a function of the proportion of acid detergent lignin (ADL) in the cell wall of pelleted rations and sagebrush (*A. tridentata*, circled points) they consumed. Data for ruminants, large hindgut fermenters, large hindgut fermenters, and small hindgut fermenters are provided for comparison (Robbins, 1993)



Regardless of the amount of high- and low-quality pellets offered, pygmy rabbits voluntarily consumed more greenhouse sagebrush than cottontails relative to their metabolic body mass ($F = 5.72$, $P < 0.001$, Table 4). For example, pygmy rabbits ate five times more greenhouse sagebrush relative to their size than cottontails did when given *ad lib* access to both high-quality pellets and sagebrush (Table 4). However, sagebrush comprised only $8.1 \pm 2.5\%$ DM consumed by pygmy rabbits when offered with *ad lib* high-quality pellets, and $14.8 \pm 2.5\%$ when offered with *ad lib* low-quality pellets. Cottontails ate sagebrush as $0.7 \pm 0.2\%$ of their diet when eating high-quality pellets and $1.8 \pm 0.8\%$ when eating low-quality pellets. Both pygmy rabbits and cottontails ate more greenhouse sagebrush relative to their metabolic body mass when offered low-quality than high-quality pellets at 50% and 25% *ad lib* ($F = 37.85$, $P < 0.001$). For example, pygmy rabbits doubled their intake of sagebrush when offered 25% *ad lib* of low-quality pellets than when offered 25% *ad lib* of high-quality pellets (Table 4). Both rabbit species ate more greenhouse sagebrush as the amount of either type of pellet offered declined ($F = 30.22$, $P < 0.001$, Table 4).

When offered together with 50% *ad lib* intake of both high- and low-quality pellets, pygmy rabbits and cottontails ate a similar amount of greenhouse and outside sagebrush ($t < 6.48$, $P > 0.08$, Table 4). However, when no supplementary pellets were offered, pygmy

Table 3 Retention time on gas chromatograph-mass spectrophotometer (GC-MS), gas chromatograph (GC), % of dry plant tissue, and % of six major peak area of distilled oil of six terpenoid compounds found in sagebrush (*Artemisia tridentata*)

Compound name	Retention time on GC-MS (min)	Retention time on GC (min)	Outside sagebrush		Greenhouse sagebrush	
			% Dry mass	% Major peak area	% Dry mass	% Major peak area
Artemiseole	6.49	4.71	0.36	19	0.75	16
1,8-Cineole/eucalyptol	7.48	5.56	0.26	14	0.71	15
<i>E</i> - β -Santolina epoxide	7.63	5.90	0.07	4	1.02	21
<i>Z</i> - β -Santolina epoxide	7.72	5.99	0.12	7	1.05	24
Methyl santolinatate	8.96	7.26	0.20	11	0.52	11
Camphor	9.32	7.36	0.91	45	0.54	12
Total major peaks	–	–	1.92	100	4.59	100
Total minor peaks	–	–	1.44	–	4.11	–
Total all peaks	–	–	3.36	–	8.70	–

Table 4 Dry mass ($\bar{X} \pm SE$) of pellets, greenhouse sagebrush, or outside sagebrush (*Artemisia tridentata*) consumed by pygmy rabbits (PR, *Brachylagus idahoensis*) and cottontails (CT, *Sylvilagus floridanus*) when offered 4 different amounts of high- and low-quality pellets

Pellet type	Amount sagebrush consumed (g/kg ^{0.75} /d)												
	Amount pellets offered				Greenhouse sagebrush (<i>ad lib</i>)				Outside sagebrush (<i>ad lib</i>)				
	No sage		PR		CT		PR		CT		PR		CT
No pellets	-	-	-	-	62.3 ± 6.8	-	26.2 ± 0.4	-	20.0 ± 5.8	-	-	-	-
High-quality pellet 1	69.5 ± 9.0	52.8 ± 1.0	61.1 ± 8.9	5.3 ± 1.9	44.3 ± 1.4	0.3 ± 0.1	-	-	-	-	-	-	-
50% <i>ad lib</i>	-	-	29.5 ± 0.5	13.3 ± 1.3	21.5 ± 1.1	1.6 ± 0.4	30.1 ± 0.8	26.4 ± 0.7	21.1 ± 0.9	8.3 ± 2.1	-	-	-
25% <i>ad lib</i>	-	-	15.3 ± 0.4	30.4 ± 1.2	11.1 ± 0.7	4.0 ± 0.4	-	-	-	-	-	-	-
Low-quality pellet 2	106.0 ± 12.3	84.4 ± 6.1	67.4 ± 9.2	11.5 ± 0.8	71.3 ± 2.6	1.3 ± 0.5	-	-	-	-	-	-	-
50% <i>ad lib</i>	-	-	32.6 ± 1.0	25.8 ± 0.8	35.5 ± 1.8	3.9 ± 1.2	38.9 ± 1.7	33.9 ± 8.3	36.8 ± 1.8	4.5 ± 2.2	-	-	-
25% <i>ad lib</i>	-	-	18.1 ± 0.2	52.2 ± 64	18.0 ± 1.0	16.4 ± 1.5	-	-	-	-	-	-	-

rabbits ate three times more dry mass of greenhouse sagebrush than outside sagebrush ($t = 5.01, P = 0.02$, Table 4).

When consuming all mixed diets of pellets and sagebrush, pygmy rabbits acquired about twice the DEI of cottontails relative to their metabolic body mass (all $F < 12.77$, all $P < 0.02$; Fig. 6). By voluntarily eating more greenhouse sagebrush, both pygmy rabbits and cottontails achieved a higher total DEI when eating mixed diets (at 50% and 25% *ad lib*) including low-quality pellets than those that included high-quality pellets (both $F > 10.49$, both $P < 0.02$). As the DEI of pellets decreased, so did the total DEI of cottontails ($F = 25.77, P < 0.001$) when offered greenhouse sagebrush *ad lib*. However, pygmy rabbits ate enough greenhouse sagebrush to maintain their total DEI as their DEI of pellets varied from 100% to 0% of their diet ($F = 4.26, P = 0.11$; Fig. 6). However, when pellet intake was restricted to 25–50% *ad lib*, pygmy rabbits voluntarily ate enough sagebrush to meet between 67% and 110% of their requirements, and met only 30% of their requirements when eating outside sagebrush alone. In contrast, cottontails only met their daily DE requirements when given *ad lib* access to low- and high-quality pellets alone and with *ad lib* access to sagebrush (Fig 6). Cottontails met only 67% of their daily energy requirements on greenhouse sagebrush alone, and when pellets were restricted to 25–50% *ad lib*, they only ate enough sagebrush to meet 35–67% of their DE requirements. However, mass gain during the mixing trials was not consistently related to pellet type, pellet amount, sagebrush type, or rabbit species ($F = 2.05, P = 0.11$).

When offered *ad lib* access to both greenhouse and outside sagebrush together with 15% of their *ad lib* intake of high-quality pellets, pygmy rabbits ate five times more leaves of greenhouse ($\bar{X} = 50.82 \pm 3.2\text{g}$) than outside sagebrush ($\bar{X} = 10.35 \pm 1.4\text{g}$, $t = 6.73, P = 0.003$). Pygmy rabbits ate the same proportion of leaves and stems of greenhouse and outside sagebrush ($t = 1.52, P = 0.20$).

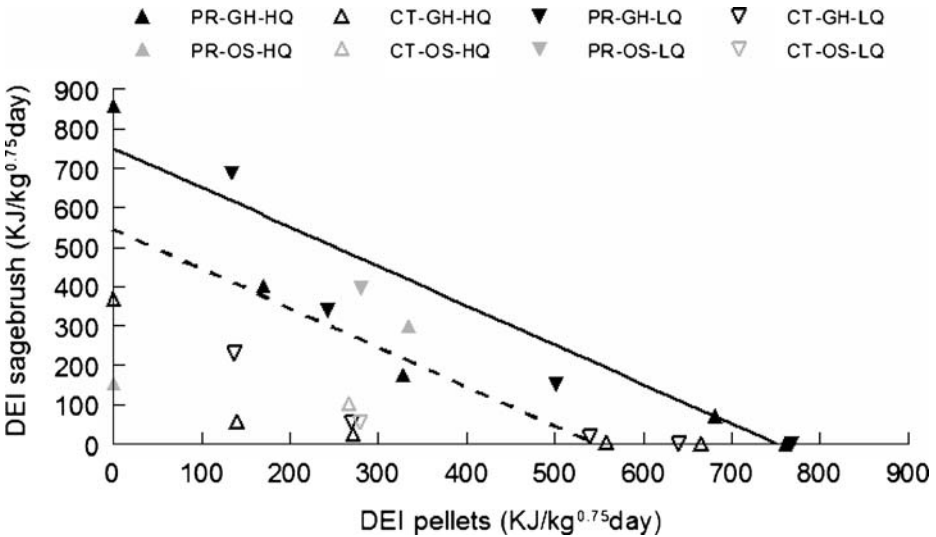
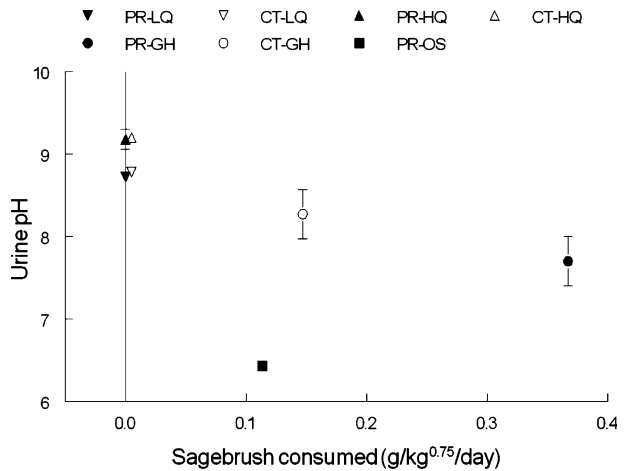


Fig. 6 The amount of digestible energy (DEI) of greenhouse (GH) and outside (OS) sagebrush (*Artemisia tridentata*) voluntarily consumed by pygmy rabbits (PR) and cottontails (CT) each day when offered different amounts of a high-quality (HQ) or low-quality (LQ) grain-alfalfa pelleted. The diagonal lines indicate the amount of additional energy in sagebrush pygmy rabbits (solid line) and cottontail rabbits (dashed line) would have to consume to meet their daily energy requirements and maintain body mass when eating pellets

Fig. 7 pH of urine when in relation to the dry mass of greenhouse (GH) and outside (OS) sagebrush (*Artemisia tridentata*) eaten by captive pygmy rabbits (PR) and cottontail rabbits (PR) when offered *ad libitum*, and when consuming diets of high-quality (HQ) or low-quality (LQ) pelleted grain-alfalfa offered *ad libitum*



pH of Urine

When consuming high-quality pellets, low-quality pellets, and greenhouse sagebrush alone *ad lib*, pygmy rabbits and cottontails had the same urine pH ($F = 2.66$, $P = 0.13$), even when including DMI of sagebrush as a covariate ($F = 0.06$, $P = 0.53$; Fig. 6). Both had a higher urine pH when eating high-quality pellets than low-quality pellets, and a lower urine pH when eating only greenhouse sagebrush than either pellet ($F = 27.38$, $P < 0.001$; Fig. 7). Despite eating about half the total mass of outside sagebrush, the urine pH of pygmy rabbits was lower when eating outside sagebrush than when eating greenhouse sagebrush ($t = 5.0$, $P = 0.01$; Fig. 7).

Discussion

Pygmy rabbits had relatively high energy and low protein requirements. Daily energy expenditure (i.e., basal metabolic rate + activity + thermoregulation + heat increment, kJ/d) tends to scale as $591.6 \text{ kJ/kg}^{0.75}$ for eutherian mammals (Robbins, 1993). When living in small cages, the daily energy requirement of our 1.2-kg cottontails ($549.2 \text{ kJ/kg}^{0.75}/\text{d}$) was consistent with this scaling, and consistent with Rose, (1973), who found energy requirements of eastern cottontails to range from $531\text{--}607 \text{ kJ DE/kg}^{0.75}/\text{d}$ when ambient temperature ranged from 10°C to 20°C . Likewise, snowshoe hares (*L. americanus*, 1.5 kg) needed only $510 \text{ kJ/kg}^{0.75}$ (Holter et al., 1974) to maintain their body mass. In contrast, pygmy rabbits (0.45 kg) required DE of $750.8 \text{ kJ/kg}^{0.75}/\text{d}$ to maintain their mass in small cages, 27% more than predicted from the scaling relationship for eutherian mammals, and 36% higher per unit metabolic mass than eastern cottontails. Similarly, when measuring oxygen consumption of pygmy rabbits under different thermal conditions, Katzner et al. (1997b) found that pygmy rabbits had a higher resting metabolic rate than other eutherian mammals relative to metabolic body mass. McNab, (1988) showed that small herbivores such as lagomorphs tend to have higher basal metabolic rates relative to their size than other mammals, and that energy requirements for small folivores scale with body mass^{0.545}. The lower critical temperature for pygmy rabbits is $15\text{--}20^\circ\text{C}$ (Katzner et al., 1997b); thus they were always expending 18% more energy than they would at thermal neutrality during our

feeding trials, which were conducted at 0–5°C. Because of their larger size, the thermal neutral zone of cottontails is predicted to be two times wider than that of pygmy rabbits (Katzner et al., 1997b); thus cottontails may not have had to expend as much energy to keep warm in our trials.

Not only do pygmy rabbits have a higher energy requirement relative to body mass than similar mammals, their behavior and habitat demand that they acquire additional energy from their food, especially in winter. They do not hibernate, and they live in cold, exposed sagebrush-steppe rangelands in the northwestern United States. Additional activity and cold temperatures increases the energy expenditure of cottontails by 30–60% (Rose, 1973) and pygmy rabbits by up to 60% (Katzner et al., 1997b). Sagebrush, despite its terpene concentration, has a high DE content, especially relative to grass, one of the few other foods available in the winter. For example, NDF content of two common grass species in pygmy rabbit habitat in the Columbia Basin of Washington ranged from 67% in summer to 78% in winter, whereas sagebrush in the same area contained only 36–47% NDF (Thines et al., 2004). However, the detoxification of terpenes and other PSMs in sagebrush likely increases energy demands further, as evidenced by the high energy content of urine while eating sagebrush. Sorensen et al., (2005) found that woodrats ate more, increased energy intake, and reduced their basal metabolic rate and activity when eating high-terpene juniper diets. Therefore, many herbivores that specialize on plants with high levels of PSMs have developed energy conservation methods, such as low reproduction (e.g., woodrats, *Neotoma* spp.; Meyer and Karasov, 1991) and low metabolic rates, requiring them to eat less (e.g., arboreal marsupials, Cork and Foley, 1991). On the other hand, the small body size of pygmy rabbits is advantageous in eliminating PSMs from the body. The steady-state plasma concentration of toxins scales with body mass^{0.39}; thus, small animals maintain lower concentrations of toxins in their bodies than large animals and can ingest more relative to their size (Freeland, 1991). Therefore, the size of pygmy rabbits, the smallest leporid in North America, may be an adaptation to excreting toxins, thus allowing them to consume large amounts of sagebrush.

Although pygmy rabbits have a relatively high energy requirement, they seem to have a lower N requirement (306.5 mg N/kg^{0.75}/d) than many other mammals. Their N balance falls below the mean for eutherian mammals (582 ± 235 mg/kg^{0.75}/d; Robbins, 1993), such as blacktailed jackrabbits (950 mg/kg^{0.75}/d; Nagy et al., 1976). However, herbivorous marsupials that specialize on terpene and tannin-containing foods, such as ringtail possums (290 mg/kg^{0.75}/d) and koalas (275 mg/kg^{0.75}/d), have similar N requirements to pygmy rabbits (Robbins, 1993).

Pygmy rabbits have an MFN of 7.5 g N/kg feed, which is within the normal range for mammals (1–9 g N/kg feed; Robbins, 1993). This MFN is also within the range found in other lagomorphs such as blacktailed jackrabbits (4.6 g N/kg feed; Nagy et al., 1976), domestic rabbits (*Oryctolagus cuniculus*, 8.0 g N/kg feed; Slade and Robinson, 1970), and snowshoe hares (9.1 g N/kg feed; Holter et al., 1974). EUN was 22.9 mg/kg^{0.75}/d, lower than that of the average nonruminant eutherian mammal (160 ± 22 mg N/kg^{0.75}/d), such as blacktailed jackrabbits (128 mg/kg^{0.75}/d; Nagy et al., 1976). However, the lowest excretion of urinary N we measured on pygmy rabbits eating low N diets was 97 mg/kg^{0.75}/d (Fig. 3). Thus, the standard method for estimating EUN may have underestimated EUN for pygmy rabbits.

When eating more than 9 g DM/d, pygmy rabbits require a diet of at least 7% CP, which is similar to the minimum dietary protein requirements of nonruminant eutherians and browsing ruminants (Robbins, 1993). The National Research Council, (1977) recommends that domestic rabbits be fed a diet with a CP content (6.25 N) of 13–18% for maintenance,

and a dietary level of 16% CP is considered adequate for growth and reproduction. Sagebrush in the greenhouse (18%), outside (15.6%), and in pygmy rabbit habitat in the Columbia Basin (9.5–11.4%; Thines et al., 2004) had at least twice the CP content of grasses (3.6–5%) available in native habitat (Thines et al., 2004.), and is adequate to meet the requirements for domestic and pygmy rabbits.

The smaller pygmy rabbits generally digested foods less efficiently than the larger cottontails, large hindgut fermenters, and ruminants (Fig. 5). Because smaller herbivores have a higher metabolic rate relative to their fermentation capacity (i.e., size of ceca), they have a faster throughput of ingesta and less efficient digestion of plant fiber (Demment and Van Soest, 1985). Fiber digestibility of pygmy rabbits and cottontails in our study was generally consistent with that of European rabbits (*O. cuniculus*; Kuijper et al., 2004) and snowshoe hares (Holter et al., 1974).

Despite terpenes in sagebrush, both pygmy rabbits and cottontails digested the NDS of sagebrush and pelleted diets equally, and pygmy rabbits digested the NDS of greenhouse and outside sagebrush similarly (Table 2). Furthermore, both digested the NDF of greenhouse sagebrush better than the pelleted diets without terpenes or other PSMs, even relative to the indigestible portion of the cell wall (Table 2, Fig. 5). Outside sagebrush was poorly digested, yet had a lower content of terpenes overall. Because sagebrush did not precipitate BSA, the biological activity of any condensed tannins in sagebrush was likely too low to affect digestibility. These findings suggest that terpenes and other PSMs in sagebrush do not reduce fiber or soluble digestion in either rabbit species, and that pygmy rabbits, which “specialize” on sagebrush, do not seem to have developed a mechanism for digesting this high-terpene food better than the generalist cottontail. Specific monoterpenes are known to be toxic to rumen microorganisms *in vitro* at 0.7–1.3 $\mu\text{l/ml}$ and reduce *in vitro* and *in situ* digestibility (Schwartz et al., 1980; Welch and Pederson, 1981). However, nearly 80% of terpene concentration of sagebrush is lost by the time it reaches the stomach of pygmy rabbits (White et al., 1982b) and the rumen of mule deer (Cluff et al., 1982), likely through evaporative losses during chewing and eructation. Terpenes may also be absorbed into the bloodstream from the digestive system before they can influence digestibility of forages. Therefore, concentrations of monoterpenes in the digestive system of pygmy rabbits feeding on sagebrush are likely not high enough to reduce digestibility (White et al., 1982b; Gershenson and Croteau, 1991).

On the other hand, urine pH of both cottontails and pygmy rabbits declined and urinary energy increased when sagebrush was added to the diet, indicating that consuming sagebrush incurs a metabolic cost to rabbits. However, the specialist pygmy rabbit did not have a consistently higher urine pH or lower urinary or fecal energy than the generalist cottontail, suggesting that pygmy rabbits may not have an increased capacity to avoid or metabolize terpenes or other PSMs. They had a lower urine pH when consuming solely outside sagebrush, which had a lower overall monoterpene content but a higher content of camphor, which is known to deter feeding by lagomorphs eating conifers (Rodgers et al., 1993). Outside sagebrush may also have had higher levels of unmeasured PSMs.

Both pygmy rabbits and cottontails were able to meet their DE requirements by increasing their DM intake of pellets as the nutritional quality of pellets declined. To do this, pygmy rabbits consumed more relative to their body mass than cottontails did, increasing DM intake from 9.6% of body mass on high-quality pellets to 14.0% on low-quality pellets, whereas cottontails increased from 5.3% to 8.2% of their body mass when shifting from high- to low-quality pellets (Table 2). When the amount of either type of pellet was restricted, both chose to eat more sagebrush, but cottontails did not consume

enough additional sagebrush to meet their energy requirements (Fig. 6). Regardless of the amount and quality of pellets offered, pygmy rabbits ate more sagebrush overall, and in relation to energy requirements, than did cottontails. However, when allowed free access to pellets containing a DE content similar to sagebrush, they selected pellets over sagebrush, which comprised only 8% of their diet. Under the same conditions, sagebrush comprised only 0.7% of the cottontails' diets. When offered low-quality pellets containing only half the protein and 60% of the DE content of sagebrush, pygmy rabbits increased their use of sagebrush to 15%, and cottontails to 1.8%, but sagebrush still did not make up a substantial portion of the diet of either rabbit. Likewise, Stephen's woodrats, a terpene specialist, consumed twice as much juniper (27% of diet) as did whitethroat woodrats (*N. albigula*), a generalist (10%), when given *ad lib* access to a control diet with a low N content (1.25%) and high ADF (23%) similar to juniper (Dearing et al., 2000).

Like our captive rabbits, wild pygmy rabbits in native habitats expand their use of sagebrush from 11–50% in early summer, when alternative forages are more available and of higher nutritional quality, to 72–98% in the winter, when grasses and forbs are rare and less nutritious (Green and Flinders, 1980b; Thines et al., 2004). In contrast, domestic lambs (generalist foragers) actually ate more (Villalba and Provenza, 2005) or the same amount (Burritt et al., 2000) of a terpene-containing food as the energy and protein content of an alternative food increased. Therefore, the presence of high-quality foods in the environment may either decrease or increase diet breadth used by generalist and specialist herbivores.

Despite the lower fiber content of the outside sagebrush, pygmy rabbits chose to eat less of it when offered alone and when offered with 50% *ad lib* high- and low-quality pellets than of greenhouse sagebrush. In addition, pygmy rabbits ate five times more greenhouse sagebrush than outside sagebrush when they were simultaneously offered. Pygmy rabbits may have preferred greenhouse sagebrush because it contained nearly twice the protein content and was more digestible, despite its higher fiber content (Tables 1 and 2). Although the greenhouse sagebrush had higher monoterpene levels overall than outside sagebrush, and had higher levels of all specific terpene compounds identified except camphor, the specific effects of these terpenes and other PSMs on preference and digestion in pygmy rabbits is unknown. White et al., (1982a) found no relationship between total monoterpenoid content and dietary preference of sagebrush by pygmy rabbits, but pygmy rabbits did prefer plants from individual populations, and preference was negatively correlated with the monoterpene α -thujone (not found in our sagebrush samples). However, urine pH was lower when our pygmy rabbits ate solely outside sagebrush than solely greenhouse sagebrush, suggesting that the terpenoid compounds or other PSMs in the outside sagebrush required more detoxification, and thus may have been a less desirable food choice. Similarly, snowshoe hares preferred mature white spruce (*Picea glauca*) over juvenile white spruce that contained four times more camphor, and camphor added to pellets deterred feeding by captive hares (Sinclair et al., 1988).

The terpenoid content of both types of sagebrush we fed our pygmy rabbits likely differed to some degree from sagebrush consumed in their native habitat. Monoterpene concentration in sagebrush varies greatly with climate, soils, age, season, genetics, and plant part (Welch and McArthur, 1981; Gershenzon and Croteau, 1991; Zhang and States, 1991). Total terpene content measured in wild sagebrush varies from 0.37% to 3.7% DM, and the composition of terpenoids, such as α -thujone, camphor, α -pinene, 1,8-cineole, β -thujone, and terpineol, differs among populations, individuals, and plant parts (Welch and McArthur, 1981; Cluff et al., 1982; White et al., 1982a). For example, camphor content of oils distilled from sagebrush varies from 0% to 70.3% dry mass (Welch and McArthur, 1981). The reproductive structures and new growth, which we fed to pygmy rabbits in our

experiments, generally have higher levels of monoterpenoids than older, structural components (Gershenzon and Croteau, 1991). Our greenhouse sagebrush was fertilized with N, whereas the outside sagebrush was not, which might explain, in part, the higher N content and the different terpenoid composition (Gershenzon and Croteau, 1991). Similarly, when white spruce was grown in a nursery, it had about 30% less camphor than wild white spruce seedlings (Rodgers et al., 1993). Snowshoe hares selectively ate more nursery-grown white spruce than naturally regenerated spruce seedlings, and in most cases avoided the naturally regenerated spruce all together. Plants growing in low light and poor soil are expected to invest in carbon-based defenses, whereas those grown in fertile soils and high light (e.g., greenhouses) are more likely to invest in N-based defenses (Rodgers et al., 1993).

Because pygmy rabbits sacrificed protein and DE in their diet when they selected low-quality pellets over sagebrush in our experiments, and because wild pygmy rabbits decreased their use of sagebrush when other nutritious forages were available in the spring (Thines et al., 2004), pygmy rabbits do not seem to be constrained by the type of obligatory relationship with sagebrush that animals like koalas have with *Eucalyptus* spp. (Marsh et al., 2003). Sagebrush clearly exacts a cost to its specialist forager. However, because pygmy rabbits ate more sagebrush than cottontails and were able to meet their DE requirements while feeding exclusively on sagebrush, they have likely adapted mechanisms to keep the costs of ingesting terpenes relatively low. Adaptations for dealing with PSMs include avoiding ingesting them, reducing their absorption from the gut, and detoxifying them in the liver (McArthur et al., 1991).

Nonspecialist and specialist foragers alike often avoid PSMs by carefully selecting individual plants and plant parts (Zhang and States, 1991; Lawler et al., 1998; Dearing et al., 2000; Marsh et al., 2003). For example, small herbivores such as mountain hares (*L. timidus*), black-tailed jackrabbits, and woodrats often discard young leaves and eat the stems of plants, which have lower concentrations of PSMs but also have lower N and DE content (Gershenzon and Croteau, 1991; Meyer and Karasov, 1991; Palo et al., 1992). Pygmy rabbits ate leaves and stems in proportion to availability when eating greenhouse and outside sagebrush, but when given the opportunity during pretrials, we noticed that cottontails tended to eat stems and discard leaves of sagebrush.

Specialist herbivores may have more transporter proteins or transporter proteins more specific to a particular PSM, which move PSMs out of cells and into the intestinal lumen, thus reducing absorption of PSMs into the circulation (Sorensen et al., 2004). For example, the specialist Stephen's woodrat had lower blood levels of α -pinene, a terpene in juniper, and excreted 40% more in the feces than did the generalist whitethroat woodrat (Sorensen and Dearing, 2003; Sorensen et al., 2004). If absorbed, PSMs can be eliminated from the bloodstream through enzymatic reactions in the liver that add a functional group or conjugate the PSM to change its solubility (McArthur et al., 1991; Dearing et al., 2005). However, even specialist herbivores that encounter PSMs in their normal diet have a threshold, albeit higher than the generalists, to the amount of PSMs that can be processed and will not voluntarily ingest terpenes above a crucial daily amount (Boyle et al., 1999; Marsh et al., 2003; Boyle and McLean, 2004). This threshold is caused, in part, by the finite tolerance of the kidney for excreting acidic metabolites produced during detoxification (Freeland and Janzen, 1974; Foley, 1992; McLean et al., 1993; Foley et al., 1995). Therefore, urine pH is a general indicator of overall detoxification processes. Dearing et al., (2000) found that urine pH declined from about 8.7 when a generalist and specialist woodrat consumed a nonterpene control diet, to about 7.7 on a juniper diet. The specialist, which had a slightly higher pH when consuming juniper, may have produced metabolites that were less acidic, or were able to buffer the acidic metabolites better than the generalist. In our study,

urine of both pygmy rabbits and cottontails declined to a similar degree from a nonterpene pelleted diet to a sagebrush diet (Fig. 7). However, specialist pygmy rabbits did not maintain a higher urine pH on greenhouse sagebrush than generalist cottontails did.

In summary, pygmy rabbits have a relatively high metabolism and moderately low N requirement, and can acquire high levels of DE from sagebrush that dominates their natural habitat. However, their low voluntary intake of sagebrush in the presence of low-quality non-terpene foods and their increased excretion of acids and energy in the urine when consuming sagebrush suggests that consuming sagebrush exacts a cost even for this “specialist”. However, because pygmy rabbits voluntarily consumed more sagebrush than cottontails regardless of the quantity and quality of supplementary foods, these small rabbits likely detoxify the monoterpenes and other PSMs better than the generalist rabbit species that overlap their range. Any “obligate-like” relationship between pygmy rabbits and their deep soil sagebrush habitat (Green and Flinders, 1980b) likely occurs not only because pygmy rabbits are better competitors for sagebrush as forage, but also because this habitat provides cover suitable for thermoregulation and hiding (Katzner and Parker, 1997a; Katzner et al., 1997b), and reproductive habitat suitable for digging natal burrows (Rachlow et al., 2005). Future studies examining behavioral and physiological responses to individual monoterpenes and other PSMs in sagebrush and detoxification pathways will enhance our understanding of the tolerances and requirements of this unique rabbit.

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Introduction

The stemborers *Busseola fusca* Fuller (Noctuidae) and *Chilo partellus* Swinhoe (Pyralidae) are economically important pests on maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench) crops in Africa, causing losses in yield of 20–80%, depending on the pest population density and the phenological stage of the crop at infestation. Larvae of these species are polyphagous and attack a wide range of cultivated and wild plants belonging to three families—Poaceae, Cyperaceae, and Typhaceae (Khan et al., 1997a). Studies have shown that the high diversity of wild grasses surrounding farmers' fields in sub-Saharan Africa are important in stemborer management (Khan et al., 1997a,b; Khan and Pickett, 2004). Although most of these grasses are hosts for the stemborers and, indeed, elicit higher levels of oviposition than maize or sorghum, subsequent larval survival is poor (Khan et al., 2004, 2006). These aspects have been exploited in the development of “push–pull” strategies for stemborer control, in which gravid females are repelled from the main crop by a repellent intercrop (“push”) and simultaneously attracted to a trap crop (“pull”) planted around the field (Khan et al., 2000, 2001; Khan and Pickett, 2004). The latter comprise a number of wild hosts, including Napier grass, *Pennisetum purpureum* Schumach., which are considerably more attractive to gravid females than the crop itself (Ndemah et al., 2002; Rebe et al., 2004).

In a previous study on volatiles from maize, sorghum, and Napier grass, six key compounds were identified as mediating host location and oviposition by gravid stemborer females (Khan et al., 2000). However, the occurrence of these compounds in all of the hosts investigated would not appear to account for the differential attraction/oviposition observed in the laboratory and field. To address this question, a comparative analysis was conducted of the electrophysiologically active components of the volatiles from four host plants, the cultivated crop plants maize and sorghum, and two wild grasses, Napier grass (*P. purpureum*) and blue thatching grass (*Hyparrhenia tamba* (Steud.) Stapf).

Methods and Materials

Insects

Pupae of *B. fusca* and *C. partellus* were obtained from cultures maintained at the International Centre of Insect Physiology and Ecology's (ICIPE) Thomas Odhiambo Campus, Mbita Point, Kenya, that originated from colonies collected from maize and sorghum stubbles in farmers' fields in that vicinity. They were maintained at a temperature of 20°C with a 16-hr photophase and an 8-hr scotophase. Newly emerged moths were separated from pupae everyday to keep track of the age of adults and provided with 10% sugar solution as a food source. Females (2–7 d old) were used in all experiments.

Plants

Plant species used were maize, *Z. mays* (Western Seed Company Hybrid 502), sorghum, *S. bicolor* (var. Gadam), Napier grass, *P. purpureum* (originally obtained as cuttings from

Kenya), and blue thatching grass, *H. tamba* (obtained as seeds from South Africa). Seeds were sown individually for maize and sorghum, or in a group for *H. tamba*, in plastic pots filled with compost. *P. purpureum* was grown from cut stems planted in compost, 2–3 plants in a pot. Plants were grown in a glasshouse under controlled conditions, with a 16-hr photophase and an 8-hr scotophase; relative humidity was 60% and temperature was 27–30°C during the photophase and 22–25°C during the scotophase. Natural lighting was supplemented with sodium lamps (SON-T, 600 W) that gave photosynthetically active radiation of approximately 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ at bench height. Plants used in experiments were 19–27 d old, although the ages of plants from the same species were within 1–2 d of each other. Comparably sized plants of each species were used.

Chemicals

Synthetic compounds were purchased from commercial sources (Aldrich, Fluka, or Lancaster Synthesis) or were samples of compounds that had been previously synthesized at Rothamsted by methods described in the literature—(*E*)-ocimene (Gaoni 1977; Chou et al., 1984); (*E*)- β -farnesene (Kang and Kim, 1986); (*E*)-4,8-dimethyl-1,3,7-nonatriene; (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Maurer et al., 1986). All were $\geq 95\%$ as the designated geometric isomer.

Collection of Volatiles from Intact Plants

Volatile compounds were collected by entrainment of intact plants by using portable equipment developed at Rothamsted (Agelopoulos et al., 1999; Birkett et al., 2003). To avoid causing plant stress by transfer, all entrainments were performed at the site where the plants were being grown. Part of the plant was enclosed in an open-bottomed glass cylinder (100 mm o.d. \times 600 mm length), and the bottom was closed with two semicircular aluminum plates that fitted closely together except for a hole in the middle that fitted, but not tightly, around the plant. The holes were circular openings (diam 2 cm for *H. tamba* and 1 cm for *P. purpureum* and *S. bicolor*) or an elliptical opening of (2 \times 1.3 cm (for *Z. mays*). Into any large gaps between the plates and the stem(s) was packed silanized and baked glass wool. Air, purified by passage through an activated charcoal filter, was pumped into the vessel, through a port in one of the aluminum plates at 1.2 lmin^{-1} , and volatiles were collected on Porapak Q (0.05 g) packed into a glass tube (80 mm \times 5 mm o.d.) inserted into a collection port at the top of the vessel. Air was drawn out through the collection tube by another pump at 1 lmin^{-1} , the differential flow rates eliminating the risk that unfiltered air could be drawn into the vessel from outside, while obviating the need for an injuriously tight seal around the plant stem(s). In general, volatiles were collected for 10–12 hr starting at the 4th hr of the photophase. All connections were made with PTFE tubing and ferrules, and as much as possible the equipment, especially the glassware, was heated at 180°C for at least 2 hr before use. Porapak Q tubes were washed with redistilled diethyl ether and conditioned at 140°C with filtered nitrogen pumped through for 10 hr before use.

Gas Chromatography of Volatiles

Collected volatiles were eluted from the adsorbent with freshly distilled diethyl ether (500 μl) and concentrated to 50 μl under a slow stream of nitrogen. These solutions of plant volatiles were stored at -10°C until analysis. Gas chromatography (GC) of the solutions was carried out on both polar (DB-wax, 30 m \times 0.23 mm i.d. \times 0.5 μm film thickness) and

nonpolar (HP-1, 50 m×0.32 mm i.d.×0.52 μm film thickness) capillary columns using an HP6890 GC (Agilent Technologies, UK) fitted with a cool-on-column injector and a flame ionization detector (FID). The oven was kept at 30°C for 1 min, heated at 5°C min⁻¹ to 150°C, and then 10°C min⁻¹ to 250°C (220°C for the wax column), where it was maintained for 20 min. Tetradecane (10 ng μl⁻¹, 1 μl) was added to 10 μl of the sample as an internal standard, and three to four aliquots were analyzed.

Electrophysiology

Electroantennogram (EAG) recordings were made from four to nine *B. fusca* and *C. partellus* females for each compound tested. The Ag–AgCl glass electrodes were filled with saline solution (composition as in Maddrell 1969, but without glucose). An antenna was excised and suspended between the two electrodes. The tip of the terminal process of the antenna was removed to ensure a good contact. Responses were measured in mV deflections, and the signals were passed through a high-impedance amplifier (UN-06, Syntech, The Netherlands) and analyzed with a customized software package (Syntech).

Stimulus Delivery

The delivery system, with a filter paper in a disposable Pasteur pipette cartridge, has been described previously (Wadhams et al., 1982). The stimulus (2 sec duration) was delivered into a purified airstream (1 lmin⁻¹) flowing continuously over the preparation. Solutions (10⁻¹ g ml⁻¹ and decadic dilutions) of pure synthetic compounds were made in distilled hexane, with the exception of indole, which was dissolved in diethyl ether, and applied (10 μl) to a filter paper strip. The solvent was allowed to evaporate for 45 sec before the strip was placed in the cartridge. The control stimulus was hexane (10 μl) or diethyl ether (10 μl) in the case of indole. Presentation of compounds was randomly ordered, and fresh cartridges were prepared immediately before each stimulation. A standard of 4-allylanisole (10⁻⁵ g) was applied at the beginning and at the end of each experiment and between groups of five to six compounds, to monitor the condition of the antennal preparation. Responses were normalized with respect to the standard by subtracting the value for the hexane control and expressing this result as a percentage of the response to the standard.

Coupled Gas Chromatography-Electroantennography (GC-EAG)

The coupled GC-electrophysiology system, in which the effluent from the GC column is simultaneously delivered to the antennal preparation and the GC detector, has been described previously (Wadhams 1990). Separation of the volatiles was achieved by injecting 1–2 μl of sample onto a nonpolar column (HP-1, 50 m×0.32 mm i.d.×0.52 μm film thickness) by using an AI 93 GC equipped with a cold on-column injector and an FID. Oven temperature was maintained at 40°C for 2 min and then programmed at 5° min⁻¹ to 100°C, and then at 10°C min⁻¹ to 250°C. The carrier gas was hydrogen. Simultaneous records of the EAG and FID responses were obtained with commercial software (Syntech, Netherlands) installed on a computer.

Coupled Gas Chromatography-Mass Spectrometry A capillary GC column (50 m×0.32 mm i.d., HP-1) fitted with an on-column injector was directly coupled to a mass spectrometer (VG Autospec, VG Analytical, UK). Ionization was by electron impact at

70 eV, 250°C. Oven temperature was maintained at 30°C for 5 min and then programmed at 5°C min⁻¹ to 250°C. Tentative identification by gas chromatography-mass spectrometry (GC-MS) was confirmed by peak enhancement with authentic samples (Pickett 1990).

Statistical Analysis

Statistical analysis was run by using SPSS for Windows (SPSS 1989–2001) with values of $P < 0.05$ accepted as significant. Mean EAG responses (% of response to standard) to the tested compounds were calculated, and significant differences between *B. fusca* and *C. partellus* were established by performing *t*-tests.

Results

GC-EAG analysis of the volatiles from all four host plant species, using both insect species, detected a total of 43 electrophysiologically active components (Table 1), of which 41 were chemically characterized by at least two of the following methods: (1) GC-MS analysis, (2) comparison of Kovat's index (KI) on both nonpolar (HP-1) and polar (DB-wax) columns with the indices of synthetic standards, (3) coinjection with authentic standards on both columns. A GC-EAG trace showing responses of *B. fusca* to volatiles from *H. tamba* is shown in Fig. 1. Electrophysiological activity, in either *B. fusca* or *C. partellus* female antennae, of 37 of these compounds was confirmed by EAG with authentic standards (Fig. 2). Authentic samples of α -pinene (**11**), limonene (**20**), linalool (**26**), and β -caryophyllene (**39**) used in these studies were racemic.

The majority of physiologically active compounds detected by GC-EAG were common to all four plant species, although there are significant differences in the levels produced by the different hosts (Table 1). Of particular note are the large variations in the amounts of (*Z*)-3-hexen-1-yl acetate, methyl salicylate, (*E*)-4,8-dimethyl-1,3,7-nonatriene, β -caryophyllene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Two of the compounds eliciting GC-EAG activity were not identified (**2** and **9**). Neither of them was present in the headspace of *Z. mays*. Compound **2** was shown by GC-MS to be present in trace quantities in *P. purpureum*, *H. tamba*, and *S. bicolor*, although GC-EAG responses to the volatiles of these plants were obtained only from *B. fusca*. Compound **9**, detected only in the *H. tamba* and *S. bicolor* samples, elicited GC-EAG responses from both stemborers.

The identified compounds fall into several distinct groups: aliphatic compounds, which include the well-documented green leaf volatiles; aromatic compounds; and terpenoids, which can be subdivided into monoterpenoids, sesquiterpenes, and tetranorterpenes. The relative amounts of all identified compounds in each plant species and grouped within these chemical classes are shown in Table 1. Overall, wild hosts produced significantly higher levels of physiologically active compounds than either *Z. mays* or *S. bicolor*. These differences relate particularly to the elevated production by the wild hosts of the green leaf volatiles, hexanal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, and indole. Only in the cases of α -pinene (**11**), (*E*)-ocimene (**22**), and 4-ethylbenzaldehyde (**30**) did either of the cultivated hosts (in both instances, *Z. mays*) show higher levels than the wild hosts.

Dose–response studies with 4-allylanisole, over the range 10⁻⁶ to 10⁻³ g, showed no significant differences in absolute EAG amplitudes between *B. fusca* and *C. partellus* females (Fig. 3). This compound was therefore chosen as the standard for all subsequent

Table 1 Quantities (ng) of EAG active components in the headspace of host plants of the stemborers *Chilo partellus* and *Busseola fusca*

Compound	<i>P. purpureum</i> (N=5)	<i>H. tamba</i> (N=3)	<i>S. bicolor</i> (N=4)	<i>Z. mays</i> (N=2)
Green leaf volatiles and other aliphatic compounds				
1 (<i>E</i>)-2-Pentenal	0.2±0.1	0.5±0.1	0.4±0.2	nd
4 Hexanal	5.0±5.0	1.3±0.4	0.9±0.1	0.3±0.3
5 (<i>E</i>)-2-Hexenal	4.7±3.8	0.9±0.5	0.3±0.1	1.2±0.2
6 (<i>Z</i>)-3-Hexen-1-ol	12.3±11.3	3.3±3.0	1.7±0.4	1.3±0.4
7 3-Methylbutyl acetate	0.4±0.2	1.9±1.0	0.4±0.1	0.8±0.0
8 Pentyl acetate	0.4±0.4	0.5±0.3	0.3±0.2	nd
13 6-Methyl-5-hepten-2-one	2.4±1.1	3.9±2.3	1.2±0.7	1.2±0.5
14 Octanal	3.4±2.0	14.7±10.2	2.0±1.1	5.8±2.3
16 (<i>Z</i>)-3-Hexen-1-yl acetate	120±117	23.1±8.6	3.3±1.5	1.3±0.7
17 Hexyl acetate	1.6±0.8	3.4±2.0	1.3±0.6	2.2±0.1
25 Nonanal	4.5±3.6	9.4±6.7	5.4±3.5	<0.1
34 Decanal	8.7±7.2	8.0±6.8	7.7±5.8	<0.1
Aromatic compounds				
3 Toluene	0.4±0.3	0.7±0.1	2.0±0.6	1.7±0.4
10 Benzaldehyde	0.3±0.3	3.5±1.9	0.1±0.1	0.7±0.1
12 Propylbenzene	1.2±0.8	3.6±3.1	0.4±0.2	2.7±0.6
18 Phenylacetaldehyde	1.7±1.3	11.9±6.1	0.2±0.1	1.1±0.4
21 Acetophenone	3.0±1.8	1.1±1.1	0.3±0.3	3.2±3.2
23 Methyl benzoate	0.9±0.5	3.3±2.0	1.0±0.5	0.9±0.5
28 2-Ethylbenzaldehyde	nd	0.3±0.3	nd	nd
29 3-Ethylbenzaldehyde	2.0±0.9	3.2±1.8	4.4±2.9	0.3±0.3
30 4-Ethylbenzaldehyde	0.1±0.1	0.5±0.5	0.6±0.2	6.9±6.9
31 Naphthalene	0.8±0.6	2.9±2.2	0.8±0.4	nd
32 Methyl salicylate	3.2±2.1	12.3±8.3	0.8±0.4	10.5±10.5
33 4-Allylanisole	0.1±0.1	0.4±0.2	0.2±0.2	nd
35 Indole	27.1±20.7	1.6±0.8	0.2±0.2	2.7±2.7
36 4-Ethylacetophenone	3.5±1.9	1.3±0.7	nd	2.3±2.3
37 Eugenol	0.6±0.2	0.3±0.3	0.3±0.3	nd
Terpenoids				
11 α-Pinene ^a	1.8±1.1	1.8±1.3	0.3±0.2	5.1±0.1
15 Myrcene	0.7±0.7	2.2±1.9	0.3±0.1	1.8±1.8
19 1,8-Cineole	nd	1.8±1.3	nd	1.5±0.2
20 Limonene ^a	1.4±0.7	4.1±2.3	2.7±1.6	2.0±0.7
22 (<i>E</i>)-Ocimene	0.6±0.3	1.7±1.3	nd	6.2±1.4
24 α-Terpinolene	nd	0.1±0.1	nd	nd
26 Linalool ^a	1.5±0.9	14.2±9.8	2.0±0.9	0.6±0.1
27 (<i>E</i>)-4,8-Dimethyl-1,3,7-nonatriene	55.3±19.1	21.0±10.8	39.6±11.6	2.9±2.1
38 α-Copaene ^a	0.4±0.4	1.1±1.0	0.7±0.4	1.4±1.4
39 β-Caryophyllene ^a	21.5±14.1	5.7±2.7	19.5±6.3	0.4±0.4
40 Bergamotene ^a	0.9±0.8	0.3±0.1	2.3±0.6	0.4±0.2
41 (<i>E</i>)-β-Farnesene	0.3±0.3	1.3±0.1	4.4±1.5	0.2±0.2
42 α-Humulene	1.3±0.9	0.5±0.3	0.8±0.3	nd
43 (<i>E,E</i>)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	3.0±0.5	14.5±5.7	0.8±0.1	1.35±1.35
Unknowns				
2 KI=743	0.5±0.1	0.3±0.1	0.2±0.1	nd
9 KI=911	nd	1.6±1.0	0.3±0.2	nd

Table 1 (Continued)

Compound	<i>P. purpureum</i> (<i>N</i> =5)	<i>H. tamba</i> (<i>N</i> =3)	<i>S. bicolor</i> (<i>N</i> =4)	<i>Z. mays</i> (<i>N</i> =2)
Total volatiles	294.8	186.5	103.4	70.3

Compound numbers refer to the order in which they were eluted from an HP-1 GC column. Samples were collected for 10–12 hr (starting from the 4th hr of the photophase). The amounts of volatiles are estimated based on the total area under the reported peaks compared with the peak area of the internal standard (100 ng). nd: below the detection limits of 0.05 ng.

^aThe optical isomers of these compounds were not characterized.

electrophysiological studies with authentic samples. Mean EAG response to the 4-allylanisole standard (10^{-5} g) was, for *B. fusca*, $-0.75 \pm \text{SE } 0.05$ mV, and for *C. partellus*, $-0.79 \pm \text{SE } 0.07$ mV. Of the electrophysiologically active compounds, the aldehydes octanal, nonanal, and decanal elicited the strongest relative EAG response (Fig. 3). This was particularly the case for *B. fusca*, and significant differences in relative EAG responses between *B. fusca*

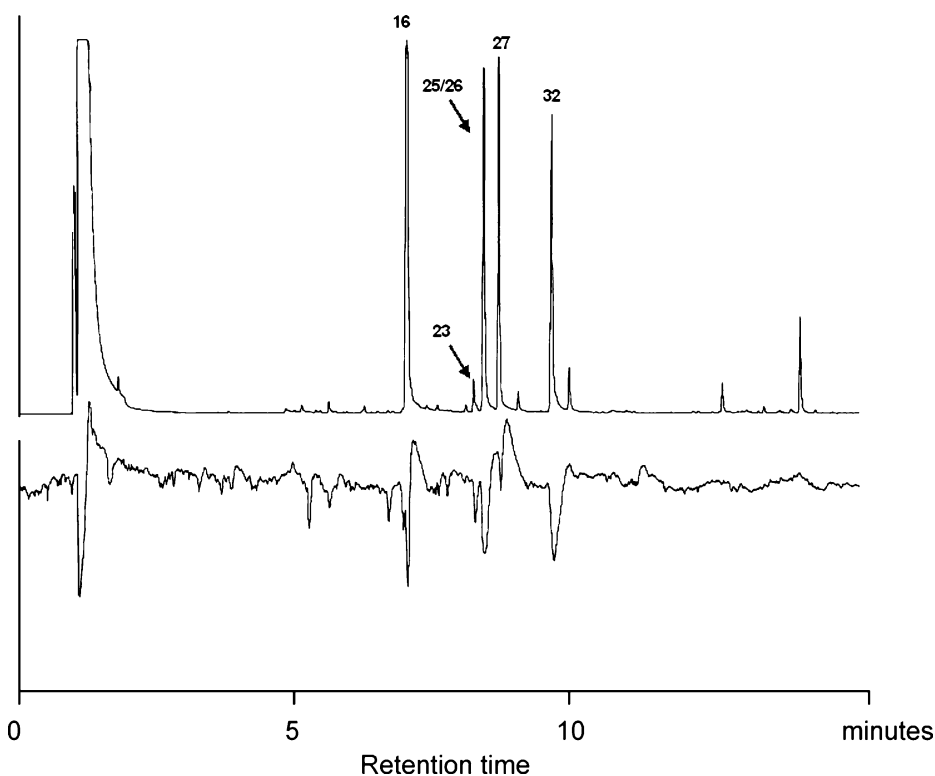


Fig. 1 Coupled GC-EAG with female *Busseola fusca* using volatiles entrained from *Hyparrhenia tamba*. Upper trace: GC of *H. tamba* volatiles; lower trace: EAG response from *B. fusca*. Major EAG-active peaks are marked (see Table 1)

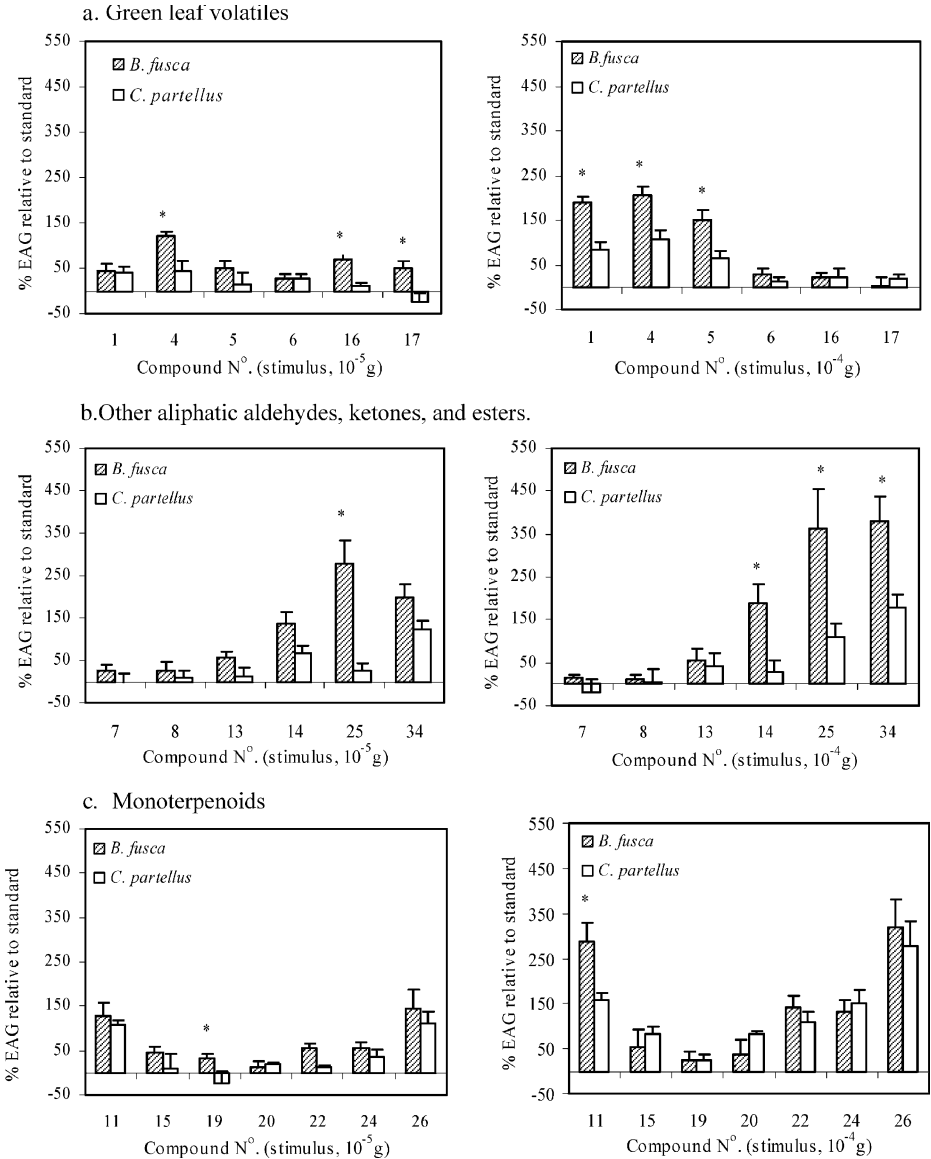


Fig. 2 Electrophysiological activity for *Busseola fusca* and *Chilo partellus*, relative to that of 4-allylanisole (10^{-5} g), of authentic compounds; asterisk indicates a significant difference between the responses of the two species ($P < 0.05$)

and *C. partellus* were observed for these aldehydes and for the green leaf volatiles hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, and hexyl acetate. In all cases, with the exception of α -humulene, where significant differences in relative EAG responses were observed between the two stemborers, the strongest responses were elicited from *B. fusca* (Fig. 2).

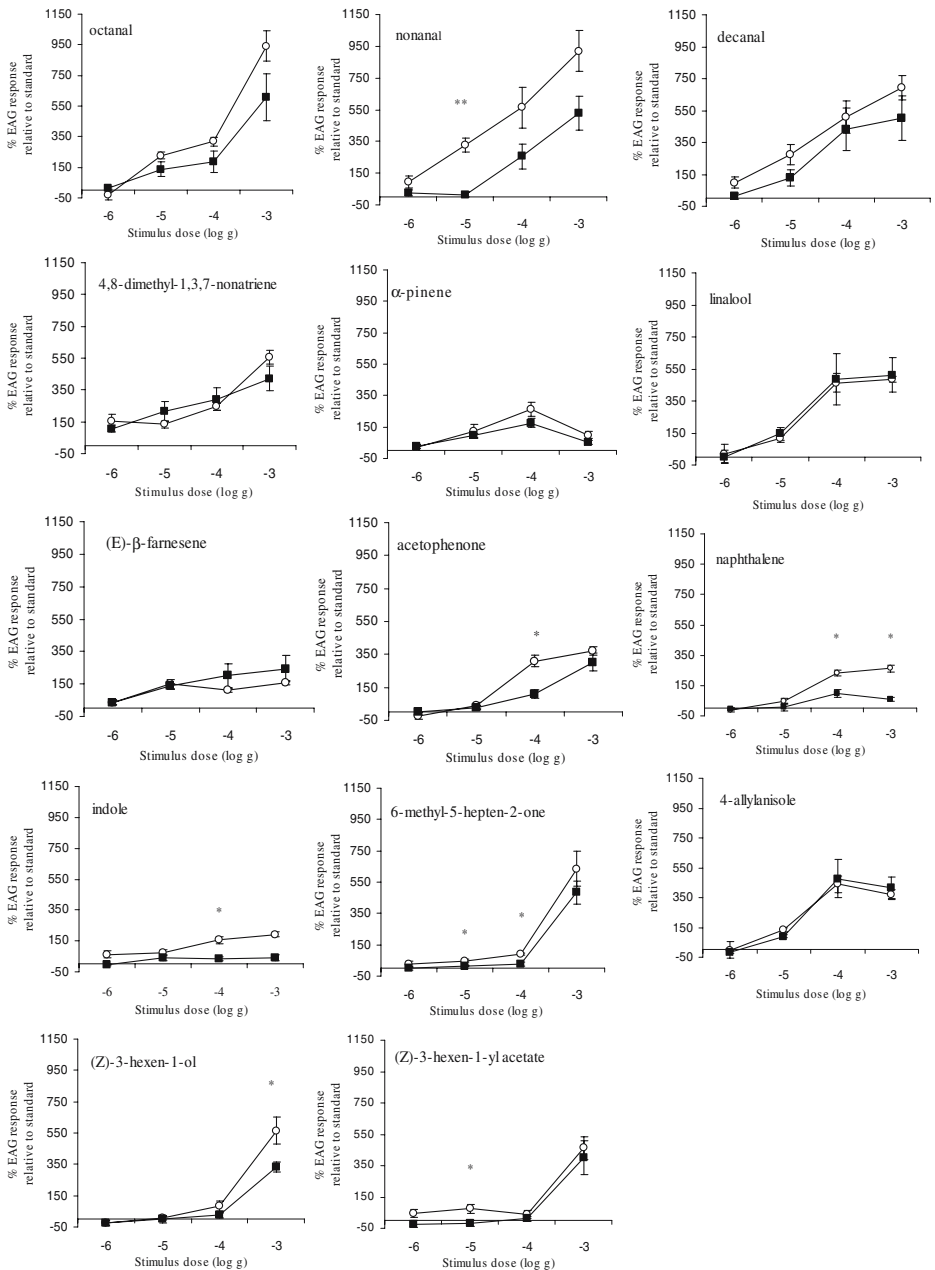


Fig. 3 Dose–response curves of electrophysiological activity, relative to that of 4-allylanisole (10^{-5} g), for *Busseola fusca* (○) and *Chiloptartellus* (●); asterisk indicates a significant difference between the responses of the two species ($P < 0.05$)

Discussion

The differential oviposition preference of *C. partellus* and *B. fusca* for the wild host grasses, used as trap crops, over the cultivated crop plants is essential in the “push–pull” system of stemborer control. While an earlier investigation identified six compounds used in host location by these stemborer moths (Khan et al., 2000), the results did not explain the insects’ preference for the wild hosts. The results of this more comprehensive study may assist in our understanding of the reasons for that behavior. However, differences between the climatic and soil conditions in East Africa and in a UK glasshouse may affect the quantities and ratios of emitted volatiles, although it is unlikely that such differences would cause qualitative changes in secondary metabolism.

Coupled GC-EAG showed the presence of 43 active compounds (Table 1), 41 of which were subsequently identified by GC-MS and cochromatography. The activities were confirmed by EAG with authentic compounds, which showed significant differences in the relative responses of the two species of stemborer to a number of compounds. Of particular interest are the differences in response to some of the green leaf volatiles (Fig. 2a), especially (*Z*)-3-hexen-1-yl acetate, which is one of the major components in the volatiles from the two wild hosts, particularly *P. purpureum* (Table 1). The aliphatic aldehydes **4** and **5** (Fig. 2a) and **14**, **25**, and **34** (Fig. 2b) also produce significant differences between the responses of the two species of insects. In all cases, it is *B. fusca* that is more sensitive. In fact, in all cases where there was differential peripheral sensitivity, with the exception of α -humulene (**42**), compounds elicited a greater relative EAG response in *B. fusca* than in *C. partellus* (Fig. 2). It is possible that the coevolution of *B. fusca* with three of the four plant species has resulted in a greater sensitivity to the volatiles from them than in the introduced species, *C. partellus*, which has coevolved with none of them.

Six key compounds, octanal (**14**), nonanal (**25**), linalool (**26**), naphthalene (**31**), 4-allylanisole (**33**), and eugenol (**37**), were identified in a previous study (Khan et al., 2000) as mediating host location and oviposition by gravid stemborer females. However, this is the first time that volatiles from the wild host, *H. tamba*, have been investigated and shown to include the same compounds. In the present work, electrophysiologically significant levels of octanal (**14**), nonanal (**25**), and linalool (**26**) were found in the volatiles obtained from all cultivated and wild hosts (Table 1). However, electrophysiologically significant levels of 4-allylanisole (**33**) and eugenol (**37**) were found only in the wild hosts, *P. purpureum* and *H. tamba*, and *S. bicolor*. EAG activity associated with naphthalene (**31**) was not found in the *Z. mays* samples. Although these results are in contrast to previous studies, which showed GC-EAG activity in volatile samples from *Z. mays*, *P. purpureum*, and *S. bicolor* to be associated with 4-allylanisole (**33**), eugenol (**37**), and naphthalene (**31**) (Khan et al., 2000), this difference may simply reflect differences in overall levels of volatiles used in the two GC-EAG studies. Authentic samples of all three compounds were found to elicit significant EAG responses from both *C. partellus* and *B. fusca* antennae (Fig. 2d).

Within the four host plant species, only the semiochemicals produced by *Z. mays* have been studied in any detail, and largely from plants under stress, either biotic or abiotic. A number of studies have investigated the induction of volatiles in *Z. mays* by herbivore damage (Turlings et al., 1998a; Ngi-Song et al., 2000; Degen et al., 2004), simulated herbivore damage (Bernasconi et al., 1998; Turlings et al., 1998b), or mechanical damage (Ngi-Song et al., 2000) and compared them with those from undamaged plants. In this context, it is particularly interesting to find the production, from undamaged plants, of large quantities of (*E*)-4,8-dimethyl-1,3,7-nonatriene (**27**) and (*E,E*)-4,8,12-trimethyl-1,3,7,11-

tridecatetraene (**43**), and from *Z. mays*, (*E*)-ocimene (**22**), as these compounds are normally associated with stress (Table 1) (Loughrin et al., 1994; Takabayashi et al., 1995), although there were no other signs of stress in any of the plants. (*E*)-4,8-Dimethyl-1,3,7-nonatriene (**27**) has been shown to reduce oviposition by gravid stemborer females and also to attract parasitoids (Turlings et al., 1990, 1991, 1995; Khan et al., 1997b; Gouinguéné et al., 2001), which makes its high production by the wild trap crop hosts surprising.

Most of the large difference in the overall production of electrophysiologically active volatiles (Table 1) between cultivated and wild hosts was associated with the production of green leaf volatiles (Table 1), possibly suggesting a key role for these compounds in host location. Although the EAG responses to these compounds were relatively low (Fig. 2a and 3), they are present in the volatiles at higher concentrations than most others (Table 1). The green leaf volatiles, in particular, hexanal (**4**), (*E*)-2-hexenal (**5**), (*Z*)-3-hexen-1-ol (**6**), and (*Z*)-3-hexen-1-yl acetate (**16**), also show a marked diel periodicity, extremely large increases in their production occurring during the 1st hr of the scotophase (Chamberlain et al., 2006). This also supports the hypothesis that they could be important compounds in locating oviposition sites for stemborers, since this is the period when most host-locating flights take place (Päts 1991).

The overall trend for *B. fusca* to be more responsive to the compounds than *C. partellus* is highlighted in the case of the unknown compound with a Kovats Index of 743 (**2**). Although this was shown by GC-MS to be present in all the hosts except *Z. mays* (Table 1), it only gave a GC-EAG response with *B. fusca*, indicating a significant differential acuity between the two stemborer species. In this context, *B. fusca* has co-evolved with *P. purpureum*, *H. tamba*, and *S. bicolor*, whereas *C. partellus* is an introduced species in Africa. Work will continue on elucidation of the structure of both unknown compounds.

Although plant volatiles are known to play a key role in host location by phytophagous insects (reviewed in Bernays and Chapman, 1994; Dethier 1982; Visser 1986, 1988; Pickett et al., 1998; Bruce et al., 2005;), the ability to detect a kairomone at the peripheral level does not necessarily mean that the component is associated with host plant recognition. To address this issue, further laboratory behavioral assays and field studies with these compounds are required. Nevertheless, this study provides insights into possible host location kairomones used by these two species of stemborers and into the differential attraction/oviposition between cultivated and wild hosts observed in the field. In particular, it provides some essential scientific input required for the sustainability of the “push–pull” strategy.

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mimicking natural overwintering conditions, are essential to our long-term goal of using this technology to detect and identify the aggregation pheromone of *H. axyridis*.

Keywords β -Caryophyllene · *Harmonia axyridis* · gas chromatography/mass spectroscopy (GC/MS) · terpene · solid-phase microextraction (SPME) · whole air sampling · Racemic

Introduction

The multicolored Asian lady beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), is an effective predator of aphids and other soft-bodied insect pests in pecan, apple, and citrus ecosystems (Tedders and Schaefer, 1994; Brown and Miller, 1998; Michaud, 2002; Brown, 2004) during the spring and summer. Lady beetles play an important role as biological pest control agents. Nonetheless, they are considered a nuisance pest when they overwinter in North American homes. In autumn, adults leave feeding sites in agricultural and urban landscapes and search for overwintering sites. Attics, wall voids, and other inaccessible locations in houses and man-made structures are often selected (Nalepa et al., 1996; Schaefer, 2003). Odors emanating from beetles in infested locations can lead to allergic rhinoconjunctivitis (Yarbrough et al., 1999; Ray and Pence, 2004). As a defense, *H. axyridis* adults secrete hemolymph from their joints when roughly handled (King and Meinwald, 1996; Laurent et al., 2002). This secreted hemolymph can stain furniture and draperies, and gives off an unpleasant odor.

In recent years, *H. axyridis* has become a concern to grape growers and the wine industry. The inadvertent crushing of beetles with grapes releases defensive compounds (e.g., pyrazines) resulting in off-taste wines (Cudjoe et al., 2005). Production of beetle-tainted wines has been reported in several wineries in the northeastern United States and southern Canada (Pickering et al., 2004, 2005).

A potential approach to managing this useful, but problematic insect is by implementing a push–pull strategy. Such an approach relies on using environmentally friendly repellents to push beetles away from structures or grape clusters during the fall, and attractants (possibly aggregation pheromones) to pull beetles into outdoor traps (Riddick et al., 2000; Riddick and Aldrich, 2004). To begin the process of detecting and identifying volatiles that function as attractants or aggregation pheromones, research was initiated to compare airborne chemicals released from living male and female lady beetles.

Gas chromatography/mass spectrometry (GC/MS) is a common analytical technique used to identify volatile analytes and MS offers sensitivity for the detection of compounds that are in trace amounts. However, GC and GC/MS alone can not always handle sample matrices directly, and sometimes extraction and preconcentration is necessary prior to analysis. To study the chemical profile of beetle odors, we utilized two sample introduction techniques. Solid phase microextraction (SPME) was developed in the early 1990s as a means of extracting and preconcentrating pollutants in water samples (Arthur and Pawliszyn, 1990; Zhang and Pawliszyn, 1993), but has also been used for the study of airborne insect pheromones (Malosse et al., 1995). SPME involves absorption/adsorption of volatiles and semivolatiles in a sample matrix to be fused onto a chemically coated fiber. Direct desorption of the extracted compounds into the GC column occurs in the heated injection port of the GC system. In contrast, trapped whole air sampling, a modified EPA TO-14 type methodology (Environmental Protection Agency, 1989), is similar to SPME. However, a variable volume of air is removed from the sample and preconcentrated by

absorption/adsorption depending on the chemical composition of the trapping material in the trap. The trap temperature is rapidly raised to allow desorption, and a series of valves allows the carrier gas to sweep through the trap that is now in line with the GC column. Both of these techniques allow living subjects to be tested in a more natural, less-stressed environment. The solvent-free environment is unique to these techniques, and their sensitivity allows reduced sampling time over traditional methods. Both whole air and SPME extraction are time-saving procedures, which reduce stress to the insects and allows the investigation of their normal pheromone production. Traditional methods often involve solvent extractions and direct injection into a GC port via a syringe (Malosse et al., 1995; Agelopoulos and Pickett, 1998; Al Abassi et al., 1998; Robbins et al., 2003; Zhang and Aldrich, 2003; Cudjoe et al., 2005).

The primary objective of this study was to develop a useful, sensitive method for detection and identification of volatile chemicals emitted by live lady beetles.

Methods and Materials

Insect

Cultures *H. axyridis* adults were purchased from Rincon-Vitova Insectaries Inc. (Ventura, CA, USA) and placed at random into one of two polypropylene cages (30 × 30 × 30 cm, 24 mesh size, Bug Dorm 1™; MegaView Science Education Services Co., Ltd., Taiching, Taiwan), provisioned with food (pure honey, apple slices, lepidopteran eggs) and sterile water on cotton wads at the base of each cage. Both cages contained mixed sexes of beetles of unknown age. Cages were kept inside a plant growth chamber (at 10°C, 60–68% RH, 12-hr photophase).

Fig. 1 Environmental sampling chamber. This is a 1-l plexiglas cylindrical container, encircled with copper tubing connected to a water bath to control temperature. A small, mesh-covered, battery-operated fan is housed inside the chamber to maintain homogeneous air circulation. The chamber is equipped to accept SPME fibers and an air tube for whole air sampling



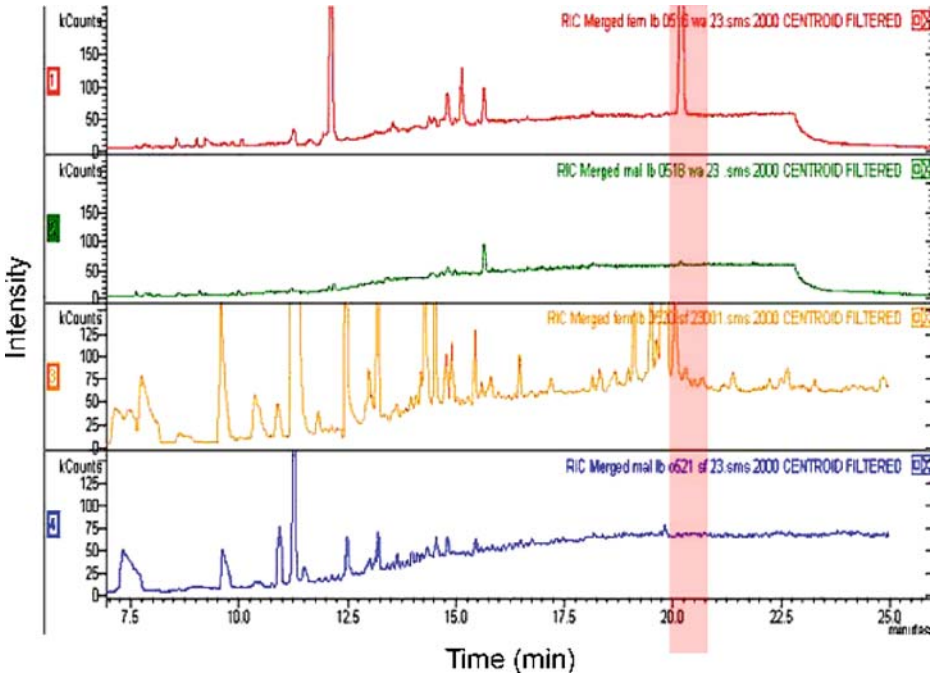


Fig. 2 Total ion chromatograms. These total ion chromatograms, obtained via GC/MS, represent air samples from with the Environmental Sampling Chamber containing 3 g lady beetles. (1) Whole air sampling of females, (2) whole air sampling of males, (3) SPME sampling of females, and (4) SPME sampling of males. Shaded peak shows an area of difference between males and females and major analyte was identified as β -caryophyllene

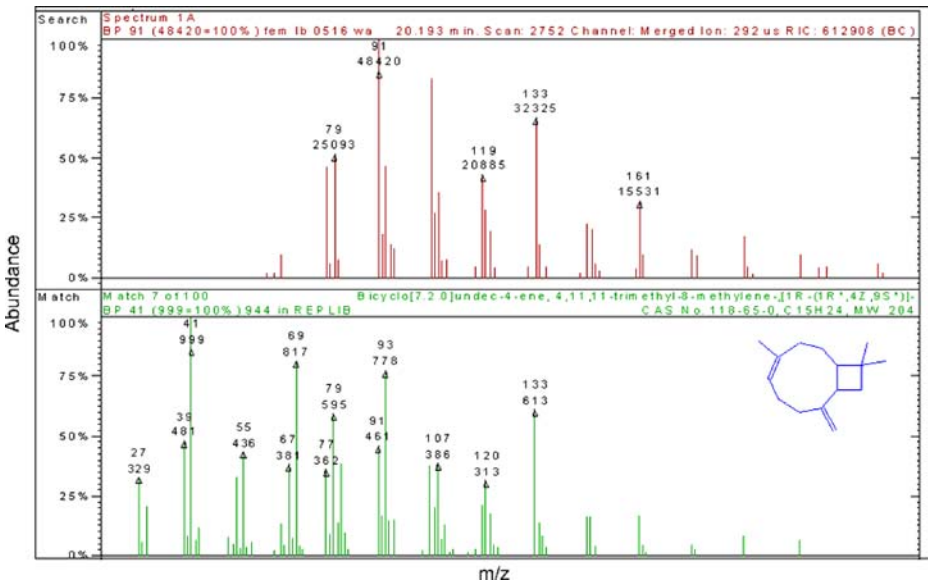


Fig. 3 Spectral match of the shaded peak to the NIST Library using data acquired on ion trap MS

Sample Preparation

H. axyridis adults were separated by gender, and approximately 3.0 g (101 males, 83 females) of insects were placed in the environmental sampling chamber for analysis. Mass was used to normalize weight discrepancy between sexes. The sampling chamber was essentially a modified, plexiglass (1 l) capillary syringe (Fig. 1). Once placed in the chamber, beetles were allowed to acclimate for 8 hr. All GC/MS sampling was performed at 24°C; temperature was kept constant throughout by circulating tap water from a water bath and through copper tubing, encircling the chamber. A small battery-operated fan was placed inside the chamber to maintain constant air circulation. A screen was placed around the fan to prevent beetles from contacting the fan blades.

A 1.0 mg/ml stock standard solution of pure β -caryophyllene dissolved in acetone was prepared and used for confirmation of analyte identification. Aliquots of these solutions were immediately sealed and kept in the refrigerator. Fresh standards were always used prior to each analysis, and all chemicals were obtained from Sigma-Aldrich.

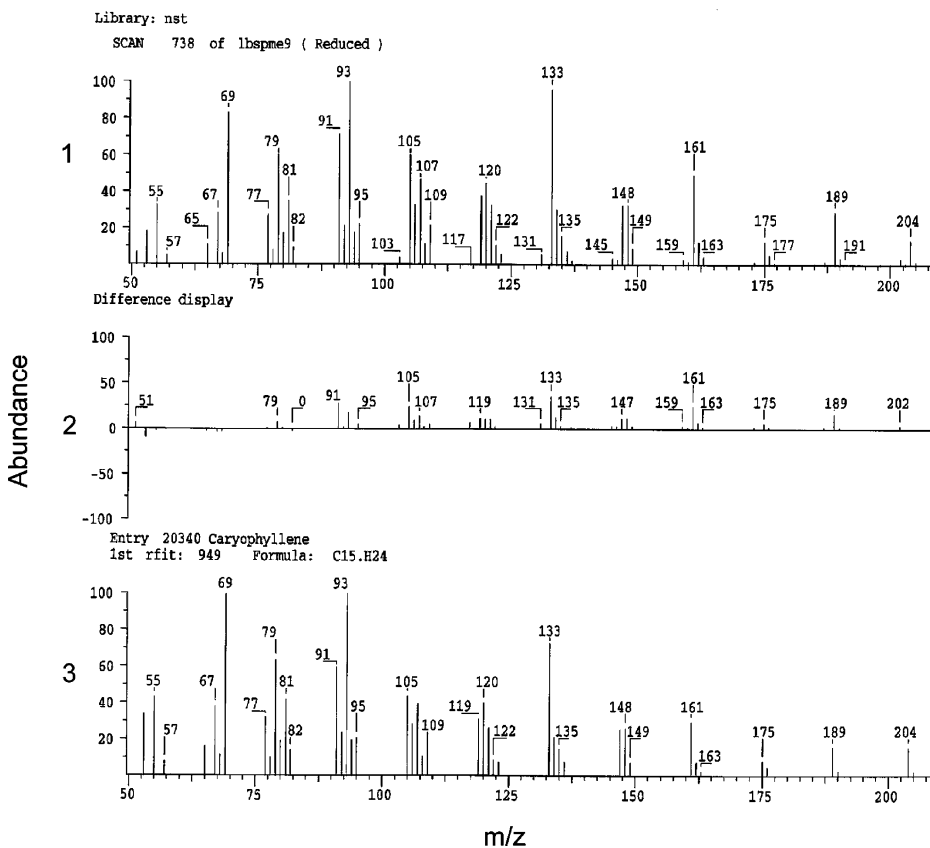


Fig. 4 Spectral identification confirmed using NIST Library and data acquired on Magnetic Sector MS. (1) Raw MS spectra of female-specific analyte corresponding to the peak represented in Fig. 2. (2) Spectral difference between the raw MS spectra and the NIST Library match. (3) NIST Library match spectra identifying and confirming the compound as caryophyllene

Sample Collection

A 75- μm carbonex/polydimethylsiloxane fiber (SPME, SupelcoTM), 1 cm in length, was used for volatile sequestration. Prior to extraction, the fiber was conditioned for 30 min in the injection port of the GC at 250°C. The fiber was allowed to absorb at 24°C for 2 hr and desorb for 3 min at 200°C.

A Tenax-G-Trap (Varian Chromatography Systems, Walnut Creek, CA, USA) was used for the whole air sampling. The trap was held at 4°C for 12.5 min, while 40 ml/min of sample air were collected yielding a total volume of 500 ml. Desorption of the trap was at 180°C for 2.5 min and then baked at 200°C for 20 min.

GC/MS Separation

A Varian Star 3600 GC with helium as a carrier gas passing through a Restek Rtx-Volatiles column (60 m \times 0.32 mm, with a 1.5- μm film) coupled with the Varian Saturn 2000 GC/MS with wave board technology, which has the NIST library installed, was used for identification. Regardless of the sampling technique, the initial GC oven temperature (40°C) was held for 3 min and then ramped to 260°C at 20°C min⁻¹, and then held for another 11 min. Electron impact ionization (EI) or chemical ionization (CI) of the analytes was achieved by using the Saturn 2000, with a scan range of 60–250 m/z and scan time of 0.45 scan/sec. For confirmation and identification of the analytes, a Finnigan MAT 95 XL Magnetic GC/MS operating at 5000 resolution was utilized based on this instrument's extreme sensitivity and spectral resolution. The column in this magnetic sector GC/MS was a Phenomenex ZB5 (30 m \times 0.25 mm, with a 0.25 μm film). Helium was used as the carrier gas, and the system was equipped with the NIST library. The higher resolution system used electron impact with a MS scan range of 50–250 m/z and a scan time of 1 sec/decade.

Chiral Separation

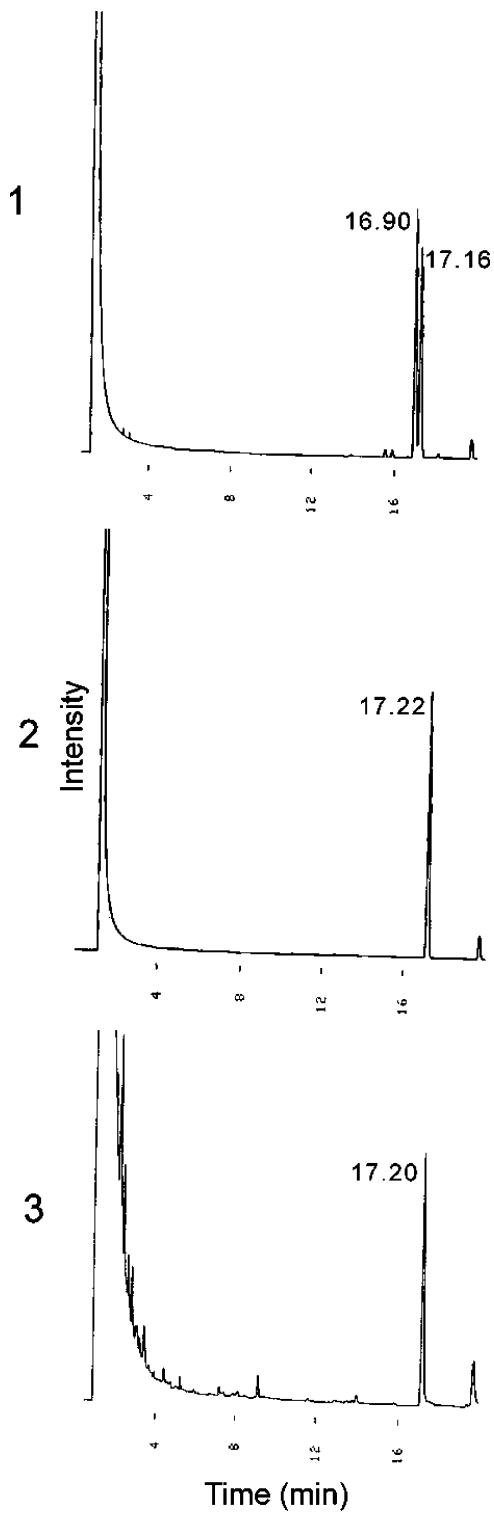
Racemic β -caryophyllene was resolved by GC on a Cyclodex-BTM capillary column (30 m \times 0.25 mm i.d.; 100°C for 3 min, to 117°C at 1°/min; Advanced Separation Technologies, Inc., Whippany, NJ, USA). Since neither the plus enantiomer nor the racemic β -caryophyllene are

Table 1 Retention times (min) for caryophyllene peaks

	Trial 1	Trial 2	Trial 3	Average	SD	SEM
Whole air sampling (EI)						
Females	20.193	20.186	20.195	20.191	4.74×10^{-3}	2.74×10^{-3}
Males	NA	NA	NA	NA	NA	NA
Caryophyllene standard	20.189	20.194	20.191	20.191	2.52×10^{-3}	1.45×10^{-3}
SPME (EI)						
Females	19.879	19.816	19.742	19.812	6.86×10^{-2}	3.96×10^{-2}
Males	NA	NA	NA	NA	NA	NA
Caryophyllene standard	19.790	19.789	20.123	19.901	0.192	0.111
SPME (EI) high resolution						
Females	11.123	11.135	11.121	11.126	7.57×10^{-3}	4.37×10^{-3}
Males	NA	NA	NA	NA	NA	NA
Caryophyllene standard	11.102	11.112	11.134	11.116	1.64×10^{-3}	9.45×10^{-3}

EI: Electron impact ionization; NA: not applicable.

Fig. 5 Enantiomeric composition of β -caryophyllene from *Harmoinia axyridis*. (1) Racemic mixture of β -caryophyllene yielding two peaks (+) 16.9 min and (-) 17.2 min, (2) pure (-)- β -caryophyllene, (3) Sample of ~500 adults



commercially available (Fricke et al., 1995), it was necessary to prepare racemic β -caryophyllene. This was accomplished by converting α -humulene [(*E,E,E*)-2,6,6,9-tetramethyl-1,4,8-cycloundecatriene; Fluka Chemical Corp., Ronkonkoma, NY, USA] to (\pm)- β -caryophyllene according to Greenwood et al. (1968).

Results

A comparison of volatiles emitted by male and female live beetles was achieved by using SPME and trapped whole air sampling techniques (Fig. 2). Differences in the total ion chromatograms between sexes, independent of the sampling technique, were evident. The shaded area in Fig. 2 highlights one of these areas of difference and indicates a compound that is present only in females. The identification of the analyte was obtained by a combination of comparisons with the NIST Library (Figs. 3 and 4) and by comparing the retention time with known standards (Table 1), which were analyzed by ion trap and magnetic sector GC/MS (Fig. 4). The analyte was β -caryophyllene. β -Caryophyllene (bicyclo[7.2.0]undec-4-ene 4,11,11, trimethyl-8-methylene) has a molecular weight of 204. The gaseous emissions from the same lady beetle population were analyzed three times at 2-d intervals. The average retention time run on the Ion trap MS was 20.191 ± 0.005 min for the whole air sampling and 19.812 ± 0.070 min for the SPME sampling. When pure caryophyllene was run with the same methodology, average retention times were 20.191 ± 0.003 and 19.901 ± 0.192 min, respectively, for whole air and SPME. There is greater variation in the retention times for the SPME method compared to whole air methodology. This can be attributed to the automation of the whole air method that reduces the human error and increases precision when compared to manual SPME technique.

The mass spectrum (Fig. 3) shows a fragmentation pattern with major ions of m/z 161, 133, 105, and 91, and gives a spectral probability of 79.3%. The match with β -caryophyllene also shows other terpene compounds that share these ions, and therefore higher-resolution GC/MS was utilized for absolute identification. The Magnetic Sector, equipped with the NIST library, confirmed β -caryophyllene as the analyte when compared with pure standard at retention time of 11.126 min. Figure 4 reveals little spectral difference between the raw analyte MS spectra and the NIST reference.

The racemic mixture of β -caryophyllene was resolved with near-baseline separation, exhibiting two peaks eluting at 16.9 and 17.2 min. ($-$)- β -Caryophyllene eluted at 17.2 min, establishing that the earlier eluting enantiomer was ($+$)- β -caryophyllene (Fig. 5). Coinjection experiments of an aeration sample of ~ 500 male and female *H. axyridis* adults (73 h/75 μ l CH_2Cl_2) with (\pm)- β -caryophyllene and ($-$)- β -caryophyllene established that caryophyllene from *H. axyridis* consisted solely of the ($-$)-enantiomer with no trace of ($+$)- β -caryophyllene.

Discussion

This work demonstrates an innovative approach in which compounds emitted by live laboratory reared beetles, in an environmentally controlled chamber, can be analyzed with two different sampling methods. Environmental chamber design along with the development and sensitivity of the sampling techniques allows for the analysis of beetle odors without subjecting the insects to undue stress. Traditional methods have involved solvent extractions that use charcoal filters, with lady beetles packed in a small cylindrical tube for

several days (J.R. Aldrich, unpublished data). These tight quarters, in the absence of food and water, can cause unintentional die-off of some of the test beetles. The induced stress may impact the profiles of emitted volatiles. Instead of several days, our sampling time is reduced to a matter of hours, and the sample size can be reduced to ensure a more natural production of emitted volatiles.

The sampling methods removed only a small aliquot of air from the environmental chamber and did not comprehensively deplete air supply. The sensitivity of the procedure can be enhanced by withdrawing larger volumes of chamber air. This was a variable volume system in which the flow rate and/or time could be changed to facilitate the desired sensitivity of the analysis. In contrast, SPME fibers could be introduced into smaller chambers, reducing headspace, and thereby concentrating the volatiles and increasing sensitivity. This increase in sensitivity will play a greater role as we identify more peaks in the chromatography profile in order to isolate the aggregation pheromone.

The sampling methods produced similar chromatograms that showed marked differences between laboratory-reared male and female beetles. A combination of retention time, GC/MS analysis with NIST library searching, complemented by higher-resolution GC/MS allowed for the identification of one analyte. This was identified as β -caryophyllene and was exclusively emitted by the females. Preliminary data from field collected beetles exhibit similar results (A.E. Brown, unpublished data).

Isoprenoids play a major role in governing the behavior of other insects (Birkett and Pickett, 2003). For example, the boll weevil is attracted to cotton plants (Hedin et al., 1973), and the female is sexually attracted to the male by airborne volatiles (Tumlinson et al., 1969). In both cases, the attracting compounds are terpenes. β -Caryophyllene has been isolated from female boll weevils and serves as an attractant under certain conditions (Minyard et al., 1969). β -Caryophyllene serves as an attractant in other species, including the Colorado potato beetle (Khalilova et al., 1998), the carrot fly (Guerin et al., 1983), the damson hop aphid (Campbell et al., 1993), and the green lacewing (Flint et al., 1979). Plant-derived (–)- β -caryophyllene inhibits the response of the lady beetle, *Coccinella septempunctata* L., to aphid alarm pheromone [(E)- β -farnesene] in olfactometer bioassays (Al Abassi et al., 2000).

The detection of the (–)-enantiomer of β -caryophyllene in airborne volatiles from *H. axyridis* females was determined by chiral column GC and by comparison of the elution sequence of the racemic mixture. The minus enantiomer is commonly found in a variety of plant species (Nazaruk et al., 2002).

This study demonstrates that volatiles emitted from live laboratory-reared beetles can be analyzed by using SPME or whole air sampling coupled with GC/MS. These sampling techniques show differences in volatile and semivolatile organic chemicals emanating from *H. axyridis* males and females. Future studies might include lowering the temperature of the environmentally controlled chamber to mimic the natural conditions within overwintering aggregations. Although SPME fiber desorption appeared to be more sensitive than whole air sampling, the latter is presently more compatible with automated real-time analysis. Unattended automated analysis will allow us to study the physiological responses of *H. axyridis* to changes in temperature, light, and humidity.

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Keywords *Tetranychus kanzawai* · *Phaseolus lunatus* · Induced indirect defense · Jasmonic acid · Salicylic acid · Herbivore-induced plant volatiles (HIPVs)

Introduction

Spider mites are common plant pests that feed mostly on leaves. A typical symptom of spider mite feeding is the presence of small, slightly colored scars. The color of scars induced on kidney bean leaves by the spider mite *Tetranychus kanzawai* Kishida (Acari: Tetranychidae) can be categorized as red or white (Yano et al., 2003). After applying bidirectional selection to the color of the leaf scars in two strains of this mite, Yano et al. (2003) found that the scars became uniformly red in one strain and white in the other (hereafter called “Red” and “White” strains). Reciprocal and backcrosses between the strains suggested that the Red genotype was fully dominant over the White, and that the color of leaf scars induced by *T. kanzawai* was controlled by a single locus (Yano et al., 2003).

Herbivory by spider mites induces several defensive responses in infested plants, including the emission of volatiles that attract natural enemies of the mites (e.g., Sabelis et al., 1984; Dicke, 1994), and the expression of defensive genes in the plant (e.g., Ozawa et al., 2000). Differences in the color of leaf scars by the Kanzawa spider mite would also be a result of differences in the induced responses of kidney bean plants.

Jasmonic acid (JA) is one of the endogenous signals involved in eliciting induced direct defense against herbivores and pathogen in plants (Creelman and Mullet, 1997; Wasternack and Parthier, 1997). It is also considered to be one of the important factors involved in induced indirect defense, i.e., the production of herbivore-induced plant volatiles (HIPVs) that attract carnivorous natural enemies of the herbivore (Boland et al., 1995; Dicke et al., 1999; Gols et al., 1999; Ozawa et al., 2000, 2004; Schmelz et al., 2003). The other plant-signaling pathway involved in induced defense is the salicylic acid (SA) signaling pathway, which is known to be involved in induced direct defense against pathogens (Ryals et al., 1996; Durner et al., 1997). Ozawa et al. (2000) showed that both the JA and SA signaling pathways are involved in the production of lima bean plant volatiles induced by the two-spotted spider mite, *Tetranychus urticae*.

The objective of this study was to determine whether intraspecific variation in *T. kanzawai* differentially affects induced defensive responses in lima bean plants. Using Red and the White strains from a field-collected population of *T. kanzawai*, we studied whether the two strains differentially induce defensive responses. We investigated the expression of genes coding pathogenesis related (PR) proteins that are under the control of either the JA or SA signaling pathways. The amounts of SA in intact leaves and those infested by either the Red or the White strains were measured. We also analyzed the headspace volatiles from lima bean leaves infested by each strain to investigate whether the induced defensive response of leaves against each strain differentially affects the production of HIPVs.

Methods and Materials

Plant and Mites Lima bean plants (*Phaseolus lunatus* L.) were grown in soil in a climate-controlled greenhouse (25 ± 2°C, 60–70% RH, 16L–8D). We used plants with two fully expanded primary leaves (10–13 d old) for all experiments.

Herbivorous Kanzawa spider mites (*T. kanzawai*) were collected from hydrangea, *Hydrangea macrophylla* (THUNB. Ex MURRAY) SER. f. *macrophylla*, at Ami, Ibaraki (36°1'N, 140°12'E), on April 27, 2004. Collected mites were transferred onto detached lima bean leaves (ca. 100 mites per leaf), and the leaves were pressed onto water-saturated cotton in Petri dishes (90 mm diam, 14 mm deep; hereafter referred to as “leaf disks”). We placed the disks in transparent plastic containers under controlled conditions ($25 \pm 2^\circ\text{C}$, 60–70% RH, 16L–8D).

Bidirectional Selection of Pure Mite Strains To obtain pure mite strains that induced either red or white leaf scars, we selected individual mites based on the color of the scars that they induced on lima bean leaves, following the method of Yano et al. (2003) with minor modification. The population collected from *H. macrophylla* was used as a base population for the following selection experiment. Adult females (100–120) were randomly selected from the base population and individually isolated on 10×10 mm bean leaf squares on water-saturated cotton in Petri dishes. These squares were cut from intact lima bean leaves. Females were allowed to feed for 4–6 d. The observed scar colors were classified as either red or white. The 10 females that induced the most distinctive red scars and the 10 females that induced the most distinctive white scars on the leaf squares were selected. The selected females were grouped according to the induced scar color and transferred to a fresh leaf square for oviposition. Generated mites were allowed to randomly mate and produce the next generation that became the base population for the second selection. We repeated this procedure for more than eight generations. They were called Red strain and White strain.

Mode of Inheritance To examine the mode of inheritance responsible for scar color variation and gene induction, we conducted reciprocal crosses according to the method of Yano et al. (2003). Three randomly selected teleiochrysalis females of each strain were separately introduced onto a 10×10 mm leaf square, together with an adult male of the opposite strain. We randomly selected female hybrids. The color of the scar induced by the hybrid female was determined, as described above. For gene expression analysis, we placed 20 hybrid females on each detached primary leaf. After 24 hr of infestation, total RNA was isolated from the leaf by the same procedure as described in the “RT-PCR Analysis” section, below.

Performance Index of Adult Females The number of eggs produced within a certain period is used as a performance index in *T. kanzawai* (Gomi and Gotoh, 1996). Teleiochrysalis females from each of the Red and White strains were collected and after 1 d the newly emerged females were individually transferred onto a 10×10 mm leaf square. We counted the number of eggs laid by each female within 1, 3, and 7 d. We also conducted the same experiment with adult females that were randomly collected from the mite colony, because we used such mites in the following experiments.

RT-PCR Analysis We detached a primary leaf with the petiole from intact lima bean plants (10–13 d old) and immediately transferred it into a vial (10 ml) containing distilled water. We separately placed 20 adult females of Red and White strain on a detached leaf and allowed them to feed for 24 and 72 hr. We also placed 120 females of White and 5 females of Red strain separately on a detached leaf and allowed them to feed for 24 hr, in order to compare the induction levels of the two strains. Total RNA was isolated from leaves using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized from 1 µg of total RNA by using AMV reverse transcriptase XL (TaKaRa, Kyoto, Japan)

and Oligo dT-Adaptor Primer (TaKaRa) according to the manufacturer's instructions. Negative controls were used in experiments in which the reverse transcriptase was omitted. The first-strand cDNA was amplified using *TaKaRa Ex Taq* DNA polymerase (TaKaRa) and the following gene-specific primers (Arimura et al., 2000): for the acidic chitinase gene, 5'-AGCAACAACGTTAATGTTGC-3' and 5'-CTTGCACCATCTATCTCTTC-3'; for the basic chitinase gene, 5'-CTCAGCGCCCTCATATCCAG-3' and 5'-GATGCGGTCTTG AACCTGC-3'. Polymerase chain reaction (PCR) conditions were as follows: 95°C for 30 sec; 30 cycles (acidic chitinase) or 20 cycles (basic chitinase) of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. The PCR products and total RNA (4 µg) were separated in a 1.5% agarose gel by electrophoresis and detected by staining with ethidium bromide. No PCR product was amplified in control amplifications that lacked reverse transcriptase.

Measurement of SA and Salicylic Glycoside (SAG) We placed 20 *T. kanzawai* females on each detached leaf and allowed them to feed for 24 hr. SA was then extracted from the leaves and quantified according to the method of Malamy et al. (1992), with minor modification. Half a gram of lima bean leaves was ground in 5 ml of 90% (v/v) methanol and centrifuged at 10,000 × *g* for 15 min. The pellet was back extracted with 5 ml of 100% methanol and centrifuged again. Methanol extracts were combined and dried at 40°C under vacuum. Extracts were resuspended in water at 80°C. The extracts were equally divided into two tubes: one for SA and the other for SAG measurements. An equal volume of 0.2 M sodium acetate (pH 4.5) was added to each tube. For the SAG measurement, 5.5 units of β-glucosidase (Sigma) were added into the tube and incubated at 37°C overnight, whereas for the SA measurement, the sample was incubated under the same conditions but without the enzyme. After digestion, samples were acidified to pH 1–1.5 with HCl. Free SA was extracted into 2 volumes of cyclopentane/ethyl acetate/isopropanol 50:50:1. The organic extract was dried and analyzed with a high-performance liquid chromatography (HPLC) system equipped with a spectrofluorescence detector (model RF-550A; Shimadzu, Kyoto, Japan) under the following conditions: column, Inertsil ODS-3 (GL sciences, 4.0 × 150 mm); column temperature, 40°C; mobile phase, 23% (v/v) methanol in 20 mM sodium acetate (pH 5.0); flow rate, 1 ml/min; excitation wavelength, 313 nm; emission wavelength, 405 nm.

Recovery rate of SA was determined by using SA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) standard that was added into the crude methanol extracts (SA standard sample). As a negative control, methanol extracts without the SA standard were also prepared. After the partial purification described above, the amount of SA was analyzed with HPLC. Recovery rate of SA (73.4%) was determined by subtracting SA amount in the negative control from the SA standard sample.

Analysis of Headspace Volatiles We placed 5 or 20 *T. kanzawai* adult females on a detached leaf, allowed them to feed for 72 hr, and then collected headspace volatiles from two leaves. The leaves were placed in a 2-l glass bottle with two nozzles; one was connected to an air cylinder and the other was connected to a glass tube packed with Tenax TA adsorbent (100 mg, mesh 20/35). Pure air gas from the cylinder was drawn into the glass bottle, and volatile compounds from the headspace of the bottle were collected with Tenax TA for 1 hr at a flow rate of 100 ml/min. The collected volatile compounds were analyzed by GC-MS (GC: Hewlett Packard 6890 with HP-5MS capillary column: 30 m long, 0.25 mm I.D., and 0.25 µm film thickness; MS: Hewlett Packard 5973 mass selective detector, 70 eV) equipped with a thermal desorption cold trap injector (TCT; CP4010; Chrompack, Middelburg, The Netherlands). Headspace volatiles collected on Tenax-TA were released from the adsorbent by heating in the TCT at 220°C for 8 min within a flow of

He gas. The desorbed compounds were collected in the cold trap unit of the TCT (SIL5CB-coated fused silica capillary) at -130°C . Flash heating of the cold trap unit provided sharp injection of the compounds into the capillary column of the gas chromatograph to which the cold trap unit was connected. The oven temperature of the GC was programmed to rise from 40°C (5 min hold) to 280°C at $15^{\circ}\text{C}/\text{min}$. Headspace volatiles were identified by comparing their mass spectra with those of the database (Wiley7N and Wiley275) and by comparison of their retention times and mass spectra with those of authentic compounds or volatile compounds from lima bean leaves infested with *T. urticae*.

Statistics *t*-Tests were used to compare the numbers of eggs laid by the Red and the White strain ($N = 27\text{--}30$), and the ion intensities of each headspace volatile compound emitted from lima bean leaves infested with the two mite strains ($N = 6\text{--}8$). Tukey's HSD test was used to determine significant differences among treatments in the experiments for endogenous salicylic acid and salicylic glycoside (SAG) analyses ($N = 4$).

Results

Bidirectional Selection of Red and White Strains After nine mite generations of bidirectional selection based on leaf scar color, the color induced by each strain became uniformly red or white (Fig. 1). To examine the mode of inheritance responsible for the color variation, reciprocal crosses between the two strains were performed. Almost all female hybrids from the reciprocal crosses of Red female \times White males ($N = 20$) and White female \times Red male ($N = 18$) induced red scars. Only one female from each cross induced an ambiguously colored scar. These results showed that the Red genotype was fully dominant over the White genotype.

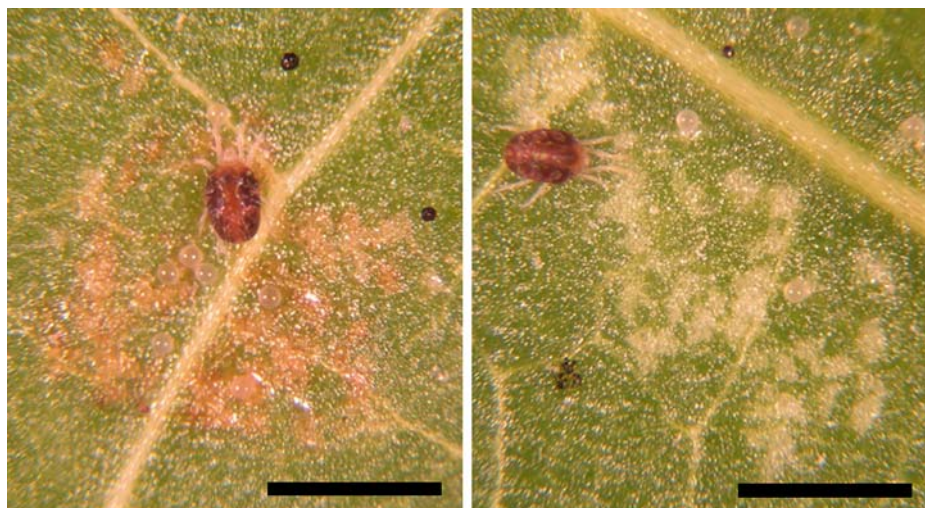


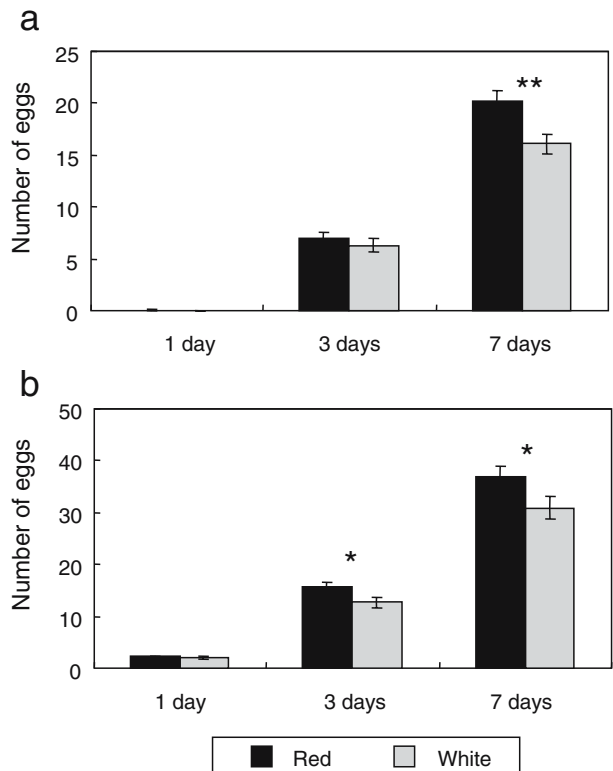
Fig. 1 Photographs showing the scar colors induced on lima bean leaves by the Red (left) and White (right) strains of *Tetranychus kanazawai*. Each stain was allowed to feed for 1 day. Bars = 1 cm

Performance Index of Adult Females The numbers of eggs laid by newly emerged females from teleiochrysalis within 1 and 3 d were not significantly different between the Red and White strains ($P > 0.05$, *t*-test; Fig. 2A), but the number of eggs laid by Red females within 7 d was higher than that of White females ($P = 0.003$, *t*-test; Fig. 2A). Similarly, the number of eggs laid by randomly collected adult females within 1 d was also not different between the two strains ($P > 0.05$, *t*-test; Fig. 2B), but the numbers of eggs laid by randomly collected Red females within 3 and 7 d were higher than those of White strain females ($P = 0.031$ for 3 d and $P = 0.049$ for 7 d, *t*-test; Fig. 2B).

Expression Profiles of Defensive Genes Induced by Red and White Strains We compared the expression of two defensive genes, acidic and basic chitinase, in lima bean leaves when infested by either the Red or the White strain (Fig. 3). In intact lima bean leaves, no expression of acidic chitinase was observed, whereas a slight expression of basic chitinase was observed. As the intact leaves were detached from the plant, the leaves had some injury stress, and this is likely to be one of the reasons why the slight induction of basic chitinase in intact leaves was detected.

The expression of the acidic chitinase gene was observed in lima bean leaves infested by 20 Red females (Red 20) for 1 d. The expression was further up-regulated after 3 d of infestation. Conversely, we could not detect the expression of acidic chitinase gene in lima bean leaves infested by 20 White females (White 20) for 3 d. The expression of basic chitinase was up-regulated in both Red- and White-infested leaves in 1 d. The expression was further up-regulated in both strains infested leaves at 3 d after damage.

Fig. 2 The total numbers of eggs laid by the Red and White strains within 1, 3, and 7 d by using newly emerged females (a) and randomly collected females (b). Two White strain newly emerged females and one Red strain and one White strain random collected females died on day 7, and so only eggs laid by these mites in 1 and 3 d were included in the data set. Error bars represent standard errors ($N = 27-30$). $**P < 0.01$, $*P < 0.05$ (*t*-test)



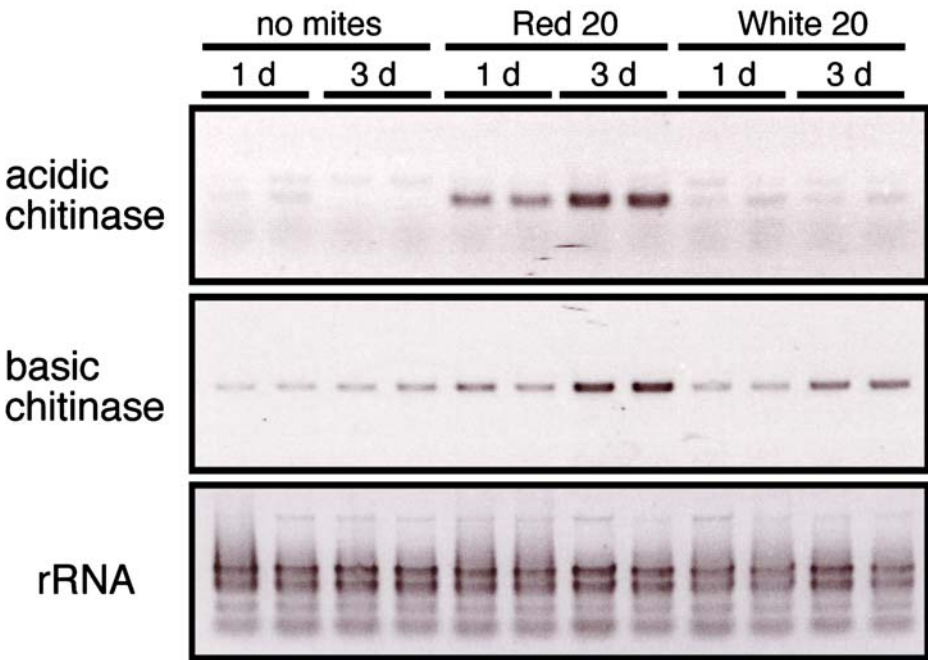


Fig. 3 Acidic and basic gene expression in lima bean leaves in response to feeding by Red and White scar strains of *Tetranychus kanazawai*. Acidic and basic chitinase gene expression in lima bean leaves was investigated by RT-PCR analysis. Twenty females of Red (Red20) and White (White20) strains were used for infestation. RNA was collected 1 (1d) and 3 d (3d) after infestation. rRNA was used as a loading control

Next, we studied the effects of the number of Red and White mites on the expression of both basic and acidic chitinase genes (Fig. 4). The expression of acidic chitinase gene was up-regulated when the number of Red mites was increased from 5 to 20. Likewise, the expression of acidic chitinase gene was also up-regulated when the number of White mites was increased from 20 to 120. The level of the expression of acidic chitinase in White 120 was lower than Red 20, based on the RT-PCR band intensity. Basic chitinase showed the same dose-dependent up-regulation in both strains. The expression of basic chitinase in White 120 and Red 20 leaves was similar according to the RT-PCR band intensity.

When lima bean leaves were infested by 20 female hybrids from the reciprocal crosses of either Red female \times White male or White female \times Red male, we observed increased acidic chitinase gene expression, which was at the same level as that in leaves infested by 20 Red females (Fig. 4).

Levels of Endogenous Salicylate The concentration of SA in leaves infested by Red females was higher than those in either intact leaves or in leaves infested by White strain ($P < 0.05$, Tukey–Kramer’s HSD; Fig. 5A). The amount of SAG showed the same trends between intact leaves, leaves infested by Red females and those infested by White females ($P < 0.05$, Tukey–Kramer’s HSD; Fig. 5B).

Headspace Volatiles of Leaves Infested by either Red or White Strains We recorded seven compounds in the headspace of lima bean leaves infested with 5 or 20 females of either Red or White strain for 72 hr. Uninfested control leaves either did not emit or barely emitted

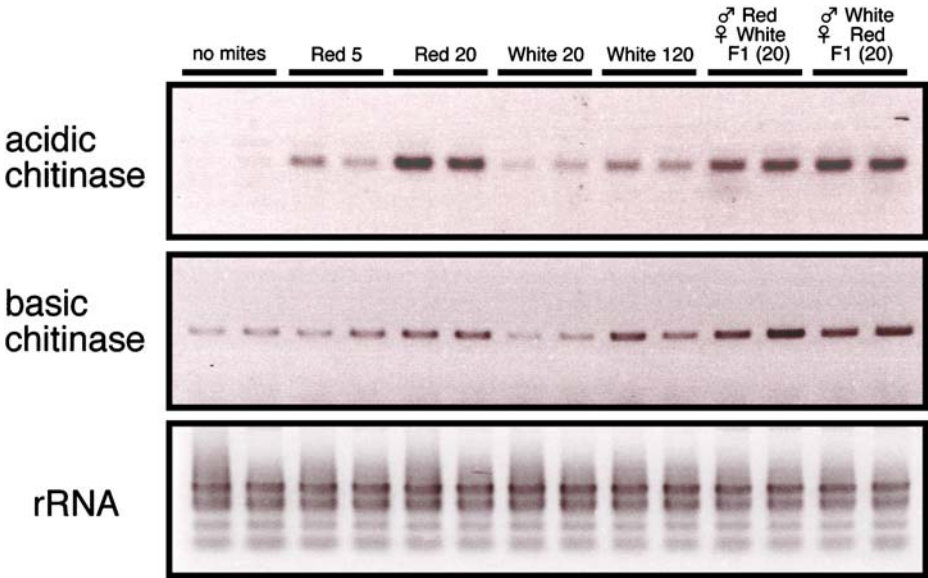


Fig. 4 Effects of the number of mites and their genetic background on defensive gene expression in lima bean leaves. Numbers after strain names and in parentheses represent the number of females used for infection. Red × White F1 and White × Red F1 represent the hybrids of reciprocal crosses between the Red and White stains. Plants were infested for 1 d. rRNA was used as a loading control

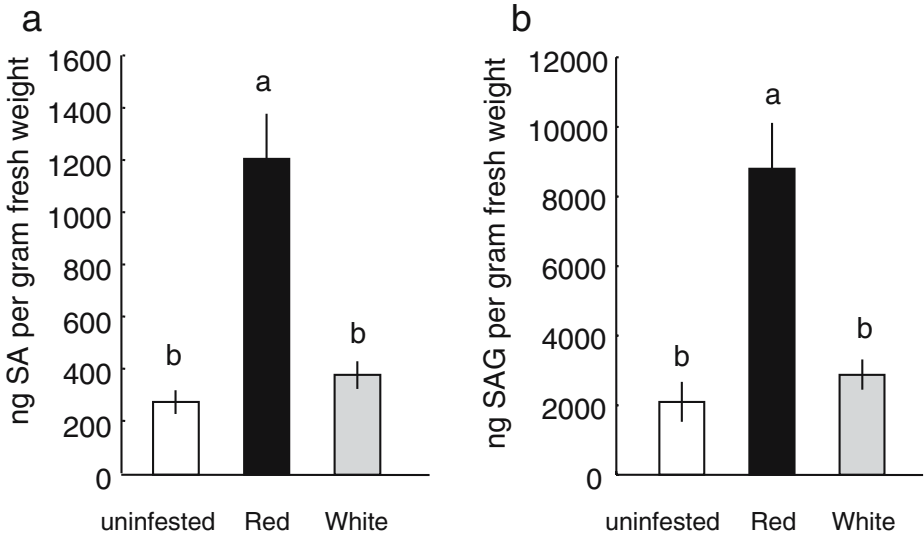
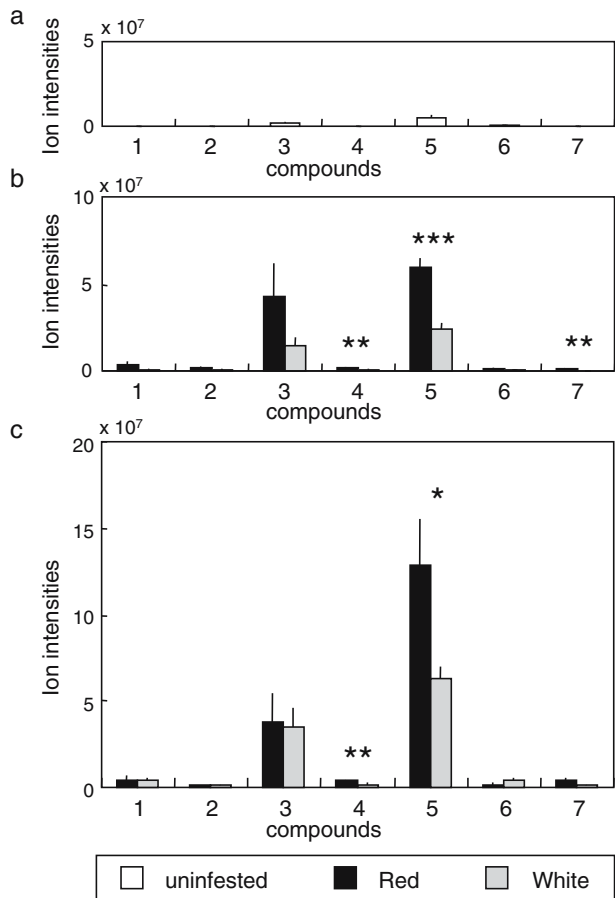


Fig. 5 Levels of endogenous salicylic acid (a) and salicylic glycoside (b) in lima bean leaves infested with Red and White strain mites. Different letters on the top of the bar in each treatment are significantly different ($P = 0.05$, Tukey–Kramer’s HSD)

Fig. 6 Ion intensities of head-space volatile compounds emitted from two lima bean leaves that were uninfested (a), or infested with Red/White strain mites: leaves infested with 5 females per leaf (b) and leaves infested with 20 females per leaf (c). Head-space volatiles were collected 72 hr after treatment. Error bars represent standard errors ($N = 4-8$); $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ (t -test). Compounds: **1**, (Z)-3-hexenyl acetate; **2**, (Z)- β -ocimene; **3**, (E)- β -ocimene; **4**, (Z)-4,8-dimethyl-1,3,7-nonatriene; **5**, (E)-4,8-dimethyl-1,3,7-nonatriene; **6**, methyl salicylate; **7**, (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene



these volatiles (Fig. 6A). When the leaves were infested with five females, significantly higher amounts of (Z)-4,8-dimethyl-3,7,11-nonatriene (Z -DMNT), (E)-4,8-dimethyl-3,7,11-nonatriene (E -DMNT), and (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) were emitted from leaves infested with Red females than in leaves with White females ($P < 0.05$, t -test; Fig. 6B). Also, the leaves infested with 20 Red females emitted higher amounts of Z -DMNT and E -DMNT than those from the leaves with 20 White females ($P < 0.05$, t -test; Fig. 6C).

Discussion

In this paper, we have shown that intraspecific variation in the Kanzawa spider mite, *T. kanzawai*, differentially affects induced defensive response in lima bean plants. From our cross experiments, we found that the color of scars induced by *T. kanzawai* was determined by mite genotype, and that the genotype of the Red strain was dominant over the White strain. Yano et al. (2003) reported the same results using kidney bean leaves as a food plant

for the mites. The performance of the Red strain mites on a lima bean leaf was slightly better than that of the White strain, and that difference was detectable at least after 7 d of damage made by newly emerged mites (Fig. 2). Interestingly, the number of eggs laid by randomly selected Red strain mites by both days 3 and 7 was significantly higher than that by White strain mites (Fig. 2), probably because average age of the randomly selected mates was higher than that of newly emerged mites. We found characteristic differences in acidic chitinase inductions between the two strains, which were randomly selected, even 1 d after infestation. Such difference in the gene induction would be the result of differences between factors injected in the leaves by the two strains, and the different response in lima bean plants against two strains would affect the performance of the mites.

It has been reported that the expression of defensive genes, such as genes for PR proteins, are induced by herbivory (Koiwa et al., 1997; Ozawa et al., 2000). Our results of gene expression analysis suggest that the damages made by Red strain mites resulted in an increased expression of both basic chitinase (*PR-3*) and acidic chitinase (*PR-4*), whereas the induction by White strain mites mainly affected the expression of basic chitinase (Fig. 4). To examine the mode of inheritance responsible for acidic chitinase induction, reciprocal crosses between the two strains were conducted (Fig. 4). Cross experiments indicate that the Red genotype was dominant over the White for the induction of acidic chitinase, suggesting that the Red strain has factors to induce acidic chitinase gene expression.

Damage made by the Red strain induced the expression of genes for both basic chitinase, which was downstream of the JA signaling pathway, and for acidic chitinase, which was downstream of the SA signaling pathway (Thomma et al., 1998; Ozawa et al., 2000). The induction of acidic chitinase and the higher accumulation of salicylate in leaves infested by the Red than the White (Fig. 5) indicate the specific activation of the SA signaling pathway in leaves infested by Red strain mites. Conversely, the JA signaling pathway would be induced by both strains, as a gene encoding basic chitinase was induced by both strains. Ozawa et al. (2000) showed that both the JA and SA signaling pathways are activated in lima bean leaves infested by *T. urticae*, and that both pathways were involved in the production of *T. urticae*-induced lima bean leaf volatiles. The major volatile compounds induced by methyl salicylate treatment in lima bean leaves are E-DMNT and TMTT (Ozawa et al., 2000). E-DMNT is also induced by the application of JA in lima bean and corn leaves (Hopke et al., 1994; Koch et al., 1999; Ozawa et al., 2000). By contrast, TMTT is not induced by JA treatment in lima bean leaves (Hopke et al., 1994; Koch et al., 1999; Ozawa et al., 2000). In this study, the emission of Z-DMNT, E-DMNT, and TMTT was higher in leaves infested with Red strain mites than those infested with White strain mites. Such differences could be partly explained by the differences between the strains in the activation of the JA and SA signaling pathways.

HIPVs have been attracting considerable attention among chemical ecologists and plant physiologists (Takabayashi and Dicke, 1996; Dicke and Vet, 1999; Kessler and Baldwin, 2002). One of the most intriguing features of HIPV production is the herbivore-species specificity: HIPVs emitted from infested plants qualitatively and/or quantitatively differ according to herbivore species, and such differences could result in the attraction of carnivorous natural enemies specific to the infesting herbivore (Takabayashi and Dicke, 1996; De Moraes et al., 1998; Dicke, 2000; Ozawa et al., 2000). Here, we show that even within the same herbivore species different strains induce strain-specific blends of volatiles. One of the mechanisms creating such strain specificity in lima bean plants would be the difference in their induction of the SA signaling pathway.

The Kanzawa spider mite is highly polyphagous, and we have found that Red strain mites made white scars on leaves of other plant species and *vice versa* (Matsushima and

Takabayashi, unpublished data). These data suggest that the response of plants against the damage made by two strains is expected to differ with the host plant species. Thus, a study is needed to clarify whether differences in the induction of the JA and SA signaling pathways, and in the production of herbivore-induced volatiles by the Red and White strains, can be observed in plants belonging to the same and different families.

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2001), which may reflect the presence of particular attractants. One preferred plant is the tropical or Indian almond, *Terminalia catappa* L. Both leaf and fruit extracts of *T. catappa* attracted mainly female flies in laboratory arena bioassays (Chien and Yaw, 2000; Siderhurst and Jang, 2006).

Female-specific attractants are a promising, if illusive, goal of fruit fly control programs because a lure for females removes both females and potential offspring (Jang and Light, 1996). Currently, food-type attractants, such as fermenting sugars, hydrolyzed protein, and yeast, provide the best attractants for *B. dorsalis* females. However, these liquid lures lack potency, have limited field life, are difficult to handle, and attract nontarget species. Plant materials such as fruits and leaves that attract female fruit flies are regarded as promising sources of attractants. Host fruits are of particular interest because some tephritid females are known to be attracted by host volatiles while searching for oviposition sites (Jang et al., 1998, 1999; Jang, 2003). However, isolation and identification of attractants from fruits is often difficult because the fruit odors are a complex blend of volatiles, which change in composition during ripening. Rapid screening of multiple compounds of interest and/or analysis of complex blends has been facilitated by coupled gas chromatography-electroantennogram detection (GC-EAD), which has been successfully used to identify fruit fly attractants from several plant sources (Cosse et al., 1995; Zhang et al., 1999; Nojima et al., 2003a,b). In particular, identification of fruit volatiles that elicited responses from antennae of *Rhagoletis pomonella* led to the development of useful lures for control of this insect (Zhang et al., 1999; Stenliski and Liburd, 2002).

The objective of the present study was to employ GC-EAD analysis to screen for volatiles of *T. catappa* that are detected by the antennae of female oriental fruit flies, and which may serve as host plant attractants. Behavioral responses to several synthetic blends of compounds that elicited antennal responses were recorded in wind tunnel, outdoor olfactometer, and field cage bioassays, with particular attention given to attraction of female flies.

Methods and Materials

Insects

Adult flies were obtained as pupae from the mass-rearing unit at the USDA-ARS Pacific Basin Agricultural Research Center, Mass-Rearing Facility in Honolulu, HI, USA. Larvae were reared on a standard wheat, sugar, and yeast diet (Tanaka et al., 1969). Pupae were shipped by air to Hilo, HI, where they were placed into 30×30×30 cm cubical aluminum screen cages containing sugar, water, and hydrolyzed protein. Flies were held at 24°C, 60–80% relative humidity, and 12L:12D photoperiod until use. Prior to testing, flies were immobilized by chilling at 5°C and separated into groups of either 50 males or females and were then held at room temperature (24°C) for at least 1 hr before use. Flies were approximately 9 to 11 d old when tested and presumed to be mated (ca. >95% of females from mixed cages are mated by day 7) (Jang et al., 1997).

Fruits

Newly ripened fruits of *T. catappa* were collected from two locations, Onekahakaha Beach Park, Hilo, HI, and Anini Beach Park, Kauai Island, HI, from October to December 2004. Fruits from Kauai were shipped by air to Hilo. Fruits were extracted fresh, or frozen at –80°C

until use. Ripening fruit in the laboratory was also attempted, but once green fruit are removed/fall from the tree they do not continue to ripen.

Sampling of Volatiles

Three different solid-phase microextraction (SPME) fibers were used for sampling of volatiles: polydimethylsiloxane (PDMS; film thickness 100 μm ; Supelco Inc., Bellefonte, PA, USA), polyacrylate (PA; film thickness 85 μm) and Carbowax/divinylbenzene (CW/DVB; film thickness 70 μm). SPME fibers were conditioned before use in a GC injector (250°C) for 30 min. Three ripe *T. catappa* fruits were placed in a 500-ml clear straight-sided jar with a screw-top lid fitted with a Teflon liner. The SPME needle was inserted through a small hole in the lid, and the fiber was exposed for 1 hr to absorb volatiles. The fiber was then removed from the collection jar and immediately inserted into the GC injection port to desorb volatiles.

Headspace sampling was also conducted by using Porapak Q (50/80 mesh; Alltech, Deerfield, IL, USA). Absorbent (1 g) was packed between glass wool plugs in a Pasteur pipette, preconditioned at 70°C for 16 hr, washed with dichloromethane, and dried under charcoal-purified nitrogen. Ripe *T. catappa* fruits (2 kg) were placed in a 4-l round-bottomed aeration chamber. Charcoal-purified nitrogen (1 l/min) was used to sweep volatiles from the chamber for 16 hr. Volatiles were eluted from the adsorbents with 1 ml CH_2Cl_2 and stored at -80°C .

Analyses

Electroantennographic responses to fruit volatiles were recorded by using an Agilent Technologies (Palo Alto, CA, USA) 6890 gas chromatograph coupled to a Syntech (Hilversum, The Netherlands) electroantennogram detector system. The GC was equipped with an HP-5 column (30 m \times 0.32 mm ID, 0.25 μm film thickness; Agilent) with helium as carrier gas at 2.3 ml/min and makeup gas at 10 ml/min, which was added before the splitter. A Graphpack-3D/2 flow splitter (Gerstel Inc., Baltimore, MD, USA) was attached to the end of the column, and the effluent was split 1:1 between the flame ionization detector (FID) and the EAD via a heated transfer line (250°C). The injector, in splitless mode, and FID were held at 250°C and 275°C, respectively. The oven was programmed from 45°C/1 min, 10°C/min to 240°C, hold 13 min. Whole fly heads were removed with forceps and secured with electrode gel (Spectra 360; Parker Laboratories, Inc., Fairfield, NJ, USA) between the electrodes of a Syntech EAG probe antenna holder. During initial runs, 1-hexanol was puffed over the antennal preparation to check sensitivity. However, this was found to be unnecessary because the majority of antennal preparations showed usable responses to *T. catappa* volatiles and immediate use increased signal strength. Antennal preparations were used only once. Humidified air (350 ml/min) was used as a carrier for effluent from the EAD transfer line to the antennal preparation. The signals generated by the EAD and FID were passed through a Syntech NL 1200 high-impedance amplifier and analyzed using Syntech GC-EAD2000 software version 2.5. Twenty runs with SPME collections (10 PDMS, 5 PA, 5 CW/DVB) and 10 with Porapak Q collections were used to determine which peaks gave consistent EAD responses. Peaks eliciting EAD responses in at least three runs were marked for identification by GC-mass spectrometry (GC-MS).

GC-MS analysis was performed on an Agilent Technologies 6890N gas chromatograph interfaced with a Hewlett-Packard 5973 Mass Selective Detector equipped with an HP-5MS column (30 m \times 0.25 mm ID, 0.25 μm film thickness). The temperature program used was

Table 1 Compounds from *T. catappa* that Elicited EAD Responses from Antennae of Female *B. dorsalis*

Compounds	SPME		Porapak Q		Prev. ref.
	% FID	EAD (mV)	% FID	EAD (mV)	
Ethanol ^{a,b}	<1	-0.14±0.05	–	–	Bd, Cc
Ethyl acetate ^{a,b}	5±2	-0.28±0.04	–	–	Cc, Rp
(<i>E</i>)-2-Hexenal	–	–	<1	-0.30±0.08	Cc
Isopentyl acetate ^{a,c}	1±4	-0.21±0.03	10±2	-0.15±0.01	Cc, Rp
4-pentenyl acetate	3±7	-0.13±0.05	12±3	-0.16±0.01	
Isopentenyl acetate ^{a,c}	3±6	-0.16±0.04	19±2	-0.30±0.07	Cc
Ethyl hexanoate ^{a,b}	2±2	-0.16±0.02	1±1	-0.07±0.01	Cc
Hexyl acetate ^{a,b}	1±1	-0.17±0.02	3±1	-0.10±0.03	Cc
Menthone ^a	<1	-0.29±0.04	<1	-0.15±0.05	
Unknown (terpinen-4-ol)	1±1	-0.24±0.01	1±1	–	
Linalyl acetate ^{a,b}	3±1	-0.14±0.03	1±1	–	Cc
2-Phenylethyl acetate ^{a,c}	4±3	-0.28±0.13	2±2	–	Cc
Ethyl nonanoate ^{a,b}	<1	-0.17±0.04	–	–	
Nonyl acetate ^{a,b}	<1	-0.27±0.07	–	–	Cc
Citronellyl acetate ^{a,c}	3±2	-0.36±0.06	1±1	–	
Eugenol ^a	10±4	-0.27±0.05	5±2	–	
Geranyl acetate ^{a,c}	25±3	-1.08±0.13	5±1	-0.15±0.03	Cc
Methyl eugenol ^a	31±1	-0.74±0.09	6±2	-0.14±0.02	Bd
(<i>E</i>)-Ethyl cinnamate ^{a,b}	<1	-0.10±0.00	–	–	
(<i>E</i>)- β -Farnesene ^{a,b}	2±1	-0.37±0.07	<1	–	Bt, Cc
(<i>E,E</i>)- α -Farnesene ^a	2±1	-0.42±0.08	–	–	Bt, Cc
(<i>Z,E</i>)- α -Farnesene ^a	<1	-0.17±0.03	–	–	Bt

Percentages reflect the percent abundance for a single compound by FID peak area (solvent peak excluded for Porapak Q). EAD reflects means of the three largest depolarizations recorded for each compound. Compounds eliciting EAG responses from other tephritids are indicated by species (*B. dorsalis*=Bd, *B. tryoni*=Bt, *C. capitata*=Cc, *R. pomonella*=Rp) (Light et al., 1988, 1992; Jang et al., 1989; Zhang et al., 1999; Hull and Cribb, 2001; Nojima et al., 2003a,b).

^a Components of the EAD SPME blend.

^b Components of the EAD minor blend.

^c Components of the EAD major blend.

45–300°C at 10°C/min with a 1-min start delay, with the injector temperature set at 250°C using helium carrier gas (1.1 ml/min). Compounds from *T. catappa* were tentatively identified on the basis of matches with literature mass spectra (NIST98 mass spectral database), and confirmed by comparison with spectra of authentic standards.

Chemicals

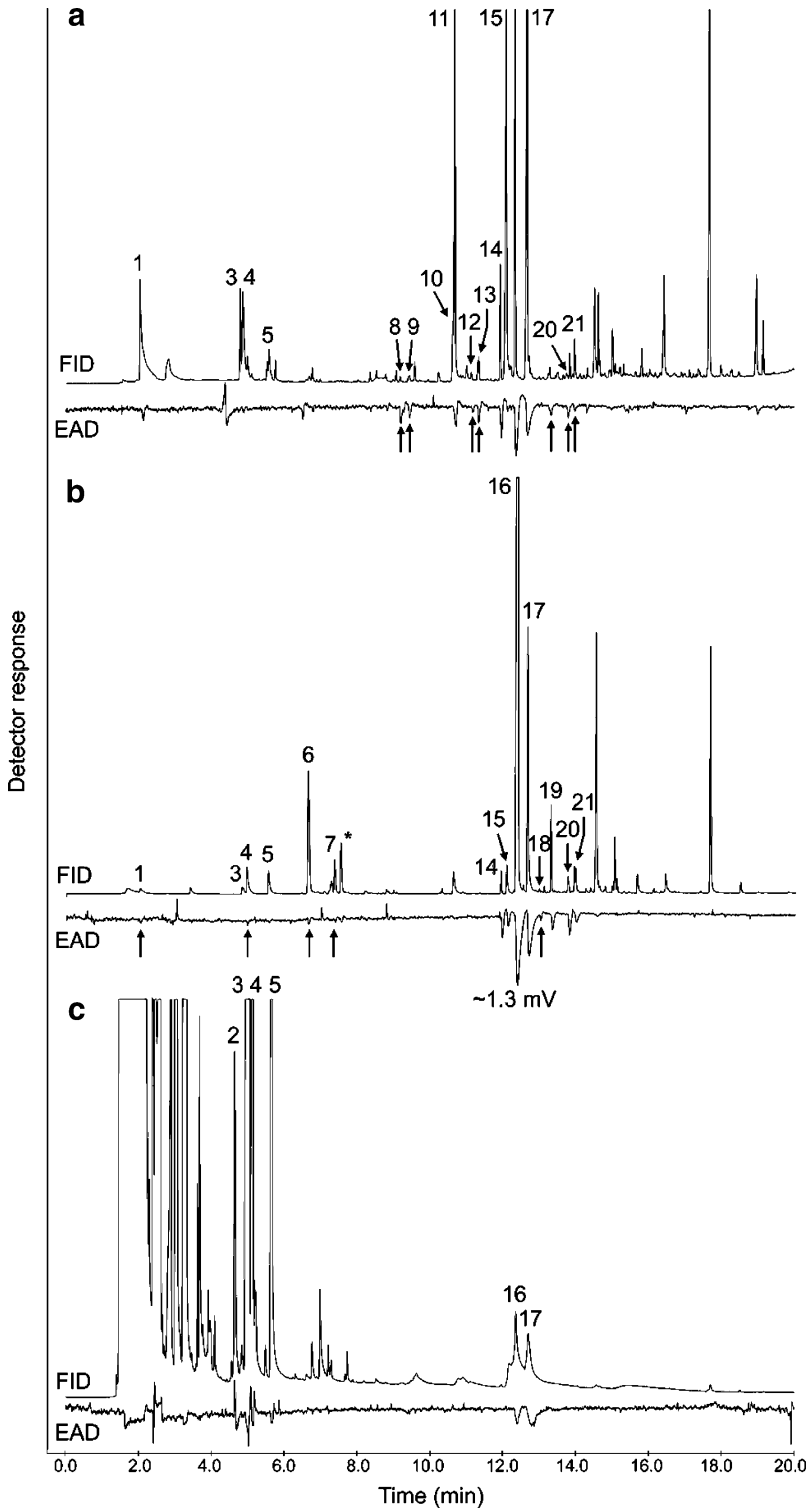
(*Z,E*)- α -Farnesene was prepared as part of a mixture of farnesenes by dehydration of *trans*-nerolidol according to Yarden et al. (1996). Separation of reaction products by column chromatography provided a mixture of farnesene isomers that were used for bioassays. A small amount of (*E,E*)- α -farnesene (<50 μ g, reference standard only) and (*E*)- β -farnesene (~100 mg, used for bioassays and reference standard) were available from previous work (Jang et al., 1989) (samples from R. Buttery, USDA-ARS-WRCC). All other synthetic compounds and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Acros Organics (Geel, Belgium). Compound purities were >98% by GC (FID). Linalyl

acetate, citronellyl acetate, and menthone were mixtures of their respective isomers. All solvents were HPLC grade. Blends of compounds that elicited antennal responses tested in bioassays were: (1) a reconstructed blend of the compounds collected by SPME that elicited EAD responses (using farnesene isomer mixture) (EAD SPME blend; Table 1); (2) (EAD major blend) isopentyl acetate, isopentenyl acetate, 2-phenylethyl acetate, citronellyl acetate, and geranyl acetate; and (3) (EAD minor blend) ethanol, ethyl acetate, ethyl hexanoate, hexyl acetate, linalyl acetate, ethyl nonanoate, nonyl acetate, (*E*)-ethyl cinnamate, and (*E*)- β -farnesene. All blends contained equal amounts of all components at 2 μ g/ml water (60 μ g/trap). These concentrations roughly reflect the concentration of methyl eugenol found in pressed *T. catappa* juice (Siderhurst and Jang, 2006).

Bioassays

Three behavioral bioassays were used to evaluate the attractiveness of blends. The first bioassays tested attraction in a more controlled, smaller-scale, short duration assay, whereas the latter bioassays were more natural, larger, and encompassed most of the day.

- (1) A laboratory glass/metal flight tunnel, consisting of a 0.9 \times 0.9 \times 2.8 m rectangular glass tunnel equipped with inlet and exit fans that produced a laminar flow of air (Jang et al., 1997) was used to compare synthetic blends against a neutral control (water). Treatments were prepared by dissolving synthetic compounds in 30 ml of water, with two drops of the nonionic surfactant Igepal CO-630 (Sigma-Aldrich), in small, invaginated glass McPhail traps. For each assay, 50 males and 50 females were released from the downwind end of the flight tunnel and allowed to respond freely for 2 hr. At the end of each test, trapped flies were sexed and counted, and the remaining flies removed. The flight tunnel was then cleaned with window cleaner and a squeegee, and allowed to dry with the fans operating. All assays were run between 13:00 and 16:00 at a temperature of 26–28°C, under fluorescent lights (2,000 lux) (Jang et al., 1994).
- (2) An outdoor multiple rotating trap olfactometer was used to evaluate attractancy on an intermediate scale, over a longer period and under seminatural conditions. This outdoor olfactometer, consisting of a 3 \times 3 \times 2.5 m rectangular wood-framed screen cage (Jang et al., 1997), was used to compare different treatments in competitive tests. A rotating hub with 62-cm-long arms was hung from the center of the cage 1.8 m from the floor. The outer arms of the motorized hub unit revolved at 1.5 rpm. Treatments were prepared in small glass McPhail traps as previously described, and all tests included a neutral control (30 ml water). In addition, some tests included torula yeast/borax (1 pellet/100 ml water), an attractant known to attract females, as a positive control. Approximately 500 flies of each sex were placed in the outdoor cage. The duration of the tests was approximately 19 hr (initiated 13:00; terminated 08:00 following day). Tests were conducted under ambient outdoor conditions (usually 23–28°C) and only natural (indirect) light was used. At the end of the test, flies were removed from the traps, counted, and sexed.
- (3) A field cage was used to evaluate attractancy in a still larger scale arena, over a longer period and under more natural conditions. This screen field cage, 2.5 \times 6 \times 15 m, containing mixed fruit trees, was used to compare treatments in competitive tests. The fruit trees, which included mango, lemon, tangerine, and guava, were stripped of fruit and blossoms before each experiment. Treatments were prepared in small glass McPhail traps as previously described and were hung in mango trees at the corners of



the cage. Some tests included a neutral control (30 ml water) and some included torula yeast/borax (1 pellet/100 ml water,) as a positive control. Trap placements was randomized within each experiment. Approximately 500 flies of each sex were placed in the outdoor cage. Tests were initiated at 10:00 and terminated at 08:00 the following day (ca. 22 hr) when flies were removed from the traps, counted, and sexed.

Data Analysis

Results of the two-choice flight tunnel tests were compared using *t*-tests (Proc TTEST) (SAS, 2000). Multiple-choice test results were analyzed by using analysis of variance (ANOVA), and means were compared using Tukey's HSD studentized range test (Proc GLM) (SAS, 2000). All analyses of significance were made at the $P < 0.05$ level of significance.

Results

GC-EAD analyses of headspace volatiles from whole *T. catappa* fruit revealed 22 compounds that consistently elicited electrophysiological responses from antennae of *B. dorsalis* females (Fig. 1). The relative amounts and percentages of compounds varied substantially with collection type, with Porapak Q showing a higher proportion of smaller, more volatile compounds such as isopentyl acetate, 4-pentenyl acetate, and isopentenyl acetate, compared to SPME fibers (Table 1). However, collections with SPME fibers induced more and stronger EAD responses. Geranyl acetate and methyl eugenol produced the largest responses, and were also the most abundant compounds in SPME collections. Slight variations in compound abundances were seen with different types of SPME fibers, but did not substantially change the profile of EAD responses observed. The largest source of variation in the volatiles was between fruits collected at different locations (Kauai and Hilo) and times. SPME collections of Kauai fruit showed higher concentrations of isopentyl acetate, 4-pentenyl acetate, and isopentenyl acetate similar to Porapak Q collections. In addition to the compounds listed in Table 1, several others were observed to stimulate intermittent, weak responses and were not analyzed further.

Compounds eliciting EAD responses were identified by GC-MS with assignment made by mass spectral database matching and subsequent comparison with authentic standards. These standards were also run on GC-EAD to confirm their electrophysiological activity. This was particularly important in the case of linalyl acetate and 2-phenylethyl acetate because these peaks overlapped. Injections of each compound alone showed that both elicited responses from female antennae. (*Z,E*)- α -Farnesene was assigned by mass spectral data, relative retention index (RI of *Z,E* isomer higher than the *E,E* isomer) and comparison

Fig. 1 GC/EAD analyses of volatiles from *Terminalia catappa* using female *Bactrocera dorsalis*. Volatile collections with PDMS fiber (A), PA fiber (B), and Porapak Q (C). 1: Ethyl acetate; 2: (*E*)-2-hexenal; 3: isopentyl acetate; 4: 4-pentenyl acetate; 5: isopentenyl acetate; 6: ethyl hexanoate; 7: hexyl acetate, 8: menthone; 9: unidentified; 10: linalyl acetate; 11: 2-phenylethyl acetate; 12: ethyl nonanate; 13: nonyl acetate; 14: citronellyl acetate; 15: eugenol; 16: geranyl acetate; 17: methyl eugenol; 18: ethyl cinnamate; 19: (*E*)- β -farnesene; 20: (*E,E*)- α -farnesene; 21: (*Z,E*)- α -farnesene. Arrows indicate consistent EAD responses. In addition, ethanol from *T. catappa* was also found to elicited EAD responses (example trace not shown). *Example of intermittent response.

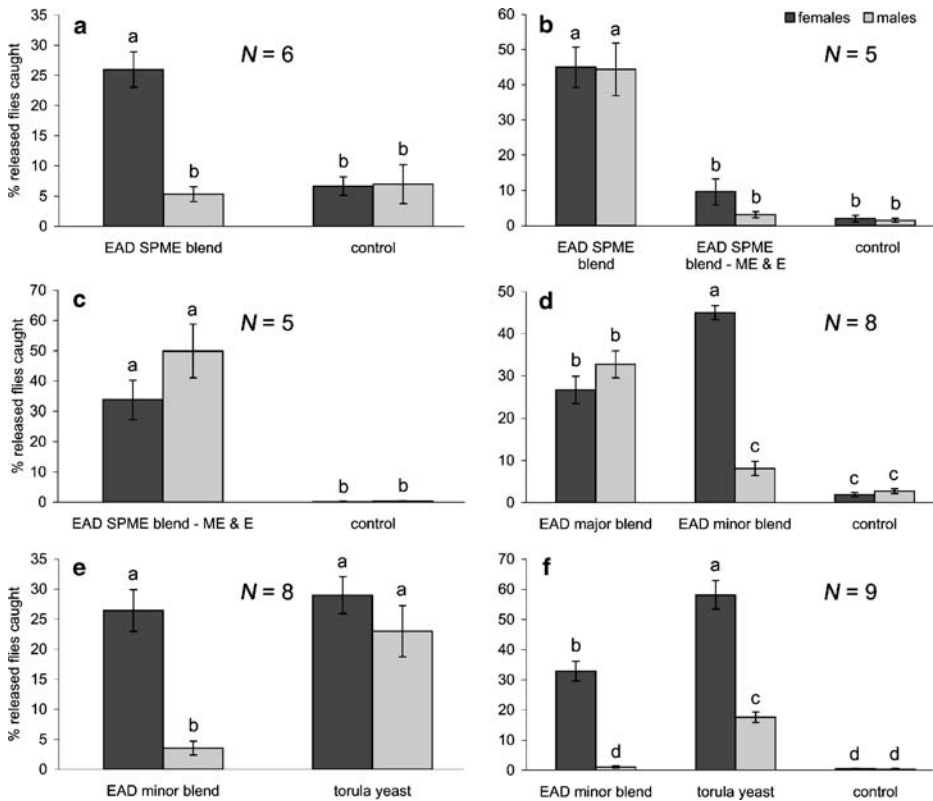


Fig. 2 Bioassays of compounds from *T. catappa* that stimulated antennae of female *B. dorsalis*. Mean percentage of released *B. dorsalis* caught per trap (\pm SE) baited with: (a) a blend of SPME collected EAD active compounds (EAD SPME) or controls in wind tunnel, (b) outdoor olfactometer tests with the EAD SPME blend the EAD SPME blend without methyl eugenol and eugenol, and controls (c) outdoor olfactometer tests with the EAD SPME blend minus methyl eugenol and eugenol, and controls (d) two sets of blend components roughly based on relative strength of EAD response and control in field cage, (e) nine-component blend of weakly EAD active compounds (EAD minor) vs. torula yeast in field cage, and (f) EAD minor blend vs. torula yeast and control in rotating outdoor olfactometer. Within each experiment different letters indicate significant differences at $P < 0.05$; Tukey's HSD (SAS, 2000).

with a mixture of synthetic farnesene isomers. Farnesene synthesis produced three isomers: (*E,E*)- α -farnesene and (*E*)- β -farnesene, which were identified by comparison with authentic standards, and a third isomer was assumed to be (*Z,E*)- α -farnesene. No attempts were made to assign the absolute configurations of compounds containing stereocenters. One consistently EAD stimulatory compound remained unidentified (MS and RI suggest terpinen-4-ol).

Individual compounds that elicited EAD responses were tested in the outdoor rotating olfactometer. There was no appreciable attraction of females to single component traps, and males were only attracted to the known sex attractants eugenol and methyl eugenol (data not shown). In wind tunnel bioassays, traps containing the EAD SPME blend showed female-biased attraction ($P < 0.05$) (Fig. 2a). In contrast, outdoor olfactometer tests with the EAD SPME blend did not show female-biased trap captures (Fig. 2b). Removal of methyl eugenol and eugenol from the blend reduced attraction of both sexes to the level of controls

in one assay (Fig. 2b) but did not restore the female-based attraction observed in the wind tunnel (Fig. 2b & c). In field cage tests, the EAD minor blend attracted more females than the EAD major blend ($P < 0.05$) (Fig. 2d). In addition, the EAD major blend attracted more male and female flies than the control, while the EAD minor blend was not significantly attractive to males ($P < 0.05$; Fig. 2d). Tests with the EAD minor blend vs. torula yeast in field cage (Fig. 2e) and outdoor olfactometer (Fig. 2f) bioassays again showed the selective attraction of females with the synthetic blend. In the field cage tests (Fig. 2e), there were no significant differences ($P < 0.05$) between the number of female flies caught in traps baited with the EAD minor blend, and males and females caught in traps baited with torula yeast. However, in the outdoor olfactometer tests (Fig. 2d), torula yeast-baited traps caught significantly ($P < 0.05$) more males and females than the respective male and female catches for the EAD minor blend. Similar to field cage results, the EAD minor blend female trap capture means were greater than male captures ($P < 0.05$), which did not differ from males captured in the control ($P < 0.05$).

Discussion

Coupled GC-EAD analysis of volatiles collected from *T. catappa* revealed consent female electroantennographic activity to 22 compounds. Identified compounds included acetates, ethyl esters, terpenoids, and phenylpropanoids—all widely found in odors of a variety of fruits and flowers. A number of the EAD stimulatory compounds identified are known to elicit electroantennographic responses from other fruit fly species. *Ceratitis capitata* (Light et al., 1988, 1992; Jang et al., 1989), *R. pomonella* (Nojima et al., 2003a,b), and *B. Tryoni* (Hull and Cribb, 2001) have shown electrophysiological activity to at least one of the following compounds identified in this study: ethanol, ethyl acetate, (*E*)-2-hexenal, isopentyl acetate, isopentenyl acetate, ethyl hexanoate, hexyl acetate, linalyl acetate, 2-phenylethyl acetate, geranyl acetate, nonyl acetate, and the isomers of farnesene (Table 1). Although the limited number of studies on these three species make comparisons difficult, it is interesting to note that *B. dorsalis* has the greatest compound overlap with *C. capitata*, a similarity which may be based in the polyphagous feeding habits of both species (Diaz-Fleischer et al., 1999).

Among the compounds previously known to elicit EAG responses from *B. dorsalis* antennae, methyl eugenol (Lee et al., 1997) and ethanol (Light and Jang, 1987) were found in odors of *T. catappa* fruit. Few electrophysiological studies have been conducted with *B. dorsalis*, and those have focused on limited classes of compounds (Lee et al., 1997, 1998; Light and Jang, 1987). The presence of methyl eugenol in *T. catappa* has been reported previously, and may partly explain why this tree is a preferred host for oriental fruit fly (Siderhurst and Jang, 2006). Because methyl eugenol is widely thought to only attract males, the observed EAD responses from antennae of females was surprising and may be supportive of a behavioral role for methyl eugenol in females of *Bactrocera* species, as suggested by Raghu (2004).

The aim of the blend formulations was to develop mixtures of compounds that would exhibit strong, female-biased attraction. Because of the variation in compound abundances observed in volatile collection, and to reduce testing complexity, all blends contained components at 2 $\mu\text{g/ml}$, roughly reflecting the concentration of methyl eugenol found in pressed *T. catappa* juice (Siderhurst and Jang, 2006). SPME collections have been used in previous research into fruit-based attractants for tephritids by (Zhang et al., 1999; Nojima et al., 2003a, b), and SPME results were the primary source for the formulation of the initial

blend (EAD SPME blend, Table 1) tested in the wind tunnel. 4-Pentenyl acetate and an unidentified compound (tentatively terpinen-4-ol) were commercially unavailable at the time of blend formulations. The female-biased attraction in wind tunnel bioassays, even with the presence of eugenol and methyl eugenol, is again somewhat surprising. However, these results are consistent with previous experiments conducted in the same arena. These previous experiments showed an ~8:1 female/male trap capture ratio with ethanol extracts of *T. catappa*, which contain methyl eugenol (Siderhurst and Jang, 2006). The meaning of these results is unclear but may suggest a female bias associated with this bioassay. All further tests were conducted in larger screen cages with fruit trees, under more natural conditions.

Tests with the EAD SPME blend in the outdoor olfactometer showed no sex-biased trap captures, presumably due to the presence of the eugenol and methyl eugenol in the lure formation, so these compounds were excluded from all further experiments. However, this blend failed to show female-biased attraction when eugenol and methyl eugenol (contained in EAD SPME blend) were removed from the lures (Fig. 2c). Two additional subset blends formulated roughly on the relative strength of EAD responses (EAD major and EAD minor) were tested in field cage trials. The blend of compounds with minor EAD responses had strongly female-biased attraction. Ethyl acetate, ethanol, ethyl hexanoate, and hexyl acetate, four of the nine EAD minor blend components, have been previously reported as components of attractive blends for females (Hwang et al., 2002). Further testing of this EAD minor blend against torula yeast in outdoor experiments showed comparable female attraction to this blend and to yeast, and much lower male attraction.

Similar analytical methodology, utilizing SPME and GC-EAD, has been applied to hosts of another tephritid, *R. pomonella*, including apple (Zhang et al., 1999), flowering dogwood (Nojima et al., 2003a), and hawthorn (Nojima et al., 2003b), and has resulted in successful identification of attractant blends. Although *T. catappa* is strongly attractive to *B. dorsalis* and since compounds that elicited EAD responses were readily identified, the formulation of a fruit odor-based lure from *T. catappa* has proven more difficult than initially expected, largely due to the number of compounds that stimulate the antennae. This difficulty may be reflective of the fundamental differences between monophagous spp., such as *R. pomonella*, and polyphagous spp., such as *B. dorsalis*. Diaz-Fleischer et al. (1999) has suggested that fruit flies with a large host range may respond to a broader range of host odors than monophagous or oligophagous species. This hypothesis is consistent with the relatively high number of compounds that elicit EAD responses from the polyphagous species *B. dorsalis* (this study) and *C. capitata* (Light et al., 1988, 1992; Jang et al., 1989), whereas *R. pomonella* showed responses to a more limited set of compounds (Zhang et al., 1999; Nojima et al., 2003a,b). With *R. pomonella*, a tight fly–host relationship would be expected to lead to olfactory/attraction mechanisms well tuned to the signature compounds/blend of the host. In contrast, *B. dorsalis* may be expected to detect and discriminate between the odors of many hosts, with receptors for a wider range of compounds and not respond as strongly to any single blend. This difference need not preclude the development of fruit odor attractants for generalist fruit fly species, as partly demonstrated in this study, but the formulation of blends may not be as straightforward as it is with specialists.

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(EAG) recordings to the (2*S*,3*R*,7*R*)-isomers of the propionates of 3,7-dimethyl-2-tridecanol, 3,7-dimethyl-2-tetradecanol, and 3,7-dimethyl-2-pentadecanol, as well as to the acetates of the tri- and pentadecanols (the acetate of the tetradecanol was not tested). The propionate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tetradecanol caught more males in the field than the corresponding isomer of tri- or pentadecanol. We suggest that the (2*S*,3*R*,7*R*)-isomer of 3,7-dimethyl-2-tetradecanol is likely the main sex pheromone precursor in *G. pallida*, with a subsidiary role for the (2*S*,3*R*,7*R*)-isomer of the tridecanol. Preparation of highly pure (2*R*,3*R*,7*R*)- and (2*S*,3*R*,7*R*)-stereoisomers of 3,7-dimethyl-2-tetradecanol, including the biologically active esters, was performed via chemoenzymatic methods and is described in detail.

Key words 3,7-Dimethyl-2-tetradecanol · sex pheromone · electrophysiology · gas chromatography · mass spectrometry · synthesis · stereoisomer · lipase · field activity · *Gilpinia pallida*

Introduction

Many species of the sawfly family Diprionidae (Hymenoptera: Symphyta) are important defoliators of northern coniferous forests (Smith, 1993). In 1976, the acetate and the propionate of the alcohol 3,7-dimethyl-2-pentadecanol (diprionol) were identified from *Neodiprion lecontei* (Fitch) and *Diprion similis* (Hartig) (Jewett et al., 1976), constituting the first examples of diprionid sex pheromones. Several other species within the family Diprionidae have been found to use these esters of diprionol or similar alcohols as sex pheromones (see reviews by Anderbrant, 1993, 1999). Usually, only the alcohol precursor is found in the females, whereas males are attracted by the corresponding acetate or propionate. Regarding chemical communication or pheromone biology, most species investigated so far belong to the large genera *Diprion* and *Neodiprion*, although *Macrodiprion* and *Microdiprion* species have also been studied (Bergström et al., 1998; Wassgren et al., 2000; Östrand et al., 2003).

Within the genus *Gilpinia*, only *G. frutetorum* F., a species introduced from Europe to North America, has been investigated. Kikukawa et al. (1982) reported a weak attraction to the acetate of a two-isomer diprionol mixture containing the (2*S*,3*R*,7*S*)- and (2*S*,3*R*,7*R*)-stereoisomers. This and several other species within this genus occur in Europe and Asia and sometimes cause severe damage to pine forests (Pschorn-Walcher, 1982). One of these is *G. pallida* (Klug), with documented outbreaks in northern, central, and eastern Europe. It has one, or in more southern areas, two generations per year and can cause considerable damage (Pschorn-Walcher, 1982). During outbreaks, it sometimes occurs with other diprionids, notably *Diprion pini* (L.) (A. Sierpinski, personal observation). Because *G. pallida* males were often caught in monitoring traps intended for *D. pini*, i.e., baited with the propionate of (2*R**3*S**)-3,7-dimethyl-2-tridecanol (Herz et al., 2000), we suspected that the *G. pallida* sex pheromone was similar to that of *D. pini*. We present here results from the investigations aimed to identify the pheromone, including analysis of female pheromone content, male antennal response and attraction in the field, and synthesis of the main pheromone component.

Methods and Materials

Insects

Females extracted for chemical analyses and males used for electrophysiological recordings came from laboratory cultures at TU-Munich and INRA, Orleans, both originating from

cocoons collected near Eching, Bavaria, Germany (Herz and Heitland, 2002). Additional insects came from cocoons collected at several locations in central and southern Finland.

Extraction

Whole bodies of 50–100 adult females were extracted in pure ethyl acetate for 72 hr at room temperature. Several extracts of females were prepared between 1996 and 2000. Females of different ages were used, from newly eclosed to about 3-d-old. Extracts were purified and fractionated by liquid chromatography (LC) (for details, see Bergström et al., 1995). Fractions 7 and 8, which contained dipironol and related precursor alcohols, were used to identify the structures and to determine the configuration of the stereoisomers.

Chemical Analysis

Purified extracts were analyzed by combined gas chromatography-mass spectrometry. A Hewlett-Packard 5890 gas chromatograph (GC) equipped with a Finnigan TSQ mass spectrometer (MS) (quadrupole) in the electron impact (EI) mode was used for the analyses. The analytical fused silica column (25 m × 0.25 mm i.d.) was a CP-WAX-58 (ChromPack, Middelburg, The Netherlands), coated with a nitroterephthalic acid-modified, chemically bonded polyethylene glycol as stationary phase (film thickness, 0.20 μm). The oven temperature program was kept isothermal at 50°C for 5 min, and then programmed to 220°C at 10°C/min. Alcohols were quantified by adding a known amount (100 ng) of 3,11-dimethyl-2-tetradecanol to the extracts as an internal standard before evaporation and LC.

Stereochemical Analysis

Two gas chromatographic methods were used to determine the configuration of the stereoisomers. In both cases, the alcohols were first derivatized for detection by specific detectors. One method was separation of isopropyl carbamate derivatives (König et al., 1982) on a XE-60-(*S*)-valine-(*S*)-2-phenylethylamide fused silica column (ChromPack; 50 m × 0.23 mm i.d., film thickness 0.12 μm) on a GC equipped with a nitrogen/phosphorous sensitive detector. Analytical conditions were as follows: isothermal at 120°C for 5 min and then programmed to 185°C at 2°C/min. Nitrogen was used as carrier gas at a pressure of 97 kPa. In this case, a Hewlett Packard 5880 GC was used. In the second method, the analysis was carried out on a Hewlett Packard 6890 GC supplied with Chemstation software and coupled to an electron capture detector ⁶³Ni. The stereoisomers were separated as pentafluorobenzoates (Wassgren and Bergström, 1995), by using a CP-Sil-88 fused silica column (ChromPack; 50 m × 0.25 mm i.d., film thickness 0.20 μm). Oven conditions were as follows: isothermal at 60°C for 3 min after injection and then raised at a rate of 5°C/min to 195°C, isothermal 40 min; carrier gas was N₂ (15 psi) and the injection technique was pulsed splitless mode with electronic pneumatic control (EPC).

Electrophysiology

One antenna was excised from each male and placed between two electrodes, consisting of thin glass capillaries filled with Ringer solution. The antennal base was in contact with one grounded platinum electrode. The signal from the electrode holding the antennal tip was connected to a high-impedance amplifier. The antenna was continuously flushed with a charcoal-filtered, moistened airstream of 0.5 m/sec. The outlet of the tube was 1 cm from

the antenna. The stimulus was injected into the airstream through an opening in the glass tube 20 cm upstream of the antenna. A stimulus was created by a stimulation device (Murphy Developments, Hilversum, The Netherlands), which delivered the stimulus in a 0.5-sec puff at a flow rate of 5 ml/sec. The stimulus originated from a Pasteur pipette containing a 5 × 20 mm piece of filter paper. The appropriate amount of synthetic sample, diluted in cyclohexane, was added to the filter paper and the solvent allowed to evaporate. During the recordings, a standard stimulus was delivered every third time, including the first and the last one. Electroantennograph (EAG) recordings were analyzed using “EAG version 2.2a” (Syntech, Hilversum, The Netherlands 1993). The EAG response to a stimulus was standardized by dividing the recorded response by the average of the responses of the standard puffed before and after. Mean responses were analyzed by ANOVA followed by Tukey’s *post-hoc* test at $P < 0.05$ using SPSS 11.0.4 for Macintosh. Data were log-transformed before analysis if necessary to obtain homogeneity among variances.

EAG recordings were performed at different times with different series of synthetic compounds. First, before the extraction and chemical analysis of the females had been completed, a number of known diprionid pheromone isomers were tested. Later, esters of several identified and available alkanol isomers were added (Table 1).

Field Tests

We were fortunate to encounter two outbreaks of this sporadic pest insect (Poland and Sweden) that provided sites for field behavioral assays. Another experiment was conducted in France at a site where *G. pallida* had been observed during previous years. Field trapping experiments were conducted in 1998 and 1999, using Lund-I cardboard sticky traps (Anderbrant et al., 1989). Traps were placed in pine trees 1–2 m above the forest floor and at least 30 m apart. Within tests, traps with baits were repositioned according to a Latin square design when at least 10 males were caught in a trap. Test compounds were weighed and diluted with a known amount of hexane or cyclohexane. An appropriate aliquot was applied to 1 × 4 cm dental cotton rolls (Celluron[®] No. 2, Paul Hartmann AG, Heidenheim, Germany) used as dispensers. The cotton roll was placed under the roof of the trap and released the substances at a logarithmically decreasing rate. The release from the 100 µg bait was approximately 3 µg/d after 5 d and 0.3 µg/d after 1 mo (Anderbrant et al., 1992).

In 1998, 100 µg of (2*S*,3*R*,7*R*)-propionates of 3,7-dimethyl-2-tridecanol, 3,7-dimethyl-2-tetradecanol, and 3,7-dimethyl-2-pentadecanol were tested. Two replicates were placed in the forest district Bielice of forest inspectorate Bydgoszcz, Poland, with Scots pine (*Pinus sylvestris* L.) stands of different ages. Trapping started on July 16 and ended on August 16. In France, one replicate was placed in a plantation of *P. sylvestris* and *P. uncinata* Miller ex Mirbel in the Hautes-Alpes near Bois du Villard (Granon) at 1990 m elevation from June 16 to July 9.

In 1999, the same experiment was repeated, but 50-µg baits were used in a young *P. sylvestris* plantation, near Arjeplog, in northern Sweden, where an outbreak had been recorded the year before. One trap baited with a mixture of all eight stereoisomers of the propionate of 3,7,11-trimethyl-2-tridecanol (800 µg, i.e., 50 µg per isomer) was added to the test. One of the isomers of this substance is the sex pheromone of the diprionid *Microdiprion pallipes*, often occurring in these northern areas (Bergström et al., 1998; Östrand et al., 2003). The traps were repositioned once. In Poland, in the same area where the test was performed in 1998, an experiment was conducted that included propionates of isomers of 3,7-dimethyl-2-tridecanol occurring in the female extracts.

Table 1 Purity and Reference to Description of Synthesis of Chemicals Used in EAG Recordings and in Field Tests with *Gilpinia pallida*

Compound Stereoisomer	Chemical Purity (%)	Stereochemical Purity (%)	Contaminating Isomer(s)
Acetate of 3,7-dimethyl-2-tridecanol			
(2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>) ^a	>97	>99.5	(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>S</i>)
(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>) ^a	>97	>99.5	(2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>)
Propionate of 3,7-dimethyl-2-tridecanol			
(2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>) ^a	>97	>99.5	(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>S</i>)
(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>) ^a	>97	>99.5	(2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>)
(2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>) ^a	>97	>99.8	(2 <i>R</i> ,3 <i>S</i> ,7 <i>S</i>)
(2 <i>R</i> ,3 <i>R</i> ,7 <i>R</i>) ^a	>97	>99.5	(2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>S</i> ,3 <i>S</i> ,7 <i>R</i>)
(2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>) ^a	>97	>99.5	(2 <i>R</i> ,3 <i>R</i> ,7 <i>R</i>), (2 <i>S</i> ,3 <i>S</i> ,7 <i>S</i>)
Propionate of 3,7,9-trimethyl-2-tridecanol ^b	>98	6.25 of each stereoisomer	16 stereoisomeric mixture
Propionate of 3,7-dimethyl-2-tetradecanol			
(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>) ^c	>99	>99.7	(2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>)
(2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>) ^d	>98	>99.7	(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>S</i>)
(2 <i>R</i> ,3 <i>R</i> ,7 <i>R</i>) ^c	>99	>99.7	(2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>S</i> ,3 <i>S</i> ,7 <i>R</i>)
(2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>) ^d	>98	>98.8	(2 <i>R</i> ,3 <i>R</i> ,7 <i>R</i>), (2 <i>S</i> ,3 <i>S</i> ,7 <i>R</i>)
Acetate of 3,7-dimethyl-2-pentadecanol			
(2 <i>S</i> ,3 <i>S</i> ,7 <i>S</i>) ^e	>99	>97.5	(2 <i>S</i> ,3 <i>S</i> ,7 <i>R</i>), (2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>)
(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>) ^c	>98	>97.5	(2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>), (2 <i>S</i> ,3 <i>S</i> ,7 <i>R</i>), (2 <i>R</i> ,3 <i>R</i> ,7 <i>R</i>)
(2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>) ^c	>98	>97.0	(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>), (2 <i>S</i> ,3 <i>S</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>S</i>)
Propionate of 3,7-dimethyl-2-pentadecanol			
(2 <i>S</i> ,3 <i>S</i> ,7 <i>S</i>) ^e	>99	>97.5	(2 <i>S</i> ,3 <i>S</i> ,7 <i>R</i>), (2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>)
(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>) ^c	>98	>97.5	(2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>), (2 <i>S</i> ,3 <i>S</i> ,7 <i>R</i>), (2 <i>R</i> ,3 <i>R</i> ,7 <i>R</i>)
(2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>) ^c	>98	>97.0	(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>), (2 <i>S</i> ,3 <i>S</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>S</i>)
(2 <i>S</i> ,3 <i>S</i> ,7 <i>R</i>) ^c	>98	>98.3	(2 <i>S</i> ,3 <i>S</i> ,7 <i>S</i>), (2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>)
(2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>) ^c	>99	>97.6	(2 <i>R</i> ,3 <i>R</i> ,7 <i>R</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>S</i>), (2 <i>S</i> ,3 <i>R</i> ,7 <i>S</i>)
(2 <i>R</i> ,3 <i>R</i> ,7 <i>R</i>) ^c	>99	>99.0	(2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>), (2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>), (2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>)
(2 <i>R</i> ,3 <i>S</i> ,7 <i>S</i>) ^c	>99	>97.4	(2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>)

^a Bergström et al. (1995).^b Larsson et al. (2001).^c This study.^d Hedenström et al. (2002a,b).^e Högberg et al. (1990).

Chemicals

Commercially available chemicals were used without further purification unless otherwise stated. *Candida rugosa* lipase was purchased from Sigma Aldrich (Buchs, Switzerland) and stored at 4°C over blue gel. Dry diethyl ether was distilled from LiAlH₄. The alkyl halides were distilled prior to use and kept under argon. When preparing and using alkyllithium, the solvent was degassed by argon for about 1 hr prior to use. Li (s) was washed with

heptane and was made thin by using a hammer and cut in very thin pieces prior to use. The (3*R*,4*R*)-(-)-*cis*-dimethyl- γ -butyrolactone used was from the same batch as that prepared by Bergström et al. (1995), i.e., *RR*: >99.7% ee and <0.03% *trans*.

Purification and Analysis of Synthetic Materials

Preparative LC was performed on straight phase silica gel (Merck 60, 230–400 mesh, 0.040–0.063 mm; Merck, Darmstadt, Germany) by employing a gradient technique that used an increasing concentration of distilled diethyl ether in distilled pentane or of distilled ethyl acetate in distilled cyclohexane (0→100%), as eluent. Thin-layer chromatography (TLC) was performed on silica gel plates (Merck 60 F₂₅₄, precoated aluminum foil) eluted with ethyl acetate (20–40%) in cyclohexane and developed by spraying with vanillin in sulfuric acid and heated at 120°C. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DMX 230 (250 MHz ¹H and 62.9 MHz ¹³C) spectrometer using CDCl₃ as solvent and TMS as internal reference. Optical rotations were measured on a Perkin Elmer 241 polarimeter using a 1 dm cell. Mass spectra were recorded on a Varian Saturn 2000 instrument, operated in EI mode, and coupled to a Varian 3800 GC. Infrared (IR) spectra were recorded from compounds applied neat between NaCl plates by using a Perkin Elmer 782 infrared spectrometer. Unless otherwise stated, conversions and purity (Table 1) were monitored by GC (Varian 3300 or 3400 CX) with a 30 m × 0.25 mm i.d., 0.25 μm film thickness capillary column coated with VA-1 (Varian, Walnut Creek, CA, USA). Carrier gas was nitrogen (12 psi) with a split ratio of 1:50. The (2*R**3*R**):(2*R**3*S**) ratio of the individual isomers of (2*R**3*R**)-OH and (2*R**3*S**)-OH were determined by GC (Varian 3300 or 3400 CX) with a 30 m × 0.25 mm i.d., 0.25 μm film thickness capillary column coated with DB-WAX 52 CB (ChromPack). Carrier gas was helium (16 psi) with a split ratio of 1:30. Boiling points are uncorrected and given as air-bath temperatures (bath temperature/mbar) in a bulb-to-bulb (Büchi-GKR-51) apparatus.

Synthesis

2-Methylnonanoic acid **rac-2** (Figure 1)

Racemic 2-methylnonanoic acid was prepared as previously described (Hedenström and Andersson, 2002) via a malonic ester sequence. The analytical and spectroscopic data were identical with those in Hedenström and Andersson (2002).

C. rugosa Lipase Catalyzed Kinetic Resolution of 2-Methylnonanoic Acid **rac-2**

A cyclohexane solution (775 ml) containing the acid **rac-2** (20 g, 116 mmol), eicosanol (26 g, 87 mmol), tetracosane (internal standard, 6.1 mg/ml), Na₂SO₄/Na₂SO₄·10 H₂O [2 eqv./1 eqv. to maintain a water activity (*a_w*) of 0.8] was stirred for 15 min in a sealed flask at room temperature (Figure 1). The reaction was started by addition of immobilized *C. rugosa* lipase (7.4 mg/ml) into the flask. The reaction was stopped at 42.5% conversion by separating the enzyme by filtration and washing of the solid with cyclohexane (350 ml) and *n*-pentane (350 ml). The combined organic phases were extracted with aqueous sodium carbonate (10%, 5 × 150 ml) and the solvent was evaporated. The (*S*)-ester **S-3**, eicosanol, and the internal standard were present in the remainder, and after LC the (*S*)-ester (22.0 g, 48.6 mmol) was obtained chemically pure with >98% ee. The combined aqueous phase, containing the remaining substrate (*R*)-acid **R-2**, was washed with cyclohexane (300 ml)

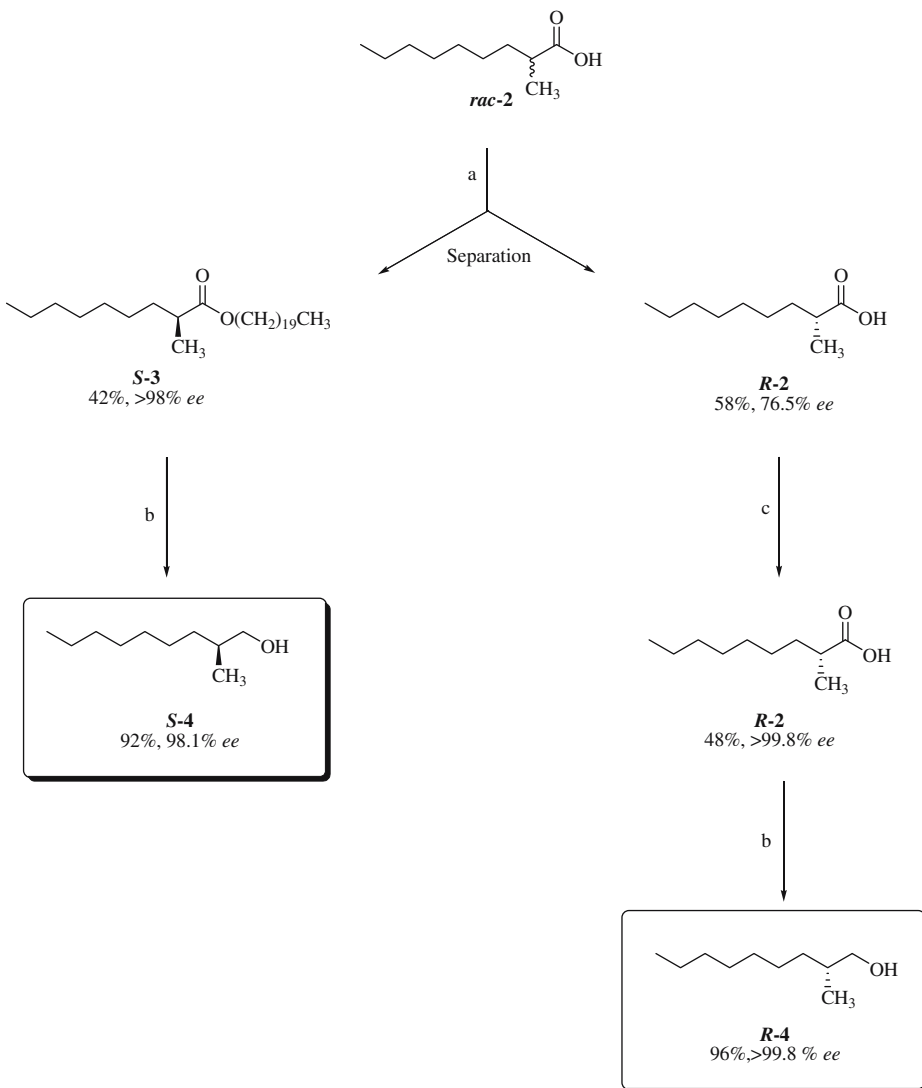


Fig. 1 (a) *Candida rugosa* lipase (CRL), HO(CH₂)₁₉CH₃, cyclohexane, water activity $a_w = 0.8$, $c = 42.5\%$. (b) LiAlH₄, Et₂O. (c) CRL, HO(CH₂)₁₉CH₃, cyclohexane, water activity $a_w = 0.8$, $c = 13\%$

and cooled to 0°C and then acidified to pH 1 with 6 M HCl followed by extraction with diethyl ether (5 × 200 ml). The combined organic phase was washed with saturated sodium chloride and dried (MgSO₄). Evaporation to dryness yielded (58%) the (*R*)-2-methylnonanoic acid **R-2** (11.5 g, 67.5 mmol) in 76.5% ee as an oil. The obtained nonanoic acid was once more esterified, as described above, but now to a conversion of 12%. The ee was continuously analyzed and found at this conversion to be >99.8%, and the reaction was interrupted, followed by the workup procedure as described above resulting in 9.4 g (55.2 mmol) of (*R*)-2-methylnonanoic acid **R-2** with >98.7% chemical purity. The analytical and spectroscopic data were similar to those reported in the literature (Levene and

Marker, 1932; Nikishin et al., 1974; Hedenström and Andersson, 2002). The ee of the (*R*)-acid **R-2** and the (*S*)-ester **S-3** were determined as described for other acids and esters (Hedenström et al., 2003) after derivatization to the corresponding phenylethyl amides obtained from enantiomerically pure (*S*)- or (*R*)- α -phenylethylamine (Hedenström et al., 2003). The diastereomeric purity of the phenylethyl amides was determined by GC by using a 30 m \times 0.32 mm i.d., 0.25 μ m film thickness capillary column coated with EC-1 (Alltech, Deerfield, IL, USA). Carrier gas was nitrogen (12 psi) with a split ratio of 1:50. The GC oven was 180°C isothermal, and retention times (min) were *SS/RR* amides-16.7 and *SR/RS* amides-18.0.

(*R*)-2-methyl-1-nonanol **R-4**

The (*R*)-2-methylnonanoic acid from above (4.0 g, 23 mmol) was reduced with LiAlH₄ as described in Hedenström and Andersson (2002) for racemic alcohol and yielded (96%) the pure alcohol **R-4** as an oil (3.5 g, 22 mmol) with >99.8% ee (Figure 2). The enantiomeric purity of the (*R*)-2-methyl-1-nonanol was determined by using a 30 m \times 0.25 mm i.d., 0.25 mm film thickness capillary column coated with β -dex 225 (Supelco Inc., Bellefonte, PA, USA). Carrier gas was helium (13 psi) with a split ratio of 1:60. The GC was temperature-programmed at 70°C for 30 min, then 0.5°C/min up to 90°C. Retention time (min) for *S*-2-methyl-1-nonanol (**S-4**): 81.6 and *R*-2-methyl-1-nonanol (**R-4**): 80.9. The analytical and spectroscopic data were similar to those reported in the literature (Högberg et al., 1992; Hedenström and Andersson, 2002).

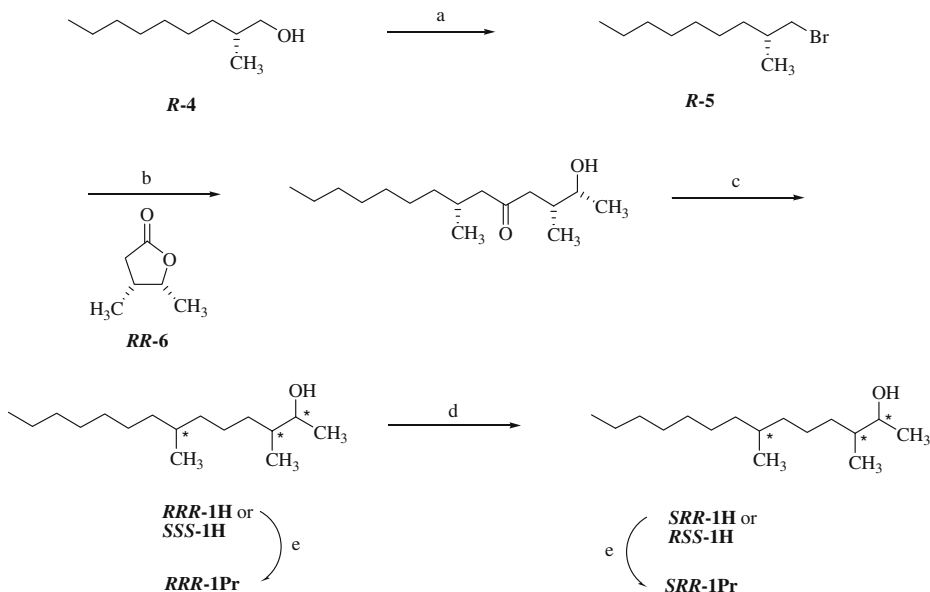


Fig. 2 (a) 1. TsCl, diethyl ether, -10°C ; 2. LiBr, acetone, Δ , 16 hr. (b) 1. Li(s), diethyl ether, -10°C ; 2. -100°C added to (*3R,4R*)-dimethyl- γ -butyrolactone (**RR-6**) and stirred for 10 hr. (c) (NH_2)₂·H₂O, KOH, diethylene glycol, 170°C 1 hr, 210°C 4 hr. (d) 1. P(Ph)₃, PhCO₂H, N₂(CO₂Et)₂; 2. LiAlH₄, Et₂O (C₂H₅OC)₂O, Pyridine

(*S*)-2-methyl-1-nonanol **S-4**

This was prepared from (*S*)-ester **S-3** in a manner similar to that described for other esters (Hedenström et al., 2003) (Figure 2). A solution of the eicosyl (*S*)-2-methylnonanoate in dry diethyl ether (22 g, 49 mmol) was added dropwise from above to LiAlH₄ (1.1 g, 29 mmol), and stirred in dry diethyl ether (32 ml) at room temperature. After 1 hr, the mixture was quenched with water (0.5 ml), 15% NaOH (0.5 ml), and 1.0 ml H₂O. After refluxing for 1 hr, the mixture was filtered and dried (MgSO₄). After evaporation of the diethyl ether, tetradecane was added to the remaining oil and fractional distillation (100–101°C/1 kPa) furnished after LC the pure title *S*-alcohol **S-4** (7.1 g, 45 mmol) with no loss in ee (>98.1% ee). The enantiomeric purity of the (*S*)-2-methyl-1-nonanol was determined by GC by using a 30 m × 0.25 mm i.d., 0.25 mm film thickness capillary column coated with β-dex 225 (Supelco Inc.). Carrier gas was helium (13 psi), with a split ratio of 1:60. The GC was temperature-programmed at 70°C for 30 min then 0.5°C/min up to 90°C. Retention time for *S*-2-methyl-1-nonanol (**S-4**): 81.6 and *R*-2-methyl-1-nonanol (**R-4**) was 80.9 min. $[\alpha]_D^{25} = -13.1^\circ$ ($c = 1.0$, MeOH). The analytical and spectroscopic data were similar to those reported in the literature (Högberg et al., 1992).

(*R*)-1-Bromo-2-methylnonane **R-5**

The *R*-alcohol **R-4** from above (3.4 g, 22 mmol) was dissolved in dry chloroform (22 ml) and stirred at –5°C (Figure 2). *p*-Toluenesulfonyl chloride (6.8 g, 36 mmol) was added in one portion and the mixture was stirred for 2.5 hr at –5°C. The mixture was poured into 25 ml H₂O and 65 ml diethyl ether. The organic phase was separated and then washed with 2M HCl (2 × 25 ml), saturated NaHCO₃ (20 ml), brine (20 ml), and dried (MgSO₄). The organic solvent was evaporated off at room temperature, leaving the tosylate as an oil. This was dissolved in dry acetone (40 ml) and added to a solution of dried (at 135°C) lithium bromide (9.8 g, 113 mmol) in dry acetone (16 ml). The mixture was refluxed for 4 hr. After dilution with water (200 ml), extraction with *n*-pentane (4 × 100 ml), washing with brine (100 ml), and drying (MgSO₄), the solvent was evaporated leaving an oil, which—after LC and bulb-to-bulb distillation (90°C/1 mm Hg)—gave the title bromide **R-5** (3.4 g, 15 mmol) with >97% chemical purity (with about 3% of the corresponding chloride) as measured by GC. The enantiomeric purity of the (*R*)-1-bromo-2-methylnonane was also determined by GC by using a 30 m × 0.25 mm i.d., 0.25 mm film thickness capillary column coated with β-dex 225 (Supelco Inc.). Carrier gas was helium (13 psi), with a split ratio of 1:60. The GC was temperature-programmed at 80°C for 1 min then 0.5°C/min up to 170°C. Retention time was 61.5 min for *S*-1-bromo-2-methylnonane (**S-5**) and was 62.1 min for *R*-1-bromo-2-methylnonane (**R-5**). The analytical and spectroscopic data were similar to those described by Hedenström and Andersson (2002) for racemic 1-bromo-2-methylnonane.

(2*R*,3*R*,7*R*)-3,7-Dimethyl-2-tetradecanol **RRR**

The title compound was prepared in the same way as the other structurally similar compounds in Hedenström and Andersson (2002) (Figure 2). Lithium (0.3 g, 40 mmol) was cut into small pieces and added to dry diethyl ether (15 ml, degassed of oxygen by argon) under stirring at –8°C. Into the reaction flask, 1.19 g (5.4 mmol) of distilled (*R*)-1-bromo-2-methylnonane in dry diethyl ether (15 ml, degassed of oxygen by argon) was added slowly (1 hr) *via* a syringe at –15°C. The mixture was stirred for 1.5 hr, then cooled to –78°C, and slowly added (0.4 hr) *via* a syringe into a solution of freshly distilled (3*R*,4*R*)-*cis*-dimethyl-

γ -butyrolactone **RR** (0.39 g, 3.4 mmol) in dry diethyl ether (15 ml, degassed of oxygen by argon) at -78°C . The reaction was allowed to slowly reach room temperature during 60 hr and then quenched with 5 ml THF. Water (100 ml) was added to the resulting mixture, followed by extraction with diethyl ether (3×100 ml), washing with brine (2×100 ml), by drying with MgSO_4 , and concentration. This crude ketoalcohol was purified by LC to give 0.20 g (0.78 mmol), which was immediately reduced as follows: The oil was dissolved in distilled diethylene glycol (2.5 ml) containing KOH (0.28 g) and hydrazine monohydrate (0.138 g, 2.7 mmol), and the solution was heated to 170°C for 3 hr then for 1 hr at 210°C . After cooling to room temperature, the mixture was poured into H_2O (50 ml), followed by extraction with Et_2O (4×25 ml), washing with H_2O (25 ml) and drying with MgSO_4 . Concentration furnished the title compound **RRR** as a yellow oil, which was chromatographed and distilled bulb to bulb to give 130 mg (0.54 mmol) with $>98.7\%$ chemical purity. The analytical and spectroscopic data were identical with those in Hedenström et al. (1992).

(2*S*,3*R*,7*R*)-3,7-Dimethyl-2-tetradecanol **SRR-1H**

The title compound was prepared by using a similar procedure described in Karlsson and Hedenström (1999), but from (2*R*,3*R*,7*R*)-3,7-dimethyltetradecan-2-ol (0.11 g, 0.45 mmol), which gave 0.13 g (0.38 mmol) of a pure benzoate after LC (Figure 2). This ester was reduced (LiAlH_4) to the corresponding alcohol and 45 mg of **SRR-1H** with $>99.3\%$ purity was obtained after LC and bulb-to-bulb distillation. The analytical and spectroscopic data were similar to those in Hedenström et al. (1992).

(2*R*,3*S*,7*S*)-3,7-Dimethyl-2-tetradecanol **RSS-1H**

The title compound was prepared as above, but from (2*S*,3*S*,7*S*)-3,7-dimethyl-2-tetradecanol (Hedenström et al., 2002a,b) (Figure 2). The analytical and spectroscopic data were identical (except for the optical rotation value) with those of **SRR-1H**.

Propionates of (2*R*,3*R*,7*R*)- and (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tetradecanol

The esters were prepared with a minor modification of the method used for other alcohols (Karlsson and Hedenström, 1999) (Figure 2). To the alcohol, **RRR** or **SRR-1H** (40 mg, 0.17 mmol), in dry distilled pyridine (1 ml) propionic acid anhydride (0.4 ml) was added, and the mixture was heated to boiling point. After cooling to room temperature, the reaction mixture was poured into 2 M HCL (10 ml), followed by extraction with diethyl ether (3×20 ml), drying (MgSO_4), and concentration. The esters were furnished in near quantitative yield as yellow oils after LC and distillation with $>98.9\%$ (**RRR**) and $>99.5\%$ (**SRR-1Pr**) chemical purities, respectively.

Results and Discussion

Chemical Identification

Previously described pheromone precursors, (2*R**3*R**)-(2*R**3*S**)-isomers of 3,7-dimethyl-2-pentadecanol (diprionol), occurring in several diprionid species, were identified through GC-MS analyses of extracts from *G. pallida*. Furthermore, both (2*R**3*R**)- and (2*R**3*S**)-

3,7-dimethyl-2-tridecanol were detected. One of the ($2R^*3S^*$)-isomers (i.e., $2S,3R,7R$) of the tridecanol is previously known as a pheromone precursor in *D. pini*. The homologue 3,7-dimethyl-2-tetradecanol was also identified, and this alcohol has been reported in trace amounts in some sawfly species, e.g., in *Neodiprion sertifer* (Wassgren et al., 1992) and *D. pini* (Bergström et al., 1995). In *G. pallida*, we identified this compound as a ($2R^*3S^*$)-isomer; it was present in 50–260 pg/female, whereas only a trace of the ($2R^*3R^*$)-isomer was detected. The ($2R^*3R^*$)- and ($2R^*3S^*$)-isomers of the three homologues were present in different amounts, from traces to 750 pg/female (Table 2). Variation in amount was also observed in extracts of individuals of different ages (Table 2). Freshly eclosed German females from the 1996 collection contained only ($2R^*3R^*$)-3,7-dimethyl-2-pentadecanol. In contrast, 24–72 hr females contained other homologues. A mixture of extracts containing about 480 females from the years 1996–1999 contained the ($2R^*3R^*$)- and ($2R^*3S^*$)-isomers of the three homologues. Analysis of the exact configuration of the sex pheromone precursors showed that some of the observed ($2R^*3R^*$)- and ($2R^*3S^*$)-isomers were separated into more than one stereoisomer (Table 3). For example, ($2R^*3S^*$)-3,7-dimethyl-2-tridecanol was separated into both ($2R,3S,7R$)- and ($2S,3R,7R$)-3,7-dimethyl-2-tridecanol, with approximately 70 pg/female of each isomer.

Differences in composition between German and Finnish females were also observed, perhaps confounded by differences in ages of females from each population (German, 24–72 hr; Finnish, 24–30 hr). The main compound in Finnish females was ($2R^*3S^*$)-3,7-dimethyl-2-pentadecanol with $2R,3S,7S$ configuration, whereas stereoisomers of the other homologues were present in smaller quantities. In German females, ($2R,3S,7R$)-3,7-dimethyl-2-tetradecanol was identified, whereas in Finnish females, the $2S,3R,7R$ configuration was found. Furthermore, in German females, stereoisomers of the precursor 3,7-dimethyl-2-tridecanol, occurring as both the ($2R^*3R^*$) and ($2R^*3S^*$) forms, have a different composition. $2R,3R,7R$ or $2R,3R,7S$ configurations were identified in both materials, but we could not determine if it is the $2R,3R,7R$ - or $2R,3R,7S$ -stereoisomer, or

Table 2 Quantification^a of Compounds Identified in Extracts of Female *Gilpinia pallida*

Origin of Female Year	German 1996	German 1997	German 1998	German 1999	German 1996– 1999 ^b	Finnish 2000
Number of females	100	220	75	85	480	270
Age	0 hr	<24–72 hr	24–72 hr	24–72 hr	24– 72 hr	24– 30 hr
Compound						
($2R^*3R^*$)-3,7-Dimethyl-2-tridecanol			50–100		20	<30
($2R^*3S^*$)-3,7-Dimethyl-2-tridecanol		40	70–140	150	150	Trace
($2R^*3R^*$)-3,7-Dimethyl-2-tetradecanol					Trace	
($2R^*3S^*$)-3,7-Dimethyl-2-tetradecanol		170–200	260	50	50	<15
($2R^*3R^*$)-3,7-Dimethyl-2-pentadecanol	× ^c	200–250	170–320		20	
($2R^*3S^*$)-3,7-dimethyl-2-pentadecanol		600–750	120–180	95	95	<120
Perillenal	× ^c	5–20 ng	4 ng			

^a In pg/female, unless otherwise indicated.

^b Mixed extracts.

^c Identified, but not quantified.

Table 3 Stereoisomeric Identification and Quantification^a of Selected Compounds from Extracts of Female *Gilpinia pallida*

Origin of Female	German	German	Finnish
Year	1996–1999 ^b	1998	2000
Number of females	480	75	270
Age	24–72 hr	24–72 hr	24–30 hr
Compound			
(2 <i>R</i> ,3 <i>R</i> ,7 <i>R</i>)- or (2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>)-3,7-Dimethyl-2-tridecanol	20	<50	20
(2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>)-3,7-Dimethyl-2-tridecanol	75	70	
(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>)-3,7-Dimethyl-2-tridecanol	75	70	Trace
(2 <i>R</i> ,3 <i>R</i> ,7 <i>R</i>)- or (2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>)-3,7-Dimethyl-2-tetradecanol	Trace		
(2 <i>R</i> ,3 <i>S</i> ,7 <i>R</i>)-3,7-Dimethyl-2-tetradecanol	50	260	
(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>)-3,7-Dimethyl-2-tetradecanol			<15
(2 <i>R</i> ,3 <i>R</i> ,7 <i>R</i>)- or (2 <i>R</i> ,3 <i>R</i> ,7 <i>S</i>)-3,7-Dimethyl-2-pentadecanol	<20		
(2 <i>R</i> ,3 <i>S</i> ,7 <i>S</i>)-3,7-Dimethyl-2-pentadecanol			120
(2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>)-3,7-Dimethyl-2-pentadecanol	95	5–20 ng	4 ng

^a In pg/female unless otherwise indicated.

^b Mixed extracts.

a mixture of both. The (2*R**3*S**)-isomer of 3,7-dimethyl-2-tridecanol was present in equal amounts in each as 2*R*,3*S*,7*R*- and 2*S*,3*R*,7*R*-stereoisomers, but the Finnish females contained only traces of the 2*S*,3*R*,7*R*-stereoisomer. German females usually contained more stereoisomers of each homologue. One of the German extracts did not contain detectable amounts of (2*R**3*R**)- or (2*R**3*S**)-3,7-dimethyl-2-pentadecanol. Our study reports the first observation of compounds with 2*R* configuration in a pine sawfly species.

trans-Perillenal, known from other diprionids (e.g., Wassgren et al., 1992), was found in all extracts at a concentration of 5–20 ng per female. The concentration of perillenal decreased with female age; freshly eclosed females contained about 20 ng/female, and the amount decreased to 4 ng/female for 24–72 hr insects.

Synthesis

The main procedure (Figures 1 and 2) was similar to that used earlier for preparation of sex pheromones from other pine sawfly species (Karlsson and Hedenström, 1999; Hedenström and Andersson, 2002). Thus, racemic 2-methylnonanoic acid (*rac*-**2**) was synthesized in good yield by using a published malonic ester sequence (Hedenström and Andersson, 2002). The racemate was resolved in two successive esterifications using eicosanol in cyclohexane at a specific water activity ($a_w = 0.8$; Figure 1). After LiAlH₄ reduction of the *S*-ester (**S-3**) and *R*-acid (**R-4**), the respective **R-4** and **S-4** alcohols were recovered in high enantiomeric purity (>99.8% and >98.1% ee, respectively). The alcohol **R-4** was converted to the bromide **R-5** via the tosylate by using a standard method (Figure 2). The bromide **R-5** was reacted with an excess of lithium metal in diethyl ether to yield the corresponding alkyl lithium, which was used to alkylate (3*R*,4*R*)-(-)-*cis*-dimethyl- γ -butyrolactone. This resulted in a ketoalcohol, which was reduced to the alcohol **RRR-1H**. Mitsunobu conditions (see Figure 2 for the reaction conditions) were used to invert the configuration at carbon 2 in compound **RRR-1H** and to form the diastereomeric compound **SRR-1H**. **RSS-1H** was similarly prepared but from **SSS-1H** (Figure 2). For electrophysiological and

behavioral tests, the propionates of **RRR-1H** and **SRR-1H** were prepared in quantitative yield by stirring with propionic anhydride in pyridine.

Electrophysiology

During the first EAG experiment, antennae from male *G. pallida* responded strongly and consistently to the two higher doses of both the acetate and propionate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-pentadecanol, as well as to the acetate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanol (Figure 3). Also, when the highest dose of the propionates of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanol and of (2*S*,3*R*,7*S*)-3,7-dimethyl-2-pentadecanol was tested, the responses were significantly higher than to the blank control (Figure 3). In addition, a significant response was obtained when antennae were stimulated with the intermediate dose of the propionate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol, but as the response to the highest dose returned to levels not different from the blank, we consider this result as less relevant. Taken together, the results from these first recordings support the hypothesis of the stereoisomeric identity (2*S*,3*R*,7*R*) of the pheromone, based on catches in traps for *D. pini* (Herz et al., 2000).

A large number of compounds, as pure stereoisomers or as mixtures (Table 1), were screened in EAG recordings using one dose, 1 µg. None of the propionate stereoisomers with a 2*R* configuration elicited responses stronger than the solvent control, irrespective of alkanol chain length. This was true for males both from Finland and from Germany. The same weak activity was shown by the (2*R**3*R**) isomers tested and by the 2*S*,3*R*,7*S*-isomer.

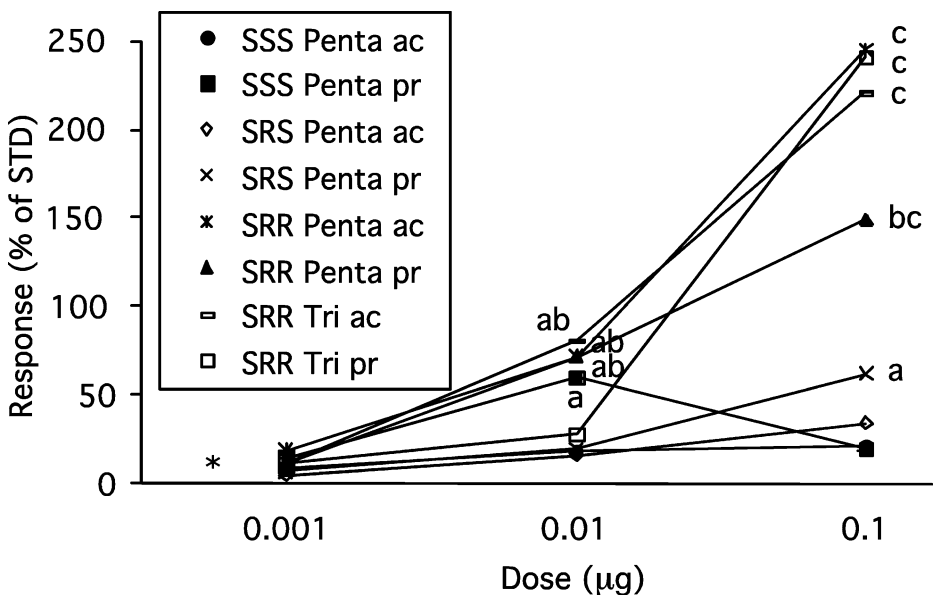


Fig. 3 Mean electroantennographic response of antennae from male *Gilpinia pallida* (German origin) expressed as percentage of standard stimulus (STD), which was the propionate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol in a dose of 0.01 µg. Standard error (SE) is not shown because of the large number of data points ($N=9$, except for 0.1 µg SSS Penta ac, where $N=8$). SRR Tri ac: acetate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanol; pr: propionate; *: response to the solvent control. Because of the large number of substances tested, only mean values different from the solvent control are shown by letters. Different letters indicate significant differences among mean responses and between the mean and the solvent control ($P=0.05$, Tukey's test on log-transformed values)

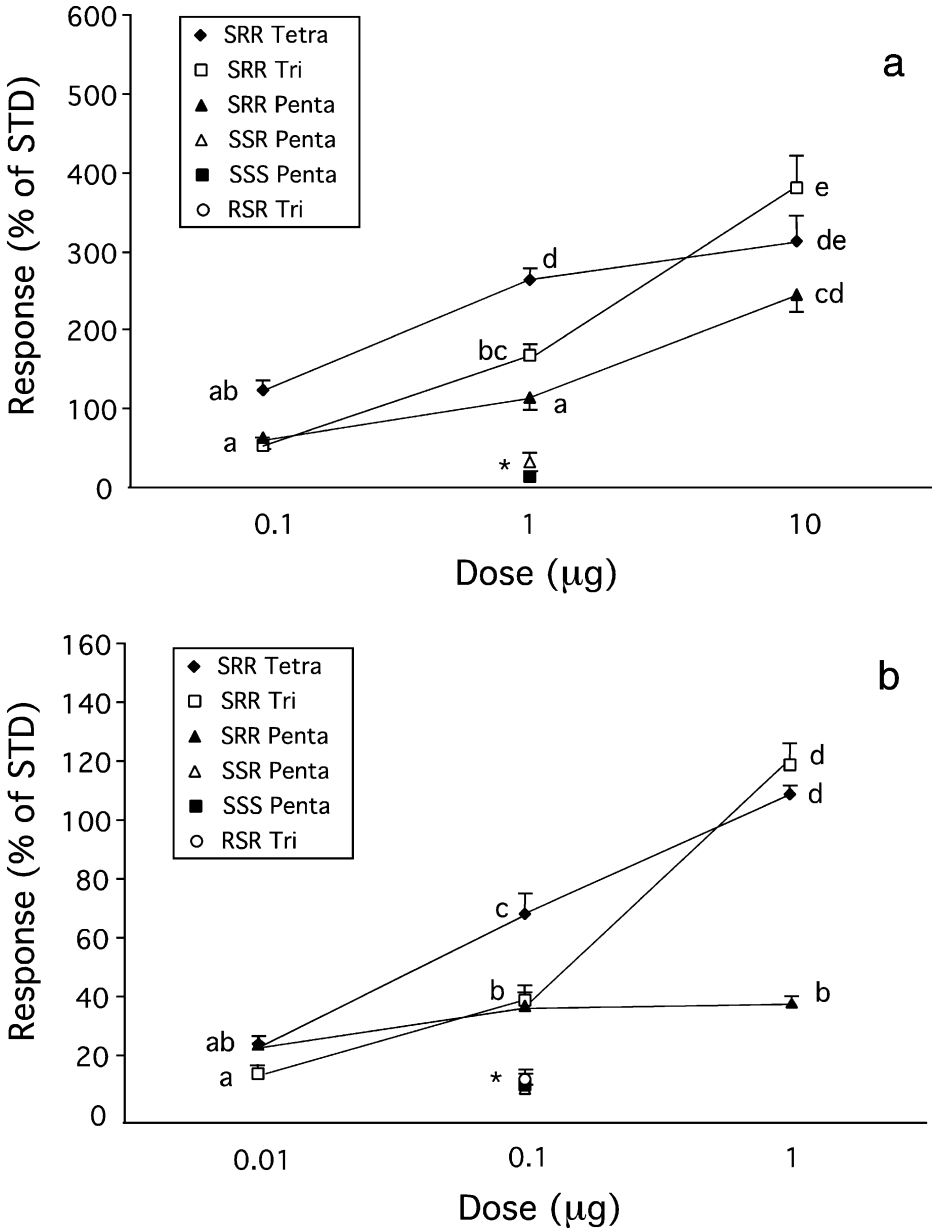


Fig. 4 Mean (+ or – SE) electroantennographic response of antennae from male *Gilpinia pallida* from (a) Germany ($N=8$) and (b) Finland ($N=5$) to some isomers of the propionates of 3,7-dimethyl-2-tridecanol, 3,7-dimethyl-2-tetradecanol, and 3,7-dimethyl-2-pentadecanol expressed as percentage of standard stimulus (STD), which was (a) 1 µg SRR Tri pr and (b) 0.1 µg SRR Tri pr. For abbreviations, see Figure 3 legend. *: Response to the solvent control. Different letters indicate significant differences among mean responses and between the mean and the solvent control ($P=0.05$, Tukey’s test on log-transformed values)

Table 4 Response of Male *Gilpinia pallida* to Traps Baited With (2*S*,3*R*,7*R*)-Propionates of 3,7-dimethyl-2-tri-, tetra- and pentadecanol (50 µg Bait), and of 3,7,11-trimethyl-2-tridecanol (800 µg Bait=50 µg per isomer), Sweden 1999^a

Bait	Total Catch	
	June 18, 1999	July 2, 1999
Blank	221	0
Propionate of 3,7,11-trimethyl-2-tridecanol	73	0
Propionate of (2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>)-3,7-dimethyl-2-tridecanol	603	1
Propionate of (2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>)-3,7-dimethyl-2-tetradecanol	1188	61
Propionate of (2 <i>S</i> ,3 <i>R</i> ,7 <i>R</i>)-3,7-dimethyl-2-pentadecanol	115	0

^a Seven *G. pallida* females were also trapped.

However, the propionate of the 2*S*,3*R*,7*R*-isomer of all three alkanols, 3,7-dimethyl-2-tridecanol, 3,7-dimethyl-2-tetradecanol, and 3,7-dimethyl-2-pentadecanol, and the acetate of the tri- and pentadecanol (acetate of tetradecanol not available) elicited antennal responses, with a clear dose–response relationship (Figure 4). This response pattern is analogous to that of *N. sertifer*, which showed strong EAG response to the acetate of the 2*S*,3*S*,7*S*-isomer of a homologous series of alkanols, i.e., 3,7-dimethyl-2-tridecanol to 3,7-dimethyl-2-hexadecanol (Wassgren et al., 1992).

Field Tests

We only trapped three male *G. pallida* during the 1998 experiment in France, but all were caught in the trap baited with the (2*S*,3*R*,7*R*)-propionate of 3,7-dimethyl-2-tetradecanol. This was also the only compound that attracted *G. pallida* in 1998 in Poland. In one replicate, 42 males were caught; whereas in the other, 30 males were trapped. The peak flight occurred at the end of July.

More than 2000 males were trapped during the 1999 experiment in northern Sweden (Table 4). Again, the (2*S*,3*R*,7*R*)-propionate of 3,7-dimethyl-2-tetradecanol was the most active homologue, although all other baits, including the blank, caught a considerable number of sawflies during the first trapping period (Table 4). Due to the short and intensive flight period and the remote location of the outbreak, the treatments were only rerandomized once, so statistical analysis of the data was not feasible. During the second trapping period, 61 of 62 males were caught in the trap baited with (2*S*,3*R*,7*R*)-propionate of 3,7-dimethyl-2-tetradecanol (Table 4).

Only 11 males were trapped during the 1999 experiment in Poland. Five responded to the trap baited with the (2*S*,3*R*,7*R*)-propionate of 3,7-dimethyl-2-tetradecanol, whereas three each responded to two traps baited with combinations of (2*R**3*S**)-isomers of propionates of 3,7-dimethyl-2-tridecanol. Taken together, the results of the field trapping indicate that the (2*S*,3*R*,7*R*)-isomer of 3,7-dimethyl-2-tetradecanol is likely the main component of the pheromone precursor in *G. pallida*, with lower, but substantial, response to the propionate of the *SRR* isomer of the tridecanol. The propionate of the pentadecanol homologue was even less active. With *N. sertifer*, only one of the 2*S*,3*S*,7*S* homologues (the acetate of 3,7-dimethyl-2-pentadecanol) caught a significant number of males, despite a strong electrophysiological response to all homologues (Wassgren et al., 1992).

We have no explanation as to why the precursor of the most electrophysiologically and behaviorally active stereoisomer was only detected in one of the samples (Tables 2 and 3).

Future studies of the actual release from females of different ages and geographical origins might shed light on this issue. It is possible that one or more of the other electrophysiologically active substances found in female *G. pallida* may also be pheromone precursors, and may function in blends with the esters of the tridecanol and tetradecanol. However, it was not possible to examine the effect of the other components because the populations had collapsed. It is also possible that male *G. pallida* respond to several esters of the main component, as was recently reported for *D. pini* (Anderbrant et al., 2005). However, the propionate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tetradecanol is a potent attractant of *G. pallida* and might serve as lure in monitoring traps.

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Introduction

The eggplant flea beetle, *Epitrix fuscula* Crotch (Coleoptera: Chrysomelidae), is an important pest of eggplants. Found throughout most of the United States, *E. fuscula* is most common in the south. It is the most prominent insect pest of eggplant in Arkansas, requiring multiple insecticide applications after transplanting for beetle control (McLeod et al., 2002). Adult beetles can be especially damaging in the spring and are capable of defoliating young eggplant transplants in as little as 12 hr (Sorensen and Baker, 1994; Andersen, 2000). Flea beetle feeding, including from *E. fuscula*, is an important cause of crop failure in eggplants grown by organic methods (Patton et al., 2003). A number of cultural practices recommended for flea beetle control include trap crops, row covers, removal of crop residues, sticky traps, and careful timing of plantings (Sorensen and Baker, 1994; Delahaut, 2001; Kuepper, 2003). An effective detection and monitoring tool, such as a pheromone, could assist in the control of *E. fuscula*.

The life history of *E. fuscula* was reported by Sorensen and Baker (1994). Briefly, adults overwinter in soil or ground debris and emerge and mate in early spring. Females lay eggs near the base of host plants. Larvae feed on roots or tubers and pupate in the soil when mature. Adult beetles emerge in about 7 to 10 days and begin to feed on foliage. In the southern U.S., the eggplant flea beetle is thought to have at least two generations per year (Sorensen and Baker, 1994; Capinera, 2001). This species has a limited host range, feeding primarily on solanaceous plants (Capinera, 2001). It causes damage to potatoes and sugar beets, and will feed on some weed species such as horse nettle and pokeweed (Sorensen and Baker, 1994). Adults cause the same type of characteristic, leaf-feeding damage associated with other flea beetles, consisting of numerous, small, irregular “shot holes” (Kuepper, 2003).

Previously, pheromones have been reported from both male and female flea beetles. Males of *Longitarsus jacobaeae* Waterhouse were reported to be attracted to cues associated with conspecific females, although no chemical compounds were identified (Zhang and McEvoy, 1994). The first report of a male-produced pheromone in a flea beetle was for *Phyllotreta cruciferae* (Peng and Weiss, 1992; Peng et al., 1999), detected from feeding beetles by laboratory and field bioassays. Subsequently, six male-specific sesquiterpenes were found in this species and fully characterized, and four of the six were synthesized (Bartelt et al., 2001; Muto et al., 2004; Mori, 2005). Soroka et al. (2005) found that a blend of these compounds was attractive in the field to both male and female *P. cruciferae* and that even greater attraction was possible when the blend was combined with allyl isothiocyanate (AITC), a previously known, host-related attractant for *P. cruciferae* (Vincent and Stewart, 1984; Pivnick et al., 1992). Tóth et al. (2005) performed a similar field trial in Hungary by using pure enantiomers of *P. cruciferae* pheromone components in combination with AITC and achieved similar results, attracting *P. cruciferae* as well as some other, congeneric species. The same male-specific compounds emitted by *P. cruciferae*, plus two additional sesquiterpene alcohols, have also been found in the volatiles emitted from males of three *Aphthona* flea beetle species, *A. flava*, *A. czwalinae*, and *A. cyparissiae* (Bartelt et al., 2001), but field bioassays have not yet been performed.

By using techniques that were employed with *P. cruciferae* and *Aphthona* spp., a male-produced pheromone was demonstrated in *E. fuscula*. The identification of six male-specific compounds and the synthesis and field activity of the two major components are reported.

Methods and Materials

Insects

In early May of 2004, overwintered *E. fuscata* adults were collected from eggplants on an organic farm located in Farmington, IL, USA. In 2005, beetles were again collected from the same site and from an eggplant research plot at National Center for Agricultural Utilization Research (NCAUR), in Peoria, Illinois. The sex was determined under a microscope, by using the abdominal characteristics described for *Phyllotreta* (Smith, 1983). The fifth abdominal sternum of females appears as a simple, smooth surface, but in males contains an apical median lobe. The beetles were used for collection of volatiles in the laboratory.

Collection of Volatiles

Volatiles were collected from groups of males, females, or mixed-sex beetles feeding on eggplant leaves, and from eggplant leaves alone, as described previously for another species and host (Bartelt et al., 2006). Briefly, beetles and foliage were placed in 45×3 cm (ID), horizontal glass tubes, equipped with Super-Q (Alltech, Deerfield, IL, USA) filters on both ends and through which air was drawn (300 ml/min) by vacuum. The inlet filter cleaned incoming air, and the second filter trapped the volatiles emitted within the tube. Typically, 10 beetles were used per tube, but numbers ranged from 3–20. A single eggplant leaf, about 9 cm in length and 5 cm at its greatest width, served as a food source and was replaced every time volatiles were collected. To keep the leaf fresh, the petiole was placed in a 5-ml glass vial containing water. A Teflon[®] seal, held in place with a ring-shaped screw cap, kept water from spilling. Collection duration was 1 to 4 d, and collected volatiles were recovered by rinsing the outlet Super Q filter with 400 µl of hexane into a vial. Collectors were kept in an incubator at 27°C with a relative humidity of about 50%. Light was provided by eight 40-W fluorescent tubes set about 0.5 m above and behind the collection tubes, and the daily light cycle was 16:8 hr light/dark.

Gas Chromatographic/Mass Spectrometric and Gas Chromatographic Analysis

All volatile collections were analyzed by coupled gas chromatography/mass spectrometry (GC–MS), and comparisons were made among collections from feeding males, females, and mixed sexes and from host plants only. The analyses were conducted on a Hewlett Packard 5973 mass selective detector, interfaced to a Hewlett Packard 6890 GC. For most analyses, a 30-m DB-5MS capillary column (0.25 mm ID, 1.0 µm film thickness, J&W Scientific, Folsom, CA, USA) was used. The temperature program was 50°C for 1 min, then rising to 280°C at 10°C per min and holding for 5 min at 280°C. The temperature of the splitless inlet was 200°C, and the transfer line temperature was 285°C. The Wiley MS library (Wiley, 1995) was installed on the data system.

Chiral GC–MS analysis was conducted for some samples (see below) by using a 30-m Cyclodex-B column (0.25 mm ID, 0.25 µm film thickness, J&W Scientific). Temperature program was 50°C for 1 min, then rising at 30°C/min to a final temperature of either 120°C or 130°C.

A Hewlett Packard 5890 GC was used for quantitation and was equipped with a DB-1 column (as above), splitless and cool-on-column inlets, and flame-ionization detector. Estimation of amounts of selected compounds in samples was by the external standard method, relative to nonadecane, by using the splitless inlet (200°C). Septum release rates

were measured using the internal standard method (nonadecane, 4.09 μg per sample) and cool-on-column injections (with inlet temperature tracking the oven temperature).

Electrophysiology

Coupled GC–electroantennographic (GC–EAD) analyses were carried out on a Hewlett Packard 6890 GC, interfaced to antennal preparations. Amplified EAD and GC profiles were obtained simultaneously and analyzed by Syntech GC–EAD software. General methods and equipment have been previously described by Cossé and Bartelt (2000).

Liquid Chromatography

Fractionation of collected volatiles on open columns of silica gel (6×0.5 cm ID, in Pasteur pipettes), followed by GC–MS analysis of fractions, gave information on compound polarity and served as an initial purification step. Elution was with hexane, then 10% ether in hexane, and finally with 25% ether in hexane (3 column volumes per solvent).

High-performance liquid chromatography (HPLC) and other techniques were applied to these silica gel fractions to gain further information about compounds **1–6** (Fig. 1). For HPLC, a Waters 515 pump (flow rate=1 ml/min) and a Waters R401 differential refractometer detector were employed. A Supelcosil LC-SI silica column (25 cm, 0.46 cm ID, 5 μm particle size, Supelco, Bellefonte, PA, USA) was used for purifying aldehydes **1** and **2** before hydrogenation and nuclear magnetic resonance (NMR), and the solvent was 10% ethyl ether (redistilled) in hexane. A silica column (Adsorbosphere Silica 5 μ , a 25×4.6 -mm ID silica column; Alltech) that had been treated with silver nitrate

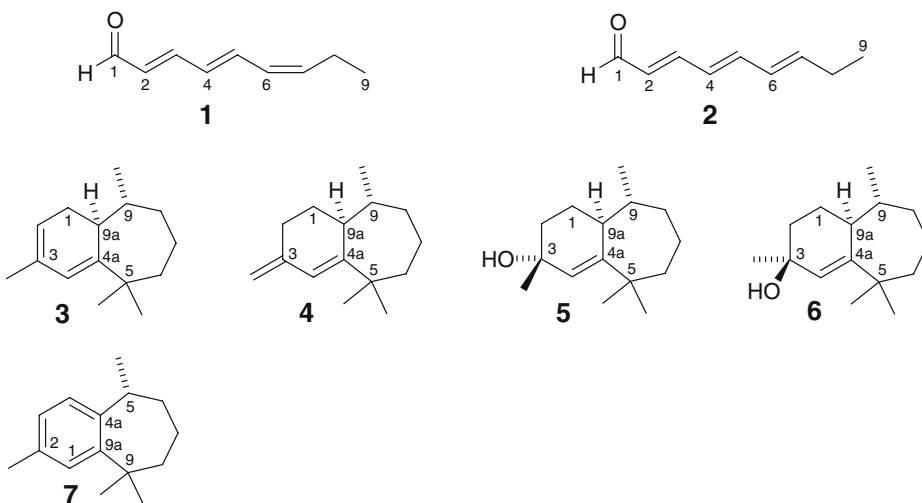


Fig. 1 Compounds **1–6**, detected during the analysis of volatiles from male *E. fuscula* feeding on eggplant leaf. Compound **7**, *ar*-himachalene, derived from *E. fuscula* **3**, to determine absolute configuration (see text). Chemical Abstracts index names: **1**, (2*E*,4*E*,6*Z*)-2,4,6-nonatrienal; **2**, (2*E*,4*E*,6*E*)-2,4,6-nonatrienal; **3**, (9*R*,9*aS*)-5,6,7,8,9,9*a*-hexahydro-3,5,5,9-tetramethyl-1*H*-benzocycloheptene; **4**, (9*R*,9*aS*)-2,3,5,6,7,8,9,9*a*-octahydro-5,5,9-trimethyl-3-methylene-1*H*-benzocycloheptene; **5**, (3*R*,9*R*,9*aS*)-2,3,5,6,7,8,9,9*a*-octahydro-3,5,5,9-tetramethyl-1*H*-benzocyclohept-3-ol; **6**, (3*S*,9*R*,9*aS*)-2,3,5,6,7,8,9,9*a*-octahydro-3,5,5,9-tetramethyl-1*H*-benzocyclohept-3-ol; **7**, (5*R*)-6,7,8,9-tetrahydro-2,5,9,9-tetramethyl-5*H*-benzocycloheptene

(Heath and Sonnet, 1980) was used for separating hydrocarbons **3** and **4**, with 0.5% 1-hexene in hexane as solvent. All effluent was collected in consecutive 1-ml fractions.

Hydrogenation

Microscale hydrogenation of HPLC-purified **1** and **2** (combined) was conducted over 10% palladium on carbon. A sample of **1** and **2** (ca. 100 ng) in a tapered vial was taken to dryness under a stream of argon and immediately redissolved in CH_2Cl_2 (100 μl). A small amount of catalyst (<1 mg, barely visible in the solution) was added. By using a fine needle, hydrogen was bubbled through the solution for 5 min at room temperature. Then, the solution was concentrated about 10-fold under argon and analyzed by GC–MS.

NMR Spectroscopy

NMR spectra were acquired on a Bruker Avance 500 instrument with a 5-mm inverse broadband probe with a Z-gradient. Proton and COSY spectra were obtained for beetle-derived **1** and **2** in CDCl_3 and for synthetic **1** and **2** in both CDCl_3 and deuterobenzene, as described below.

Chiral Analysis of Compounds **3**, **4**, **5**, and **6**

The enantiomers of **4** are known to separate on a Cyclodex-B capillary column (Bartelt et al., 2001), and the hydrocarbon silica gel fraction of the natural sample, which contained **4**, was compared with racemic synthetic **4** on that column (final temperature 130°C). The enantiomers of **3** do not separate on Cyclodex-B (Bartelt et al., 2001), but an alternative analysis was possible: Compound **3** in the hydrocarbon silica fraction was separated from **4** by HPLC on the silver nitrate column (**3** eluting 3–4 ml after injection, and **4**, at 6–7 ml), and then **3** was converted to *ar*-himachalene (**7**; Fig. 1) by treatment with 2,3,5,6-tetrachlorohydroquinone (Mehta and Singh, 1977; Bartelt et al., 2001), and the product compared with racemic **7** on Cyclodex-B (final temperature 120°C). The 25% ether hexane silica gel fraction from *E. fuscula*, which contained alcohols **5** and **6** but was free of **3** and **4**, was treated with a

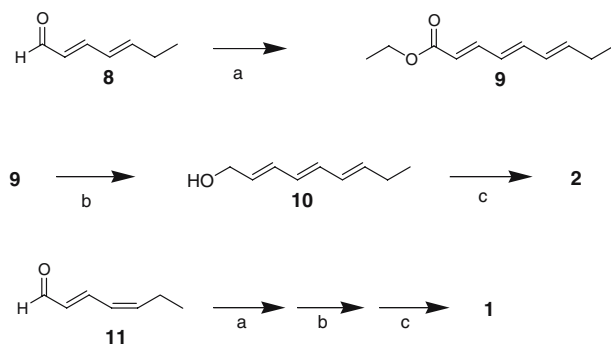


Fig. 2 Synthesis of aldehydes **2** and **1** (see text). Reagents and details for steps: (a) triethyl 2-phosphonoacetate, either in tetrahydrofuran with BuLi as base or in hexane with Li *t*-OBu as base, 0°C, warming to room temperature (RT), 1 hr, 88% yield; (b) diisobutylaluminum hydride in hexane/ether (1:1), 0°C, 1 hr, 90% yield; (c) MnO_2 in methylene chloride, RT, 16 hr, 85%. Aldehyde **1** was prepared in the same way except that (2*E*,4*Z*)-2,4-heptadienal was the starting material instead of **8**

strong cation exchanger (acidic form) to dehydrate **5** and **6** (Bartelt et al., 2001). The resulting compound **4** was analyzed on the Cyclodex-B column as above.

Chemicals

Aldehydes **1** and **2** were synthesized, and sesquiterpenes **3** to **7** were available from previous research (Bartelt et al., 2003; Muto et al., 2004).

Synthesis of **2** (Fig. 2) used commercial (2*E*,4*E*)-2,4-heptadienal (**8**) (Sigma-Aldrich, St. Louis, MO, USA) as the starting material. The chain was extended by a Wittig–Horner reaction with triethyl 2-phosphonoacetate (Boutagy and Thomas, 1974) to form the triene ester (**9**); both lithium *t*-butoxide in hexane (Petroski and Weisleder, 2001) and *n*-butyllithium in tetrahydrofuran (Bartelt et al., 1990) were satisfactory base/solvent combinations. The ester was reduced to alcohol **10**. In an initial trial, reduction was performed with LiAlH₄ (Bartelt et al., 1990), but this reagent also gave some reduction of the olefin system. Subsequent reductions were performed with diisobutylaluminum hydride (Miller et al., 1959; Kreft, 1977), and the unwanted overreduction did not occur. In either case, the resulting alcohol was oxidized to aldehyde **2** with MnO₂ (Petroski, 2003).

Aldehyde **1** was prepared by the same route (Fig. 2), but the starting material, (2*E*,4*Z*)-2,4-heptadienal (**11**), was not commercially available. Instead, this was prepared by the method of Petroski (2003). By GC–MS analysis, synthetic **1** contained 12% of **2** as an impurity immediately after synthesis. The product was diluted to a concentration of 27 µg/µl in CH₂Cl₂ and stored at –70°C until needed for field tests.

Instability of **1** was a concern, and to evaluate its lability a solution was kept at ambient laboratory conditions and periodically analyzed. Synthetic **1** (100 µg in 1.8 ml of hexane) was added to a 2-ml glass autosampler vial with crimp-top septum cap, along with undecanol (22.7 µg) as internal standard. GC analysis was performed immediately after preparing the sample (1-µl injection through the cool-on-column inlet), and subsequent injections were made every 24 hr for 3 d (the Teflon[®] lined septum top was not replaced during the test).

Field Lures

Red rubber septa (Sigma-Aldrich) were cleaned by Soxhlet extraction for 6 hr with CH₂Cl₂ and allowed to air dry overnight. To prepare septa, a solution of synthetic **1** was applied (500 µg in 20 µl), followed by CH₂Cl₂ (300 µl). After the solvent soaked in, the septa were aired 1 hr in a fume hood and then kept in a tightly capped glass jar. Septa were always used in the field the same day they were prepared and discarded after 24 hr.

The emissions of compounds **1** and **2** from freshly prepared septa were analyzed in the laboratory. Three septa were placed individually in volatile collectors, and collections of approximately 1-d duration were made for 3 consecutive days and quantitated by GC.

Field Tests

Field tests were conducted during three periods and in two areas. The three periods were late spring (7 d, May 26 to June 1, 2005), mid summer (5 d, July 11 to July 15, 2005), and late summer (10 d, August 2 to August 19, 2005). The first test area was a commercial organic vegetable farm in Farmington, IL, that contained both eggplant (var. Black Bell, Rosa Bianca, and Pingtung Long) and potatoes (var. Kennebec, Pontiac, Red Norland, All

Blue, Cranberry Red, and Onaway). The second area was a research plot located at the NCAUR that was planted only in eggplant (var. Black Beauty and Salangana hybrid).

Traps used in the field trials were yellow sticky cards, coated with adhesive on both sides (“Sticky Strips,” Olson Products, Medina, OH); these were cut in half (final size, 15×15 cm). Holes were punched on opposite sides, and the traps were secured to bamboo stakes (1 m high) with twist ties. Traps were situated so that their bottom edges were about 5–10 cm above the tops of the plants. Lures were attached with wire to the tops of traps.

A paired experimental design was used. Each pair consisted of a baited and unbaited trap, separated by about 5 m. Within the pairs, assignment of treatments to traps was by coin flip and, thereafter, was alternated each day. Traps were replaced with new ones each day, and the used ones were taken to the laboratory for examination. All flea beetles were removed from the traps (by using hexane to help dissolve the glue) and examined under a microscope to confirm identity, and the numbers of *E. fuscula* were recorded. Analysis of variance was conducted, as described with results. Sex ratios were determined from 5 subsamples of 10 beetles, each chosen randomly from both the treatment and control trap catches. Sex was confirmed by dissection (whether aedeagus was present).

Results

Volatile Collections

Comparisons of volatile collections from adult *E. fuscula* males and females revealed six male-specific peaks (1–6 in Fig. 3). The most abundant (1) was detected in 82 out of the 93 male volatile collections made in 2004 and 2005. In the 25 collections from 2005, the mean amount of 1 was 5.5 ± 4.2 (SD) ng per male per day, and the maximum was 16 ng per male per day. The minor compounds were also detected consistently whenever 1 was prominent. Typical ratios are summarized in Table 1. Compounds 1–6 were not found in the 50 volatile collections from females or in the 9 collections from eggplant leaves only. In GC traces for females or eggplant only, peaks sometimes occurred at the same, or similar, retention times as the numbered compounds from males (e.g., Fig. 3), but in these cases the mass spectra did not agree with the male compounds. Nine of 10 mixed-sex collections contained compounds 1–6 in amounts and proportions consistent with the collections from males.

Fig. 3 Comparisons of volatiles collected from male (top) and female (bottom, mirror view) *E. fuscula* beetles feeding on eggplant leaf. Compounds 1–6 are detectable only from the male volatiles. Some peaks in female collections had similar retention times to male-specific compounds (e.g., peaks 3 and 5) but differed in mass spectra. Peaks marked with asterisks are probably geometrical isomers of 1 and 2 (see text)

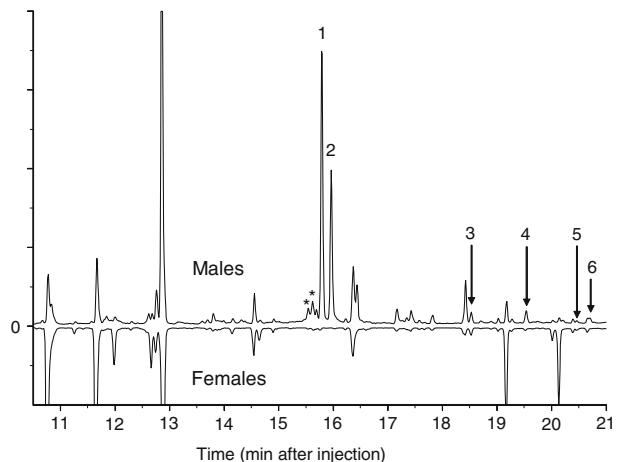


Table 1 Mean Percentage of each Male-specific Compound in the Volatile Collections Relative to Compound **1** based on GC–MS Peak Area

Male-specific compound	Mean (%) to 1±SD
1	100 ^a
2	26±13 ^a
3	7.5±3.9 ^b
4	8.2±4.2 ^b
5	0.70±0.31 ^b
6	2.2±1.1 ^b

^a *N*=23

^b *N*=12

Electrophysiological (GC–EAD) Analysis

Gas chromatographic-electroantennographic analysis of collected volatiles of male beetles feeding on eggplant foliage showed that the natural **1** and **2** were readily detected by the antennae of males (*N*=3) and females (*N*=4; Fig. 4, male example shown). Similar results were obtained for the synthetic aldehydes. Male and female antennae did not respond to **3**–**6** in volatile collections from males feeding on eggplant foliage, perhaps because of the relatively small amounts (<1 ng/compound). However, when synthetic **3**–**6** were injected at 10 ng/compound, compounds **3** and **5** did elicit antennal responses in five out of five tests. Compound **6** produced responses in two of the five tests, but no response was seen to compound **4**. Male and female antennae also responded to some foliage-specific compounds, but these were not addressed further.

Identification of Compound **1**

Compound **1** eluted from silica gel with 10% ether in hexane, a polarity consistent with an aldehyde, ketone, or ester. Silica HPLC of volatile collections rich in **1** (10 of these, combined) afforded a sample suitable for NMR spectroscopy and microhydrogenation; compound **1** (and also **2**) eluted between 8 and 9 min after injection.

The mass spectrum of **1** (Fig. 5, top) suggested a molecular weight of 136 but did not match any library spectra. An aldehyde or ketone with molecular weight 136 (C₉H₁₂O) would have four degrees of unsaturation, and an ester of the same weight (C₈H₈O₂) would have five. Hydrogenation over palladium increased the molecular weight to 142 (Fig. 5, bottom), indicating the uptake of six hydrogen atoms and, therefore, the presence of three

Fig. 4 Positive GC–EAD response (male antennae) to compounds **1** and **2** in a volatile collection from male beetles feeding on eggplants

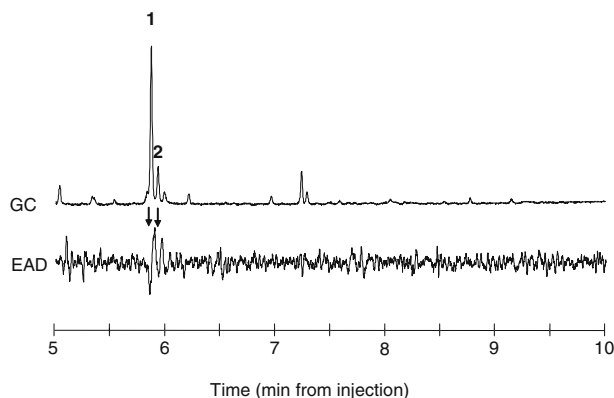
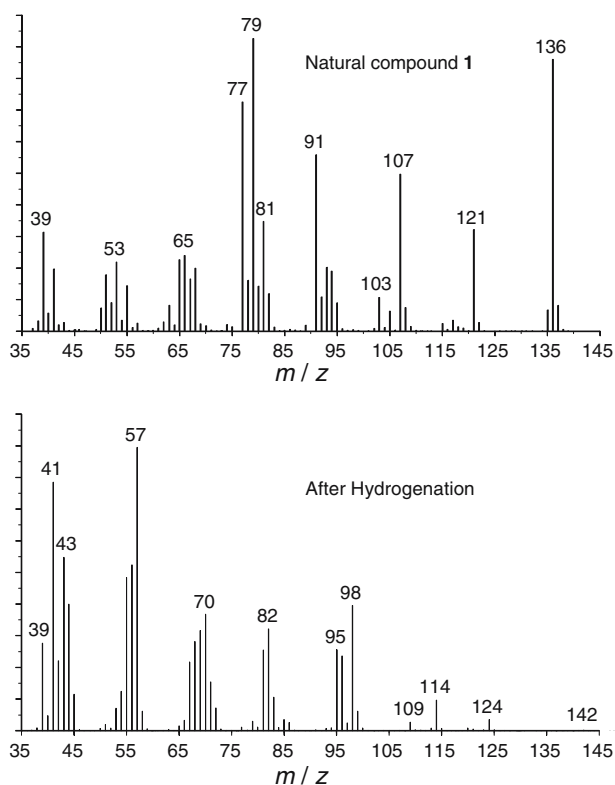


Fig. 5 Positive EI mass spectrum of compound **1** after HPLC isolation (top). Nonanal the resulting product after hydrogenation (bottom)



carbon–carbon double bonds in the original compound. The mass spectrum of the hydrogenation product gave a good library match to nonanal, and authentic nonanal had the same mass spectrum and GC retention time as the hydrogenation product. Because hydrogenation over palladium was not expected to affect an aldehyde group or the carbon skeleton, compound **1** was concluded to be a nonatrienal (the fourth degree of unsaturation required by the molecular weight being accounted for by the aldehyde carbonyl).

The ^1H and COSY NMR spectra of HPLC-purified **1** (Fig. 6; Table 2) defined the locations and configurations of the double bonds. The ^1H spectrum in CDCl_3 indicated six olefinic protons (δ 5.79–7.18), an aldehydic proton (δ 9.57), and an ethyl group (terminal methyl group at δ 1.09, split into a triplet by the methylene group at δ 2.30). The olefinic protons had to be on six consecutive carbons in the middle of the nine-carbon chain because the one-carbon aldehyde and two-carbon ethyl groups occupied the ends. Thus, compound **1** was a 2,4,6-nonatrienal. As shown in Fig. 6, the coupling involving olefinic protons was readily followed in the COSY spectrum from the aldehyde end to the alkyl end of the structure, and the olefinic proton resonances were thus assigned to specific chain positions. The key coupling constants $J_{2,3}$, $J_{4,5}$, and $J_{6,7}$ were read from the assigned ^1H spectrum as 15.2, 15.4, and 10.7 Hz, indicating configurations of *E*, *E*, and *Z*, respectively (Williams and Fleming, 1980). Thus, compound **1** was (2*E*,4*E*,6*Z*)-2,4,6-nonatrienal. Synthetic **1** matched the natural compound by mass and NMR spectra and by GC retention time.

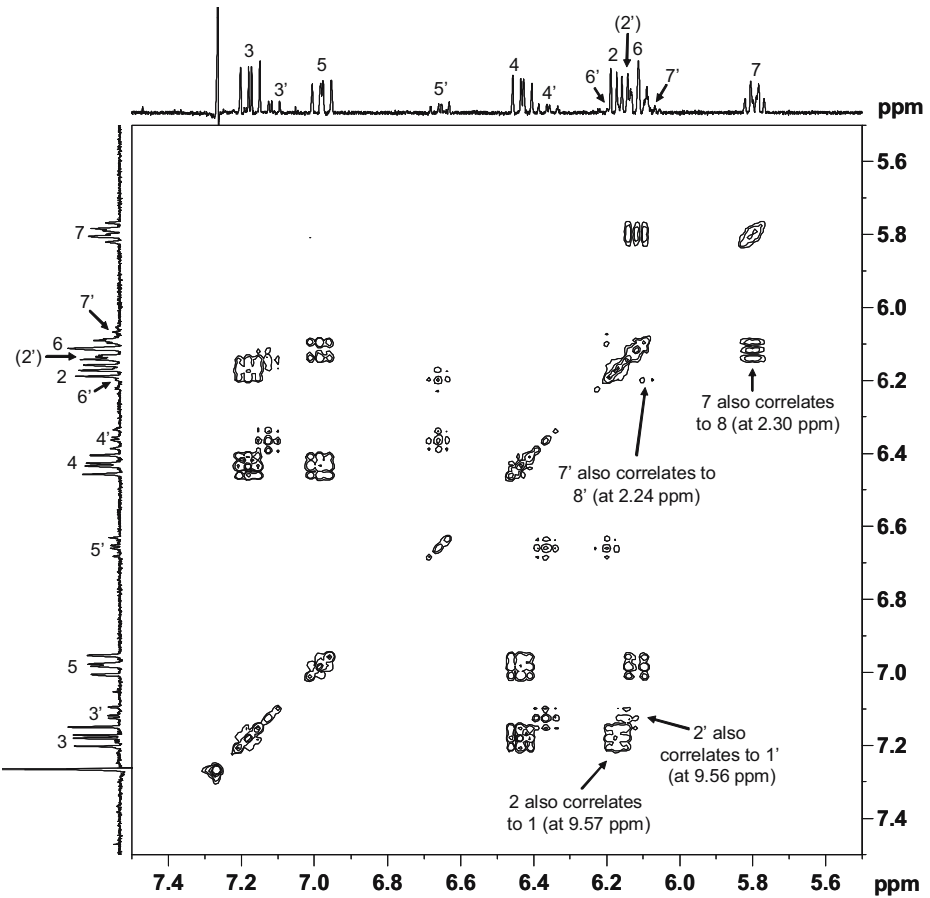
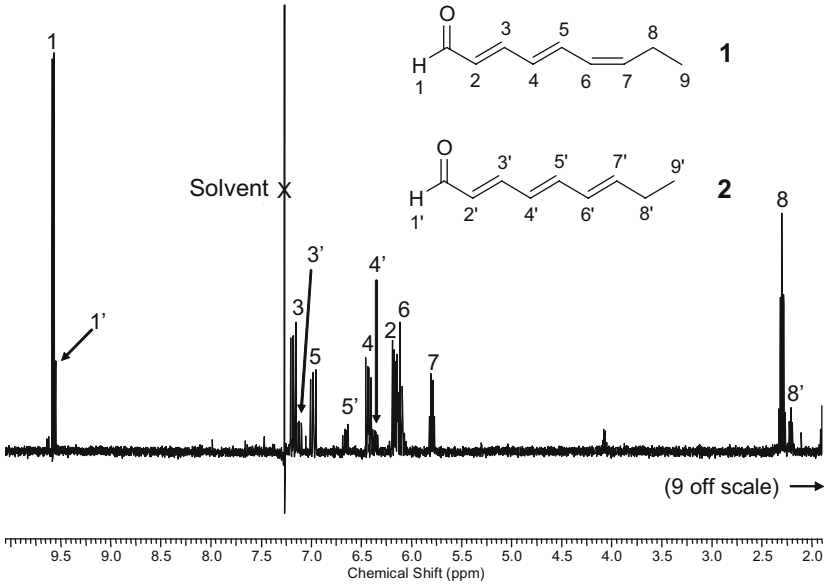


Table 2 Proton NMR Data for Aldehydes **1** and **2**

NMR property	Compound 1		Compound 2	
	CDCl ₃	C ₆ D ₆	CDCl ₃	C ₆ D ₆
Position number, description and shifts (ppm)				
1 (1H, d)	9.57	9.55	9.56	9.51
2 (1H, dd)	6.17*	6.06	6.15	6.06
3 (1H, dd)	7.18	6.56§	7.12	6.55
4 (1H, dd)	6.43	5.95*	6.36	5.89*
5 (1H, dd)	6.98	6.58§	6.65	6.21
6 (1H, dd)	6.11*	5.91*	6.20	5.91*
7 (1H, dt)	5.79	5.56	6.10	5.74
8 (2H, p)	2.30	2.08	2.24	1.96
9 (3H, t)	1.09	0.93	1.08	0.91
Coupling constants (Hz)				
<i>J</i> _{1,2}	8.0	7.8	8.0	7.8
<i>J</i> _{2,3}	15.2 ^a	15.1 ^a	15.2 ^a	15.2 ^a
<i>J</i> _{3,4}	11.2	11.4	11.1	11.1
<i>J</i> _{4,5}	15.4 ^a	15.1 ^a	14.9 ^a	14.9 ^a
<i>J</i> _{5,6}	11.6	11.4	10.6	10.7
<i>J</i> _{6,7}	10.7 ^b	11.0 ^b	15.2 ^a	15.2 ^a
<i>J</i> _{7,8}	7.9	7.6	6.5	7.4
<i>J</i> _{8,9}	7.5	7.5	7.4	7.5

Within a column, signals followed by the same symbol (* or §) partially overlap at 500 MHz.

^a Evidence for an *E* double bond ($J > 12$ Hz).

^b Evidence for a *Z* double bond ($J < 12$ Hz).

Identification of **2**

Compound **2** had a mass spectrum that was nearly identical to **1** and was believed to be a geometrical isomer. The beetle-derived NMR sample (Fig. 6) contained a small amount of **2**, and most of its resonances were visible, being somewhat offset from those of **1**. Assignment of resonances was performed from the COSY spectrum as with **1**. Coupling constants $J_{2,3}$ and $J_{4,5}$ were 15.2 and 14.6 Hz, indicating *E* double bonds, but resonances for the olefinic protons at positions 6 and 7 were obscured, so that $J_{6,7}$ could not be observed. However, given that **2** is not identical to **1**, it was concluded that the 6 double bond of **2** must be *E* and, therefore, that compound **2** was (2*E*,4*E*,6*E*)-2,4,6-nonatrienal. The mass spectrum and GC retention time of synthetic **2** were identical to the natural product, and so was the NMR spectrum, to the extent that resonances could be compared. When deuterobenzene was used as the NMR solvent instead of CDCl₃, shifts of the olefinic protons changed, but overlap of signals in **1** and **2** was not completely avoided (Table 2). Minor amounts of compounds, believed to be geometrical isomers of **1** and **2** based on mass spectra, were sometimes found in the male-produced volatiles (peaks identified with asterisks, Fig. 3), but these did not elicit antennal responses.

Fig. 6 The ¹H (top) and COSY (bottom) NMR spectra of natural sample from *E. fuscula* that contained compounds **1** and **2**. Assignment of resonances to compound position numbers are shown in upper panel; data for compound **2** are indicated with (')

Compounds 3–6

Compounds **3** and **4** eluted from silica gel with hexane (polarity consistent with hydrocarbons), whereas **5** and **6** eluted with 25% ether in hexane (polarity consistent with alcohols). Mass spectra of compounds **3**, **4**, **5**, and **6** (Fig. 1) were recognized from previous flea beetle research (compounds A, C, F, and G, respectively, in Bartelt et al., 2001). The GC retention times of the *E. fuscula* compounds also matched those of authentic standards on the achiral column.

Analysis of **4** and of derivatives of **3**, **5**, and **6** from *E. fuscula* on the Cyclodex-B GC column allowed assignment of the absolute configurations. Compound **4** from *E. fuscula* had a retention time of 20.50 min, the earlier of the enantiomers of **4** (racemic **4** gave peaks at 20.51 and 20.75 min). *P. cruciferae* and *A. flava* also have the earlier eluting enantiomer of **4** (Bartelt et al., 2001), which was shown by chiral synthesis (Muto et al., 2004) to have the (9*R*,9*aS*) configuration (see Fig. 1). *ar*-Himachalene (**7**) derived from *E. fuscula* **3** had a GC retention of 39.70 min, the latter of the enantiomers (racemic **7** gave GC peaks at 39.17 and 39.70 min). Mori (2005) established that the later-eluting enantiomer has the (5*R*) configuration (Fig. 1), and therefore the **3** from which it was derived had to have the (9*R*,9*aS*) configuration. Finally, alcohols **5** and **6** from *E. fuscula* dehydrated to give just one enantiomer of **4**, which eluted at 20.50 min, corresponding to the (9*R*,9*aS*) configuration. Thus, **5** and **6** must have been (3*R*,9*R*,9*aS*) and (3*S*,9*R*,9*aS*), respectively.

Labile Compounds

Lability of compounds **1**, **5**, and **6** made it difficult to accurately characterize the chemical blend of **1–6** at the instant it was emitted from the beetles. Isomerization of **1** into thermodynamically more stable **2** occurs over time if **1** is not carefully protected (e.g., kept in solution in a dark freezer at -70°C). For example, immediately after the sample of synthetic **1** was placed in the autosampler vial, the amount of **2** was 18% of the amount of **1**, by GC analysis. When the vial was left at ambient conditions in the laboratory, the amount of **2** as a percentage of **1** increased to 31%, 66%, and 93% after 24, 48, and 72 hr, respectively. However, the total amount of sample in the vial (**1** plus **2**) decreased by less than 3% after 72 hr (measured with internal standard), indicating most of the relative decrease in **1** must have been due to isomerization to **2**.

Isomerization of **1** was also likely in the Super Q collections from live beetles because the measured relative amounts of **2** increased as durations of volatile collections increased. For collections of 1-d duration, the amount of **2** as a percentage of **1** (mean \pm SD) was $26\pm$

Table 3 Mean Number of *E. fuscula* Adults Caught on Baited and Unbaited Sticky Traps during Three Trial Periods in 2005

Time period	Mean trap catch (<i>E. fuscula</i> /trap/d)			
	Baited	Control	N	Ratio baited:control
May 26–June 10	26.9 ^a	5.8	35	4.6:1.0
July 11–15	7.6 ^a	2.3	25	3.3:1.0
August 2–18	10.6 ^b	6.5	50	1.6:1.0

^a Significant difference between treatment and control at the 0.001 level.

^b Significant difference between treatment and control at the 0.01 level.

13 ($N=23$). For collections of 2-, 3-, and 4-d duration, the corresponding amounts of **2** were 33 ± 10 ($N=23$), 40 ± 19 ($N=29$), and 43 ± 12 ($N=13$), respectively. From linear regression analysis, the amount of **2** relative to **1** increased by 5.1 ± 1.7 (SE) percentage points per day ($t=4.38$, $P<0.001$). The predicted fitted value of **2** for time=0 d (i.e., time of emission from beetles) was $21.4\pm 3.0\%$ (SE), a value that was significantly different from zero ($t=7.12$, $P<0.001$). Thus, the apparent rate of isomerization of **1** was not high enough to account totally for the presence of **2**. It was concluded that **2** was, in fact, being emitted by the beetles.

It has been reported that compounds **5** and **6** both decompose in a heated GC inlet into compounds **3** and **4** (Bartelt et al., 2001); 50% degradation was typical, and the resulting ratio of the **3** and **4** that were formed was about 1:1. Thus, it is likely that the amounts of **3** and **4** actually in beetle emissions are considerably lower than the measured values in Table 1, and correspondingly, the real amounts of **5** and **6** from the beetles are higher than the values in Table 1.

Field Lures

Aldehydes **1** and **2** were readily released from the rubber septa, but isomerization of **1** was a problem. In 17-hr collections of emitted volatiles in the laboratory, mean amounts of compounds **1** and **2** were as follows: $27\ \mu\text{g}\pm 8.0$ (SD) and $28\ \mu\text{g}\pm 9.2$ SD, respectively, ($N=3$). Although the solution applied to the septa contained only 18% of **2**, relative to **1**, this percentage increased to 104% in the 17-hr collection, and by 72-hr collection, the amount of **2** relative to **1** was 257%.

Field Experiments

Traps baited with synthetic **1** (also emitting **2** at a level that was at least 18% as high as **1**) performed significantly better than control traps in all three trial periods (Table 3). In a three-way ANOVA (factors being treatment, seasonal period, and trap pair), the overall treatment effect was highly significant ($F=98.3$, $df=1,107$, $P<0.001$), but there was also a significant interaction between treatment and seasonal period ($F=11.5$, $df=2,107$, $P<0.001$). Thus, the relationship between the treatment and control catches changed with time of year. As shown in Table 3, the ratio between treatment and control (by using newly prepared septa each day) was 4.6:1 in late spring, but decreased to 3.3:1 in early summer and, further, to 1.6:1 in late summer. The effect of **1** and **2** was strongest in late spring, although it was significant ($P<0.01$) in all three periods. In the subsamples from treatment and control traps, captured beetles were 64% and 48% females, respectively ($N=50$ for each treatment).

Discussion

Volatile collections of male *E. fuscula* feeding on eggplant foliage showed six compounds that were not present in volatile collection from feeding females or from foliage alone. The two major male-specific compounds, (2*E*,4*E*,6*Z*)-2,4,6-nonatrienal (**1**) and (2*E*,4*E*,6*E*)-2,4,6-nonatrienal (**2**), were identified by GC-MS, NMR, and microscale hydrogenation. Both compounds were synthesized and matched natural **1** and **2** by all available criteria. The true amount of **2** emitted from the beetles, relative to **1**, is not yet known because of the tendency for **1** to isomerize into **2**, but it was concluded that at least some **2** is naturally

present. The beetle antennae respond to both compounds in GC–EAD tests. Field tests of baits containing synthetic **1** and **2** were attractive to both male and female *E. fuscula*, compared with unbaited traps. Although the blend of **1** and **2** was attractive in all three time periods, it performed best early in the season, shortly after the emergence of overwintered adults.

Aldehyde **1** has been reported previously in the volatiles from blended endive leaves (Götz-Schmidt and Schreier, 1986), and has recently been identified from oat flakes and Darjeeling black tea (Schuh and Schieberle, 2005, 2006). Aldehyde **1** has also been reported as being among the most odor-active compounds found in food, detectable to humans at an odor threshold of 0.0002 ng/l in air and with an oat-flake-like aroma (Schuh and Schieberle, 2005). In our research, it was not uncommon to detect an odor similar to bread baking when volatile collections from males were processed. Aldehyde **2** was first identified from the volatile oil of blended dry beans (Buttery, 1975), and has also been reported in the volatile constituents of endive leaves (Götz-Schmidt and Schreier, 1986) and cooked spinach (Näf and Velluz, 2000). Interestingly, the human nose is 20,000 times less sensitive to **2** than it is to **1** (Schuh and Schieberle, 2005).

Schuh and Schieberle (2005) provided proton NMR data for both **1** and **2** that generally agree with ours. One difference for **1** is that they reported the resonance for the proton at position 7 to be within a multiplet at 6.05 to 6.25 ppm, whereas we found its shift to be 5.79 ppm. Another difference is that the aldehydic proton of **1** was reported to be slightly upfield of that for **2** (9.55 vs. 9.58 ppm, respectively), whereas the reverse was true in our spectra (9.57 and 9.56 ppm, respectively). The Schuh and Schieberle (2005) synthesis of **1** resulted in a mixture of isomers, in which **1** was not the major product. This situation, along with the absence of COSY data, may have made peak assignment difficult.

Compounds **1** and **2** have not been previously reported from other flea beetle species or from any other insect species. However, it is interesting to note that males of another chrysomelid beetle, *Diorhabda elongata* Brullé, emit a homologous compound, (2*E*,4*Z*)-2,4-heptadienal, as part of their aggregation pheromone (Cossé et al., 2005).

Small amounts of other isomers of **1** and **2** were detected in the male-produced volatile collections, although their origin, exact configuration, and attractiveness are unknown. Considering the instability of compound **1**, the presence of these other isomers is not unexpected. Schuh and Schieberle (2005) also encountered additional isomeric compounds of 2,4,6-nonatrienals during synthesis of (2*E*,4*E*,6*Z*)- and (2*E*,4*Z*,6*Z*)-2,4,6-nonatrienal.

The additional four minor male-specific compounds (**3**, **4**, **5**, and **6**) matched synthetic and natural male-specific sesquiterpenes previously identified from *Phyllotreta* and *Aphthona* spp. flea beetles in GC retention times, mass spectra, and chirality (Bartelt et al., 2001; Tóth et al., 2005). The configurations of all four compounds are identical at ring positions 9 and 9a (Fig. 1), suggesting a common biosynthetic origin. The GC–EAD activity of **3**, **5**, and **6** in *E. fuscula* is consistent with, but does not prove, a pheromonal function. Field tests were attempted with racemic **3–6** during the late summer period (when results for **1** and **2** were weakest) but were inconclusive.

Pheromonal activity has been demonstrated at least for compound **3** in *P. cruciferae* (Soroka et al., 2005; Tóth et al., 2005); the males of this species emit both **3** and **4**. Males of *Aphthona flava*, *A. czwalinae*, and *A. cyparissiae* also produce **3** and **4** as well as **5** and **6**, with the proportions of each being species specific (Bartelt et al., 2001). Such a situation might be expected if the species share a common habitat and if the suites of compounds serves as pheromones, but field bioassays with synthetic compounds have not yet been conducted for the *Aphthona* species.

Further enhancement of field attractant lures may be possible once true emission rates of **1–6** from *E. fuscula* males are determined, taking into account distortions in ratios caused by chemical changes occurring before analysis (i.e., **1** isomerizing into **2**) or during analysis (i.e., **5** and **6** dehydrating into **3** and **4**). Additional compounds (i.e., other 2,4,6-nonatrienal isomers, or plant volatiles) may also increase attraction. Finally, finding a delivery system that will avoid or minimize the isomerization of **1** into **2** is also needed. In spite of these unresolved issues, a new pheromone attractant has been demonstrated to be effective in capturing *E. fuscula*, and adds to the growing list of pheromone systems identified in the flea beetle family.

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significance of avian odors has received relatively little attention. Weldon and Rappole (1997) suggested that avian odors may be indicative of chemical defense or unpalatability. Few endogenous chemical defenses have been documented, but exogenous defenses, applied to plumage and nests, may be widespread (Simmons, 1966; Clark and Mason, 1985, 1988; Ehrlich et al., 1986; Clayton and Vernon, 1993; Gwinner et al., 2000; Lafuma et al., 2001; Parkes et al., 2003; Weldon, 2004). The best known examples of endogenous chemical defense among birds are the Pitohuis and *Ifrita kowaldi* of New Guinea (Dumbacher et al., 1992, 2000). These species apparently sequester batrachotoxins, a potent class of neurotoxins, from dietary sources such as Choresine beetles (Family Melyridae; Dumbacher et al., 2005). The concentrations can be sufficient in skin and plumage to act as a deterrent against parasites and predators (Dumbacher et al., 1992, 2000; Dumbacher, 1999). However, chemical characteristics of these neurotoxins vary widely within and among species, consistent with evidence that they are acquired from an environmental source (Dumbacher et al., 2000). Two species of pitohuis (“hooded” *Polyporous dichrous* and “variable” *P. kirhocephalus*) emit a sour odor that is hypothesized to serve as an olfactory warning of the birds’ poisonous characteristics (Dumbacher et al., 1992). The chemical odors of pitohuis have not yet been characterized qualitatively or quantitatively.

Crested auklets (*Aethia cristatella*) produce a citruslike odorant dominated by even-numbered aldehydes (6–12 carbons) that may function as an ectoparasite repellent and signal of mate quality (Douglas et al., 2001, 2004; Hagelin et al., 2003). Two of the primary constituents are potent invertebrate repellents, and synthetic analogues of the crested auklet odorant repel, paralyze, and kill ectoparasites in a dose-dependent fashion (Douglas et al., 2001, 2004, 2005b). The efficacy of synthetic analogues is comparable to that of commercial arthropod repellents (Douglas et al., 2005b). Brief exposure of auklet lice to *in situ* concentrations of the odorant in tissues caused paralysis and mortality; however, suspension of pigeon lice above crested auklet feathers had no effect on survivorship compared to controls (Douglas et al., 2005a).

This report presents a method for comparison of odor production in live crested auklets that can also be applied to other vertebrates. In this method wild birds were captured and confined in a purified and regulated airstream, and the birds’ emissions were captured onto polymer traps. This method offers several advantages. First, it is not necessary to harvest tissues. Chemical concentrations vary considerably within an individual crested auklet’s plumage, and a large sample of feathers is probably necessary to obtain an accurate mean quantitative value (Douglas, 2006). This would compromise the bird’s thermal insulation and its fitness. Second, the method isolates measurements of the birds’ chemical emissions from potential environmental contamination. Third, the method yields accurate and precise duplicate measurements of specific compounds, and these measurements can be calibrated against known standards. Portable industrial instruments designed for detection of volatile organics are not sufficiently specific or accurate for this type of research application. The method described here provides a quantitative comparison of chemical emissions in live birds that does not compromise their fitness in any way.

Methods and Materials

Field Methods Research on the chemical odor of crested auklets was conducted at a colony on Big Koniuji Island, Alaska, from June 4 to July 16, 2002. In terms of phenology, this period corresponded with the onset of egg-laying to early chick rearing. The colony is situated in an ancient glacial cirque at 243 m elevation on a mountain overlooking Yukon

Harbor. The crested auklet nests in rock talus, high on the steep slopes of this cirque. It is the only seabird species that nests in the cirque. Breeding adults synchronized visitations each morning during the incubation period, gathering in a large flock in Yukon Harbor prior to visiting the colony. Birds arrived on the colony surface in a large flock and were captured in noose carpets strung over landing rocks. Each bird was measured, banded with a USFWS metal band, color bands, sampled, sexed by bill characteristics (according to Jones, 1993a), and inspected for ectoparasites.

Each bird was then placed into a glass reaction kettle, and volatile emissions were collected in a purified airstream. The flow rate of 1.0 l/min delivered oxygen at a rate of 0.2 l/min (dry atmospheric air = 20.95% oxygen; Schmidt-Nielsen, 1997), which was more than sufficient to supply the oxygen consumption needs of a 300-g nonpasserine bird (0.25 l/h at rest; Lasiewski and Dawson, 1967). Air temperature during volatile collections was 6–10°C. Duration of sampling was measured with a stopwatch. Volatile emissions were collected for 50 min on all except six birds. The period of collection for those six was reduced to 30 min. Gas chromatography (GC) peak areas for octanal were adjusted to account for the difference in collection times. The adjusted values for those six individuals lay within the range of other values in the sample. After GC-mass spectrometry (MS) analysis, peak values for all samples were normalized to a 50-min collection time (CT) with the following equation: $50 \text{ min}/\text{CT} * \text{peak area} = \text{adjusted peak area}$. Collection traps were eluted with 2.0 ml of methanol, and the elution was collected in borosilicate glass vials (3/8 oz; Fisher Scientific), sealed with Teflon-lined caps and a vapor seal (DuraSeal

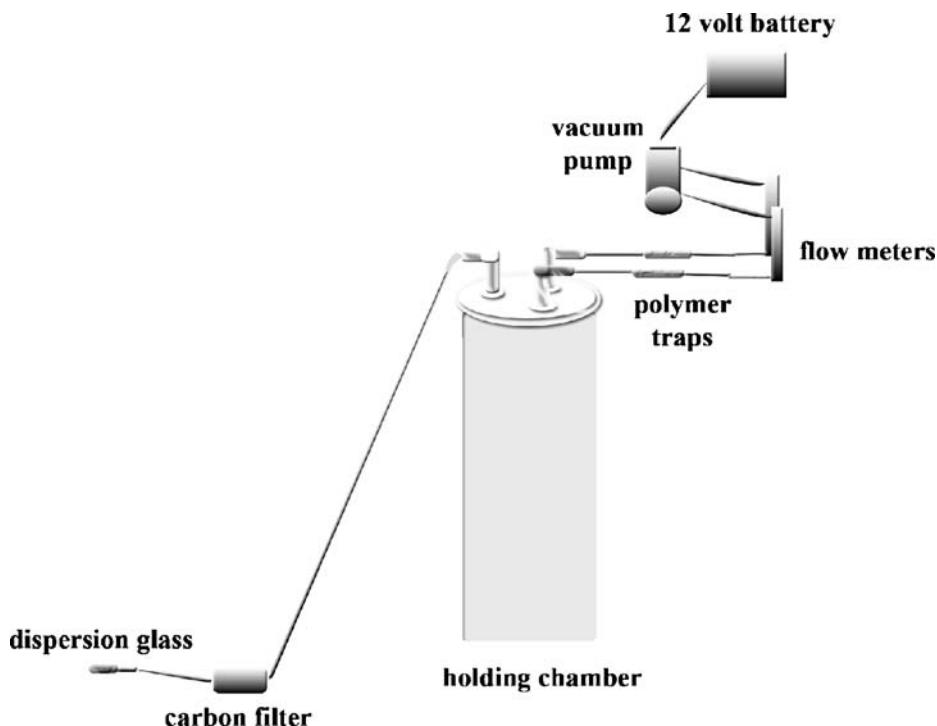


Fig. 1 Volatile collection system for measuring chemical emissions. Regulated and purified air is pulled by battery-operated vacuum pump into glass reaction kettle containing live crested auklet. Volatile emissions are captured onto polymer traps that are placed in the exiting air stream.

stretch film). The chambers were scrubbed with baking soda, rinsed with freshwater, and dried with a clean cotton towel between each sampling.

Design of Volatile Collection System The design of this system benefited from the study of similar methods used to collect plant volatiles (e.g., Turlings et al., 1991). The collection chamber (Figure 1) consisted of a 4000-ml Pyrex reaction kettle (Corning # 6947-4 L) and kettle lid (VWR 36393-051) clamped securely with a kettle clamp (VWR Cat. No. 36393-051). All tubing and fittings were glass or Teflon. The incoming airstream was filtered through a glass dispersion tube (Lab Glass UA-71801-11950, extra coarse porosity 170–220) and then an in-line charcoal filter (Whatman Carbon Cap). This purified airstream entered the chamber through a 24/40 glass elbow (LabGlass LG-1980-100). The outgoing airstream was split, exiting through two 24/40 glass elbows into two glass collection traps. The collection traps were custom manufactured by Lab Glass (Vineland, NJ, USA) and adapted from a design described in Turlings et al. (1991). Each trap was 6 cm long by 9 mm outer diam (6 mm ID). The tube was fitted with a 325-mesh stainless steel frit sealed across the diameter of the tube, 20 mm from the downstream end. Glass collection traps were packed with Super Q (80–100 mesh; Alltech, Deerfield, IL, USA) or Tenax (35/60 mesh; Alltech). These traps were conditioned under vacuum in a Bullet Dryer at 225°C for 14 hr prior to shipping to the field site. The polymer (50.0 mg) was placed on top of the frit and held in place with a small plug of glass wool. Air exiting from these collection tubes was passed through Gilmont flow meters hooked up to a battery operated vacuum source (Cole Parmer Model # 7530-25). Flow rate was regulated at 500 ml/min through each trap. Flow meters were factory calibrated prior to the field season, and calibration of the flow meters was checked again after fieldwork. This calibration was performed with Sierra 820 Mass Flowmeter, and SE was ± 0.41 ml/min. The volatile collection system was calibrated in the field by passing 3.0 ml of synthetic octanal through a bubbler, placed in line (Ace Midget Bubbler with 145–175 μm filter; Ace Glass Inc., Vineland, NJ, USA). This calibration was run exactly as performed with crested auklets, with the volatile collection system operating for 50 min. The calibration was performed once at the end of the field season to avoid contamination of equipment and samples with standards.

Chemical Analysis GC-MS was carried out in the SIM mode (selective ion monitoring) with an HP5890 Series II Gas Chromatograph equipped with a 20 m \times 0.25 mm, 5% phenyl siloxane column (Alltech), and an HP5972 Series Mass Selective Detector. The injector and detector temperatures were maintained at 250°C throughout, and the column flow was 1.0 ml/min. The instrument was programmed from 60°C to 250°C in two stages. The first stage increased at a rate of 4°C/min to a final temperature of 120°C, and remained at that temperature for 4 min. The second stage increased at a rate of 8°C/min to a final temperature of 250°C, and remained at that temperature for 2 min. Octanal was selected as an index of chemical potency because it is consistently the most abundant constituent in the crested auklet odorant (40%; Douglas et al., 2001), and it is also strongly repellent to ticks (Douglas et al., 2004). Retention time and ion abundances were obtained in EI mode from standard (99% Octanal, ACROS Organics, C.A.S. 124-13-0), and results were consistent across five replicates at different concentrations. The most abundant ions were chosen for monitoring, and dwell times (ms) were set for each ion according to its relative abundance (ion/dwell time: 43.0/100, 41.0/80, 44.0/70, 57.0/30, 84.1/30). Subsequent analyses of standards in SIM mode showed that these parameters consistently discriminated octanal from background and obtained well-defined peaks.

Quality control was assured by the inclusion of blanks, duplicates, internal standards, augmented standards, and calibration standards run at intervals in sequences at the frequency of 5–10% of total samples. Standards were made by serial dilutions in methanol (ACROS Organics HPLC grade). Undecenal (97% Undecyclic Aldehyde, ACROS Organics, C.A.S. 112-45-8) was used as an internal standard, and was added to all samples. Blanks with the internal standard were also included in the sequence. Precise quantities of standards were measured with an Eppendorf Pipette (Model 4710). Accuracy and precision of the pipette were determined by replicate weighing of 10- μ l samples of distilled water with a Mettler AE163 analytical balance ($x = 9.94 \times 10^{-3}$ g; $SE = \pm 8.38 \times 10^{-5}$; $SD = 3.25 \times 10^{-4}$).

GC-MS variability was addressed by calibrating response factors of the target analyte and the internal standard. A series of calibrations was conducted at four concentrations of octanal and undecenal (3.8×10^{-3} , 4.8×10^{-3} , 6.0×10^{-3} , and 9.0×10^{-3} μ l/ml) to obtain the regression of relative response ($R^2 = 0.96$, $P < 0.001$, $N = 22$). The following regression equation was obtained:

$$\text{Octanal Peak Area} = 7.58 * (\text{Undecenal Peak Area}) + 14135$$

For each sample, the difference between the obtained and expected GC peak areas of the internal standard was calculated. This difference in undecenal peak area was applied in the regression equation above, and the value was added to the obtained value for octanal.

Duplicate samples were collected for all birds. However, the Big Koniuji colony is located in a rugged spot, and some samples were lost due to damage to the sample vials. Some samples were expended in the testing and calibration of analytical methods. I analyzed 103 samples from 57 individuals—46 individuals with duplicate split samples and 11 individuals with single split samples. Results obtained for Tenax and Super-Q volatile traps in the 46 duplicate split samples were similar. Standard error of splits, expressed as a percentage of peak area, was 8%. In the case of duplicate split samples, average octanal peak area was used as a relative index for chemical potency. In the cases where only single splits were available, the obtained value for octanal peak area ($\pm 8\%$) was used as the relative index. Quantitative measurements, obtained from field calibration with synthetic octanal and bubbler, were used to calculate emission rates for the birds.

Evaluating Ectoparasite Abundance A visual inspection method, similar to that described in Clayton and Walther (1997), was used as a relative measure of ectoparasite prevalence and abundance. A subsample of crested auklets ($N = 12$) was fumigated with carbon dioxide (as described in Visnak and Dumbacher, 1999), and dusted with pyrethrum (as described in Clayton and Walther, 1997) to evaluate the accuracy of the visual inspection method. Ticks were identified as *Ixodes uriae* by Lance Durden and were deposited in the U.S. National Tick Collection (curated at Georgia Southern University) under accession number RML 123386. Lice were identified by Dale Clayton as belonging to the genera *Austromenopon*, *Quadriceps*, and *Saemundssonina*, and these lice were deposited into the frozen collections of the Price Institute for Phthirapteran Research at University of Utah.

Results

Chemical emission rates differed among individuals, but not between the sexes. Measurements from the field calibration obtained a mean peak area of 277,424,557 ($SE = \pm 4,586,885$)

from 3.0 ml octanal passed into the volatile collection system. Applying this calibration, the average chemical emission for crested auklets was $5.7 \mu\text{l octanal}/50 \text{ min} \pm 0.42$ (peak area = 529,800; SE = $\pm 38,800$ or 7% sample mean). There was a sevenfold difference between the highest and lowest chemical emissions. A male had the highest chemical emission at $19.9 \mu\text{l}/50 \text{ min}$ (PA = 1,842,816), and the lowest chemical emission was also a male at $2.8 \mu\text{l}/50 \text{ min}$ (PA = 262,271). Male and female auklets did not differ in chemical emission rates, $t_{(0.05)\text{two-tailed}} = 0.44$, $P = 0.66$. The mean value for male auklets was $5.6 \mu\text{l}/50 \text{ min} \pm 0.52$ (PA = 518,900; SE = $\pm 48,500$, $N = 41$) vs. $6.0 \mu\text{l}/50 \text{ min} \pm 0.68$ (PA = 557,900; SE = $\pm 62,600$, $N = 16$) for females. Measurements were also obtained for one subadult male and female with well developed ornaments. The male emitted $4.4 \mu\text{l}/50 \text{ min}$ (PA = 405,974), and the female emitted $8.8 \mu\text{l}/50 \text{ min}$ (PA = 813,506).

Most birds had no ticks, despite a high abundance of *I. uriae* ticks on the colony surface. The individual with lowest octanal emission had 14 ticks attached between the rictal plate and eye on the left side of the face. All of these ticks were in the process of obtaining blood meals. Prevalence of tick parasitism was low. Only 2 out of 96 auklets examined by visual inspection methods had ticks (just 2% of the population sampled). The second auklet had 2 ticks attached to its face. Results for the visual inspection method were similar to results for the combined methods of fumigation and dust ruffling. Only one tick was found on 12 crested auklets by the combined methods of fumigation and dust ruffling. The low abundance of ticks on auklets was remarkable considering our own encounter rate with ticks in the colony. We noted 5–10 ticks on our pant legs per hour while sitting in the colony. We counted as many as 20 ticks questing (host-seeking behavior) within small areas ($0.37\text{--}0.91 \text{ m}^2$) on the surface of large landing rocks where auklets alighted.

Visual inspection suggested that the prevalence and abundance of lice were low. Lice were found on 4 of the 96 birds inspected, an infection rate of 4.2%. Comparison of the visual inspection method with fumigation and pyrethrum dusting was in agreement with results from visual inspection. Only one louse was found by fumigation, and only one louse was found by dust ruffling on 12 crested auklets. No more than four lice were found on any bird, including specimens that were collected for dissection.

Discussion

Differences in octanal emission rates may be related to the ability to produce odor. Higher emission rates probably represent a higher expenditure of lipid reserves since the aldehydes appear to be products of fatty acid synthesis (Douglas 2006). The male with the highest chemical “potency” in this study would have expended a minimum of 0.57 ml lipid per 24 h in order to consistently maintain the same level of emissions. The male with the lowest chemical potency would have expended approximately seven times less.

The prevalence of tick parasitism on adult crested auklets in this study (2.1%) was unexpectedly low considering the high abundance of ticks in the colony. Two species of Pitohui birds (New Guinea) known for chemical defenses also exhibited a lower than expected infection rate from ticks (3.1%, $N = 32$) compared to other genera of passerines (Mouritsen and Madsen, 1994). The tick infection rate reported here for crested auklets was much lower than what has been found in other subpolar seabird colonies where parasitism by *I. uriae* ticks has been studied. Infestations in subarctic colonial seabirds have been documented in as high as 70% of the adult population (Barton et al., 1996), and intensity of *I. uriae* infestations in some subantarctic seabird colonies may be sufficient to cause mortality in adult king penguins (*Aptenodytes patagonicus*; Gauthier-Clerc et al., 1998).

Higher rates of octanal emission in crested auklets may be associated with lower incidence of tick parasitism. Aldehydes could interfere with four stages of *Ixodes* tick parasitism—engagement, exploration, penetration, and attachment (described in Kebede, 2004). Crested auklets thrust their bills and faces into the scented nape feathers of conspecifics (“ruff-sniff” behavior) during courtship and social behavior (Jones, 1993b; Hunter and Jones, 1999; Hagelin et al., 2003; Jones et al., 2004). This behavior may provide crested auklets an opportunity to evaluate chemical potency of prospective mates (Douglas et al., 2001, 2004; Douglas, 2006). Crested auklets also engage in a mutual anointment behavior that helps to distribute aldehyde secretions to body regions where crested auklets cannot self-preen (Douglas, 2006). Anointment of facial skin and plumage around the head and neck could help to deter ticks and interfere with the intraspecific chemical signaling that ticks use to locate attachment sites on the host (Sonenshine, 1985). In fact, aldehyde concentrations in crested auklet plumage are sufficient to reduce tick locomotion (Douglas, 2006). Below some threshold of chemical defense, crested auklets are likely to be more vulnerable to tick parasitism. In this study, the crested auklet with the lowest chemical emission rate (half the population mean) was parasitized by 14 ticks clustered together between the eye and rectal plate. Ticks often attach around the eyes of birds because the skin is thin, and the area cannot be preened by the host (Reed et al., 2003). Chemical potency and associated repellence of ectoparasites may be a basis for mutual sexual selection in crested auklets (Douglas et al., 2001, 2004; Douglas, 2006).

The louse infection rate among crested auklets at Big Koniuji Island, as best could be determined, was 4.2%. Similarly, there was a very low infection rate at another mono-specific colony of crested auklets. No lice were found on 80 adults at Talan Island (see Douglas et al., 2004 for details). By contrast, the prevalence and abundance of lice were much higher in a mixed-species colony that included both crested auklets and least auklets (*A. pusilla*) at St. Lawrence Island, Alaska (Douglas et al., 2005a). Among these crested auklets, there was a 100% infection rate ($N = 21$), and the intensity of infection (adult + juvenile lice) ranged from 8 to 91 lice per bird (Douglas, unpublished data). Numerical results are not directly comparable because data for Douglas et al. (2005a) were obtained by body washing, which is a more accurate method than visual inspection (Clayton and Drown, 2001). Nevertheless, a qualitative difference can be inferred. To the extent that it has been evaluated, the prevalence of louse infections in crested auklets is higher at mixed-species colonies.

This study reports on a novel research application for estimating chemical emission rates in crested auklets. The method has several advantages. Accurate quantitative estimates of chemical production and emissions can be obtained for live vertebrates without harvesting tissues that might jeopardize the animal’s fitness. The live specimen is isolated in a leak proof chamber supplied with a purified airstream. This eliminates the possibility of contamination from plant volatiles, insects, and naturally occurring materials. Flow rate is regulated, and this is critical for determining rates. The quantitative measurements can be calibrated to known standards. The methods reported here could be applied to other vertebrates to study the relationship of chemical emissions to a range of studies including hormones, mate selection, reproductive behavior, or parasitism.

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Introduction

Allelopathy is a direct or indirect effect of one plant on another through the production of chemical compounds that are released into the environment (Rice, 1984). Many allelochemicals are toxic to higher plant growth, even at low concentrations, and they can have effects on seed germination. As a consequence, allelopathy may be involved in regulating density and distribution of species in natural settings. Inhibition of seed germination and root growth in response to chemicals released by many species including sorghum (*Sorghum bicolor*), wheat (*Triticum aestivum*), and rye (*Secale cereale*) have been consistently reported in the literature (Inderjit and Callaway, 2003; Inderjit and Duke, 2003; Weston and Duke, 2003).

Sunflower (*Helianthus annuus* L.) can actively influence the growth of surrounding plants. Dried sunflower residues and leaf powder incorporated in the soil reduced the growth of sorghum, soybean, and sunflower itself (Irons and Burnside, 1982; Schon and Einhellig, 1982). Leather (1987) demonstrated selective phytotoxicity of sunflower residues towards weeds. Batish et al. (2002) observed the reduced growth of *Cyamopsis tetragonoloba*, *Pennisetum americanum*, *Zea mays*, and *S. bicolor* in fields with sunflower residues, and attributed this to phenolics released from decomposing tissue of sunflower. Macias and coworkers have isolated more than 240 natural compounds from different cultivars of sunflower (Macias et al., 2002). In commercial crops of *H. annuus*, many terpenoids including monoterpenes, sesquiterpenes lactones, diterpenes, triterpenes, and two new families of sesquiterpenoids, heliannanes and helispiranes, have been identified (Macias et al., 2000, 2002 and references therein). Most of those compounds inhibited germination of *Lactuca sativa* at concentrations lower than 10^{-8} M (Macias et al., 2001). Although the inhibitory effect of phytotoxins in sunflower leaves extracts or crop residues on germination of various seeds has been reported previously (Azania et al., 2003), their mode of action has not yet been demonstrated.

Maturing seeds of white mustard accumulate food reserves as storage oils (60%) and insoluble proteins (40%) (Bewley and Black, 1994). During seed germination, proteins are first hydrolyzed by endopeptidase to soluble peptides that are subsequently degraded to oligopeptides and aminoacids (Bewley and Black, 1994). In mobilization of triacylglycerides in seeds, the glyoxylate cycle plays a key role to effect net gluconeogenesis from the acetyl-CoA derived by oxidation. The enzymes of the glyoxylate cycle such as isocitrate lyase (ICL, EC 4.1.3.1) increase their activity during maximum fat metabolism in specialized fat microbodies (glyoxysomes) located in the storage tissue of germinating seeds. Seed germination requires a fine balance between glycolysis and the oxidative pentose phosphate pathway reject, to ensure a sufficient supply of reducing power, ATP, and carbon skeletons for further biosynthesis. High mitochondrial respiration rates occurring in catabolic phases of germination, just after imbibition, support energy supply for growth processes and result in radicle protrusion.

Since undisturbed reserve mobilization is an important requirements for germination as well as subsequent plant growth and development, we investigated the influence of water extracts of sunflower leaves on catabolic processes taking place during mustard seed germination. We measured also O₂ uptake by intact seeds and energy metabolism by determining adenylate (ATP, ADP, AMP) concentrations in tissues of germinating seeds. Additionally, changes in ultrastructure of germinating seeds provoked by the presence of sunflower phytotoxins were observed.

Methods and Materials

Plant Material and Growth Conditions Leaves from field grown sunflower cv. Ogradowy were harvested at the beginning of the flowering stage. Tissue was air-dried, and ground to obtain

a fine powder. A 10% (w/v) water extract was formulated and used as a source of phytotoxic compounds as described by Bogatek et al. (2006). Cation exchange capacity (CEC) of the resultant extract was measured with a Wescor Dew Point Microvoltmeter, model HR-33T.

Mustard seeds (*Sinapis alba* L.) were germinated in 9-cm Petri dishes (50 seeds per dish) on filter paper moistened with distilled water (control) or with a 10% (w/v) water extract from sunflower leaves at 20° C in darkness. Additional experiments were performed with seeds allowed to imbibe an aqueous PEG (polyethylene glycol) 8000 solution (28.5% w/v), characterized by CEC equal to that of the sunflower foliar extracts. Treatments were arranged in a completely randomized design with four replications. Germination percentages were determined after 18 hr (0.75 d) and then every day for 8 d. Seeds were considered germinated when the radicle visibly protruded from the seed coat.

Seed viability was estimated by using a standard tetrazolium test. Seeds were incubated in 1% (w/v) solution of 2,3,5-triphenyl tetrazolium chloride for 8 hr at 30°C in darkness. Seeds with nonstained embryos were scored as nonviable. The experiments were repeated 3–4 times.

Adenosine Phosphate Assay Adenosine phosphates were extracted from seeds according to Olempska-Beer and Bautz-Freeze (1984). ATP, ADP, and AMP contents within the extracts were measured with the bioluminescence method with a pico-ATP biophotometer (Jobin et Yvon, France), as described by Saglio et al. (1979). The results are expressed as pmol mg⁻¹ DW, and values were obtained from five independently prepared extracts. The energy charge (EC) was calculated by the ratio (ATP+0.5 ADP)/(ATP + ADP + AMP).

Gas Exchange Oxygen consumption was measured with a Hansatech gas-phase oxygen electrode unit type LD1/2 supported by the Oxygraph Plus system at room temperature in darkness. Intact seeds (50–100) rinsed with distilled water were put into a 20-ml container with an oxygen electrode, and changes in O₂ concentration were determined over the course of 30 or 60 min for dry seeds. Results are expressed as μmol O₂ g⁻¹ DW min⁻¹ and represent values obtained from at least five independent experiments.

Enzyme Extraction and Determination Seeds (1 g) were rinsed with distilled water and homogenized in 10 ml extraction medium containing: 0.05 M phosphate buffer pH 7.6, 10 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT) in an ice bath. The homogenate was centrifuged for 20 min at 15,000 g at 4°C. The resulting supernatant was filtered through a column Pharmacia 10 PD equilibrated with 0.05 M phosphate buffer pH 7.6. The resulting enzymatic extract was used immediately for the ICL assay and a protein content determination.

ICL activity (threo-D₅-isocitrate glyoxylate lyase, EC 4.1.3.1, ICL) was determined according to the procedure described by Bogatek et al. (1989). The reaction mixture contained the following: 0.05 M phosphate buffer pH 7.6, 10 mM MgCl₂, 1 mM EDTA, 5 mM DTT, 3.3 mM phenylhydrazine hydrochloride, 4 mM, D-isocitric acid (Sigma, 49.4% from threo-D₅-isocitrate), and the enzyme extract. Enzyme activity was expressed in nmol of glyoxylate released within 1 hr per mg protein.

Proteins were determined according to the procedure described by Bradford (1976) with bovine serum albumin (BSA) as a standard.

Microcalorimetric Measurements Analyses of thermal energy (μW) released from seeds were conducted in an isothermal microcalorimeter (BioActivity Monitor 2277) at 20°C for 40 hr. Twenty ml ampoules equipped with lids that enabled air exchange were used. Five seeds were put into an ampoule, heated for 25 min, and placed into the measurement

chamber. Seeds were germinated for 40 hr on filter paper moistened with sunflower foliar extract. The reference treatment consisted of an ampoule where only filter paper was moistened with extract. Seeds germinating on filter paper moistened with water only were utilized as a control. Heat emission was recalculated per 0.1 g DW. Measurements were performed with five replications.

Microscopic Observations Small blocks of tissues from cotyledons, radicles, and basal parts of the hypocotyl were fixed according to the procedure described by Karnovsky (1965) after 0, 0.75, 1.5, and 2 d of imbibition in water or in aqueous extracts of sunflower foliage. Samples were also collected from seeds that had imbibed sunflower extract for a period of 8 d. After 24 hr of postfixation in 1% osmium tetroxide, samples were dehydrated through a graded ethanol series and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed with a JEOL 1200X electron microscope.

Data Analyses Data are presented as means \pm standard errors of the means (SEM). ANOVA with Duncan's multiple range test was used to evaluate the data. Statistical analysis was performed by means of the StatSoft Statistical program.

Results

Germination Almost all (96%) untreated (control) mustard seeds germinated within 4 d (Table 1), and their prolonged culture resulted in the formation of etiolated seedlings. Aqueous extracts of sunflower leaves resulted in nearly complete reduction in germination of mustard seeds. After 8 d of treatment, only 4% of the seed population germinated, but no visible growth was observed. They continued throughout the course of the experiment to remain at a stage comparable to control seeds after 18 hr. Therefore, seeds after 18 hr (0.75 d) of imbibition in water were regarded as a potential control for comparative purposes to treated seeds due to their similar morphological and physiological growth stage. Moreover, water uptake by mustard seeds during the first 18 hr of imbibition of sunflower extracts was not statistically different from seeds germinated in water (data not shown). The CEC of sunflower extract was observed to be -1.1 MPa.

To check the influence of water potential upon germination, we used a 28.5% (w/o) PEG solution characterized by equal $\Psi = -1.1$ MPa. Reduced water potentials resulted in delayed seed germination, but more than 50% of seeds that imbibed in PEG germinated within 8 d (Table 1). At the end of second week of culture, 75% of PEG-treated seeds germinated. Moreover, almost all of them (90%) remained viable, indicating that alterations in seed germination were more likely attributable to toxicity of sunflower extracts than to water stress. The low germination of seeds in the presence of sunflower extracts was not correlated with seed viability. Only a slight decrease in seed viability was detected during the first 5 d of incubation in sunflower extracts (Table 1). Treatment longer than 2 wk was lethal, and tetrazolium evaluation showed that all seeds were dead (Table 1).

Microscopic Observations There are three types of organelles containing storage materials in white mustard embryos: (1) protein bodies with small, angular-shaped globoids embedded in densely stained, amorphous matrix; (2) myrosin grains; and (3) spherical oil bodies, with well-defined boundaries, closely packed together but never coalescing.

Table 1 Germination percentage and viability of white mustard seeds incubated at 20°C in darkness in water (Control), in 10% (W/V) aqueous extract from sunflower leaves or in 28.5% PEG 8000 water solution^a

Days of culture	0.75	2	4	6	8	10	12	14
Water (control)	0.0±0.0 ^{a,b} (99.0±1.0) ^a	78.0±3.7 ^a (100.0±5.0) ^a	96.0±4.5 ^a (100.0±6.0) ^a	98.0±2.0 ^a (100.0±5.0) ^a	100.0±1.5 ^a (100.0±2.0) ^a	100.0±1.0 ^a (100.0±1.0) ^a	100.0±2.0 ^a (100.0±1.2) ^a	100.0±1.0 ^a (100.0±2.0) ^a
Water extract of sunflower leaves	0.0±0.0 ^a (95.0±3.0) ^a	0.0±0.0 ^b (92.0±3.4) ^a	0.0±0.0 ^b (84.0±6.5) ^a	2.0±0.2 ^b (70.0±4.8) ^b	3.0±0.2 ^b (64.0±5.5) ^b	3.0±0.5 ^b (38.0±6.0) ^b	4.0±0.5 ^b (22.0±4.0) ^b	4.0±0.4 ^b (2.0±0.2) ^b
PEG 8000 water solution	0.0±0.0 ^a (97.0±2.5) ^a	10.0±2.0 ^c (96.0±4.0) ^a	30.0±4.0 ^c (95.0±4.0) ^a	45.0±4.0 ^c (93.0±5.8) ^a	52 ^c (90.0±6.5) ^a	57.0±6.0 ^c (90.0±7.0) ^a	68.0±4.0 ^c (89.0±7.5) ^a	76.0±6.0 ^c (88.0±8.9) ^a

^a Data are means of four replicates. Numbers in parenthesis correspond to percentages of viable seeds.

^b Values in column not followed by the same letter are significantly different, $P=0.05$, ANOVA with Duncan's test.

Fig. 1 Morphology of storage materials prior to imbibition. Radicle cell filled with numerous oil bodies and large protein bodies (pb)

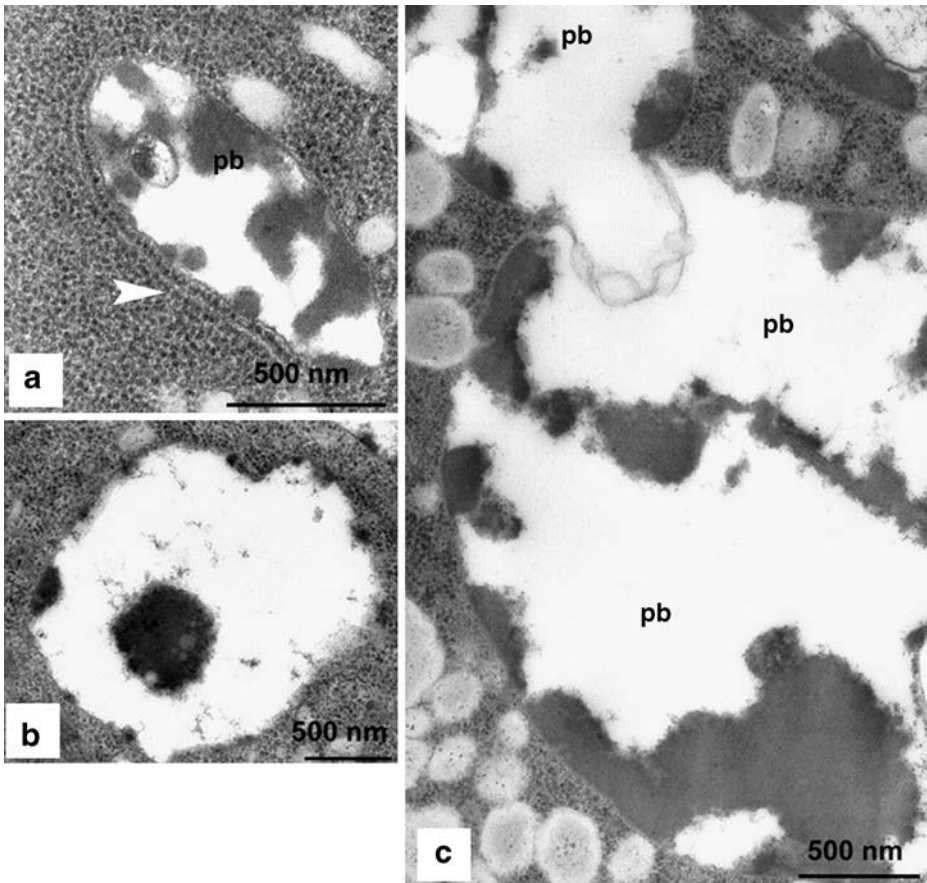
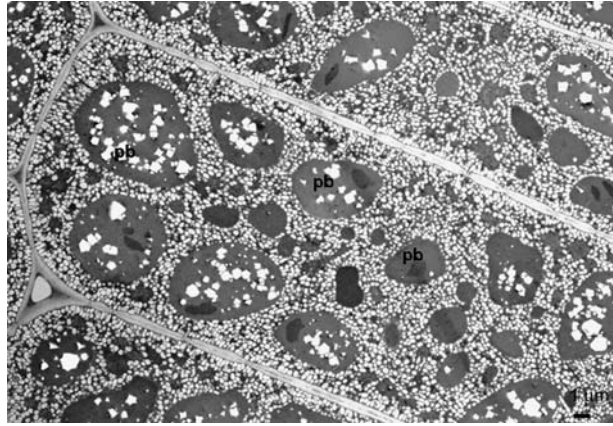


Fig. 2 (a, b) Control, 18 h incubation. After protein body (pb) digestion, arrowhead indicates cisterna of rough ER. (c) Control, 36 h incubation. Fusion of protein bodies (pb) during proteolysis

At the start of imbibition, only a few densely stained cells were observed, and indications were that cells were filled with storage materials, especially with oil bodies. Plastids and mitochondria were not distinguishable (Fig. 1). During the first 48 hr of incubation, protein and oil bodies remained undigested in the cotyledons. Mobilization of storage reserves was evident only in the axis of the embryo, both in the control and in treated seeds. Structural changes accompanying this process were visible first in the region of the root–hypocotyl boundary, and progressed acropetally towards both apices. Differences in degree of storage material digestion were also observed across the axis. Degradation of protein bodies was first initiated in the protoderm, then in the cortex, and finally in the stele.

After 18 hr of aqueous incubation, in the protoderm cells, protein bodies appeared to undergo structural changes during proteolysis. Irregular, light areas first appeared near the membrane, and then progressively increased toward the center of each protein body. The only densely stained, amorphous matrix lost its electron opacity. Remnants of storage proteins were later visible as clumps of fibrous material, which gradually disappeared. Finally, vacuoles developed as a result of proteolysis (Fig. 2). Autophagy was likely another means for vacuolar system development, which was manifested by circular cisterns of ER with sequestered cytoplasm, as the first step of autophagic vacuole formation (Fig. 4a). Protein bodies were not degraded in the embryos treated for 18 hr with the sunflower extracts. Numerous rod-shaped structures with tripartite profiles appeared near the oil bodies, and some of them were visible as connections between two oil bodies (Fig. 3a, b).

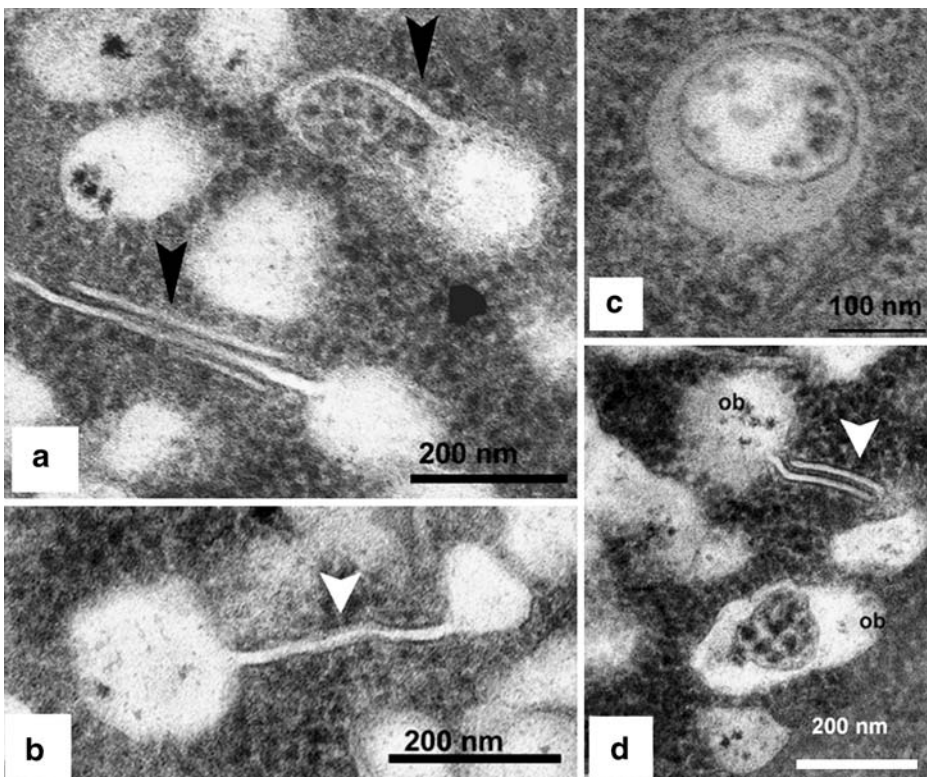


Fig. 3 (a, b, d) Sunflower allelopathic extract, (c) control, 36 h incubation. Partially depleted oil bodies (ob) so-called saccules; arrowheads indicate ring or rod-shaped membranous appendages

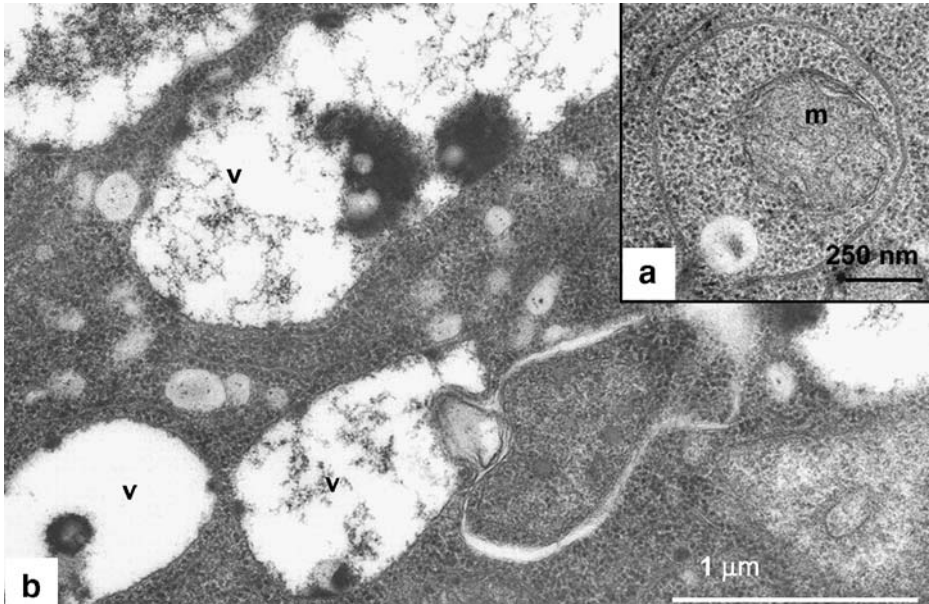


Fig. 4 (a) Control, 18 h incubation. Initial step of autophagic vacuole formation: part of cytoplasm with mitochondrion (m) and small vacuole sequestered by ER cistern. (b) Control, 48 h incubation. Large vacuoles (v) formed as the result of storage protein digestion

Morphology of mitochondria and increased amounts of rough ER cisterns, proplastids, and dictyosomes indicated an increase in metabolic activity of the cells in both the control and treated embryos during the first hours of imbibition.

As imbibition progressed, digested protein bodies also were visible in the cortex and stele cells of control embryos. Vacuoles formed from protein bodies increased in

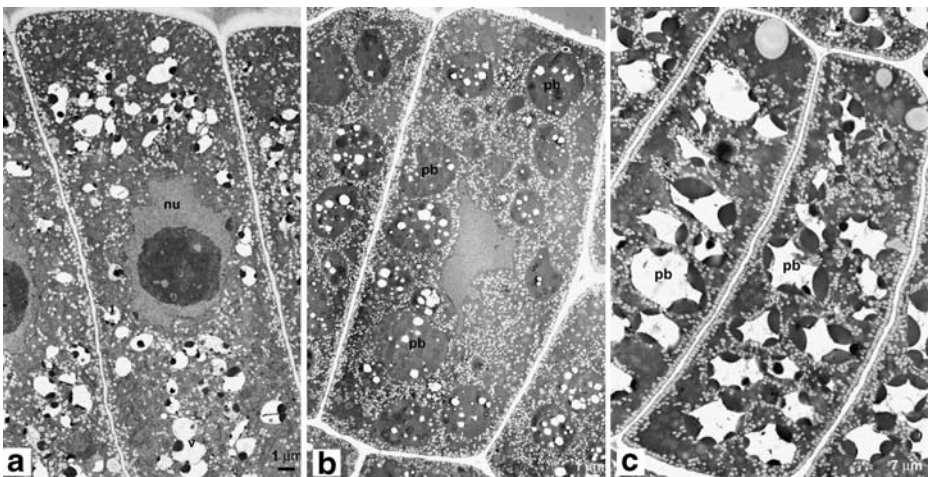
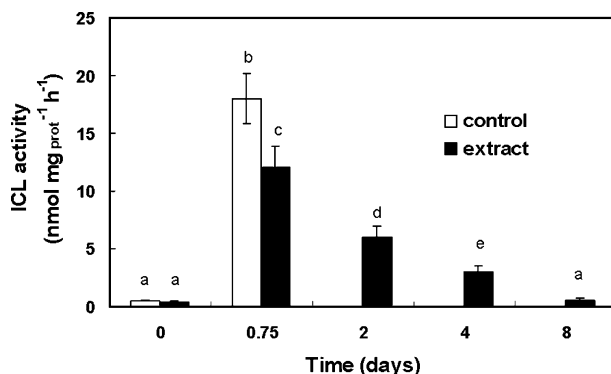


Fig. 5 (a) Control, 48 h incubation. Protoderm cell with vacuoles (v) formed by fusion of depleted protein bodies and apparently reduced numbers of oil bodies, localized along the cell walls. (b) Extract, 48 h incubation. Protoderm cell with undigested protein bodies and numerous oil bodies. (c) Sunflower extract, 8 d of incubation. Undigested protein bodies (pb) with lumps of dense material and numerous oil bodies

Fig. 6 Isocitrate lyase activity in mustard seeds germinating in water or extract of sunflower allelochemicals. Bars represent means \pm SD



numbers and fused to form large centralized vacuoles. These vacuoles were often connected with nearly initiated autophagic vacuoles (Fig. 4b). At the same time, various stages of partly depleted oil bodies were visible in the embryos of the controls as well as in treated seeds. Lipid body membranes formed interesting local invaginations leading finally to concave “sacculles”. In two dimension views, sacculles appear as ring-shaped structures (Fig. 3c, d).

Number of oil bodies, as well as connecting structures, significantly decreased in control embryos, and eventually in the protoderm cells later in all layers of the radicle (Fig. 5a). In embryos of seeds germinating in sunflower extracts, protein bodies remained unaltered and numerous. A considerable number of oil bodies were also visible in the cells of radicles and hypocotyls (Fig. 5b).

After 8 d imbibition of sunflower phytotoxins, seed coats of nearly all seeds remained unbroken, indicating complete inhibition of radicle growth. The protein bodies matrices were divided into dense lumps accumulating closely to surrounding membranes. Abnormally dense cytoplasm with weakly discernible organelles (Fig. 5c) indicated that embryos were significantly injured over time.

Isocitrate Lyase Activity Transformation of lipids to sugars, expressed as the activity of ICL, was markedly inhibited in seeds imbibing sunflower extracts. After 18 hr of germination in the presence of sunflower extracts, ICL activity in mustard seed was reduced twofold in comparison to the control. Activity decreased progressively during the experimental period, being almost undetectable after 8 d (Fig. 6).

Table 2 O₂ uptake by white mustard seeds incubated in water or aqueous extract from sunflower leaves^a

Imbibition medium	Duration of imbibition (days)	O ₂ uptake (μmol g ⁻¹ DW min ⁻¹)
–	0 (dry seeds)	0.09±0.02a ^b
Water (control)	0.75	0.47±0.04b
Water extract of sunflower leaves	0.75	0.27±0.05c
	2	0.25±0.09c
	4	0.29±0.09c
	8	0.24±0.03c

^a Data are means \pm SEM of five independent experiments.

^b Values in column not followed by the same letter are significantly different, $P=0.05$, ANOVA with Duncan's test.

Table 3 Adenylate (AMP, ADP, and ATP) concentration and energy charge (EC) in dry mustard seed and seeds imbibed in water or in 10% (w/v) aqueous extract from sunflower leaves^a

Imbibition medium	Duration of imbibition (days)	AMP (pmol mg ⁻¹ DW)	ADP (pmol mg ⁻¹ DW)	ATP (pmol mg ⁻¹ DW)	EC
–	0 (dry seed)	83.80±11.3a ^b	27.00±2.83a	8.20±1.17a	0.18±0.01a
Water (control)	0.75	55.33±9.46b	151.33±19.74b	398.34±21.64b	0.79±0.02b
Water extract of sunflower leaves	0.75	62.25±6.87b	135.25±5.07b	197.00±8.97c	0.67±0.02c
	2	56.33±1.25b	99.67±6.34b	165.00±9.63c	0.67±0.01c
	4	57.80±10.50b	132.80±14.68b	235.00±25.17c	0.71±0.02c
	8	82.00±6.48a	133.33±10.21b	228.67 ±24.24c	0.67±0.02c

^a Data are means of five measurements ± SEM.

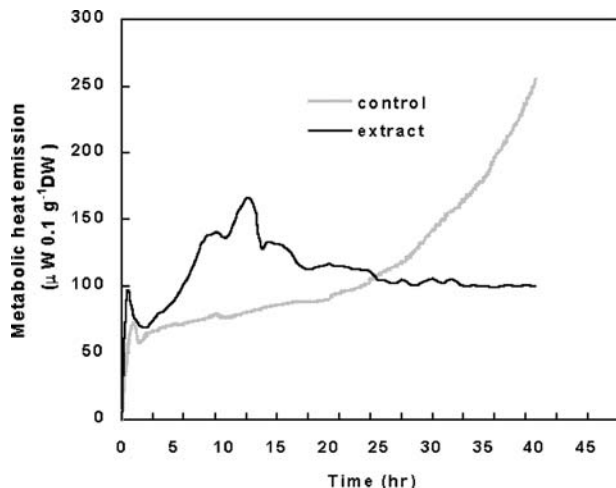
^b Values in column not followed by the same letter are significantly different, $P=0.05$, ANOVA with Duncan's test.

Gas Exchange and Energy Metabolism Oxygen uptake by intact dry seeds was low and increased during the first 18 hr of imbibition (Table 2). In treated seeds, oxygen uptake was 50% that of the control, and remained constant till the end of culture (Table 2).

Changes in ATP, ADP, and AMP concentration and the adenylate pool ($\Sigma = \text{ATP} + \text{ADP} + \text{AMP}$) during germination of mustard seeds are shown in Table 3. In dry seeds, ATP, ADP, and AMP concentrations were 8.2, 27.0, and 83.0 pmol mg⁻¹ DW, respectively, representing 7%, 23%, and 70% of the total adenylate pool. ATP concentration increased sharply during the first 18 hr of imbibition; however, in treated seeds, concentration was twofold lower than in the control, reaching 197 and 398 pmol mg⁻¹ DW, respectively, and remained about 200 pmol mg⁻¹ DW until the end of experiment. ADP concentration in both control and treated seeds increased similarly during the first 18 hr of germination, while a slight decrease in AMP concentration was detected (Table 3). As a consequence, the energy charge, which was low in dry seeds (0.18), sharply increased in seeds of both conditions of culture (Table 3). It remained about 0.67–0.70 during the 8 d of culture, although seed viability decreased (Table 1).

Metabolic Heat Production The heat efflux from seeds germinating in water was expressed as a continuous increase from 53 $\mu\text{W } 0.1 \text{ g}^{-1} \text{ DW}$ at the start to 250 $\mu\text{W } 0.1 \text{ g}^{-1} \text{ DW}$ at the

Fig. 7 Metabolic heat emission by white mustard seeds germinating in water or foliar extracts of sunflowers



40th hr of the culture (Fig. 7). Sunflower phytotoxins altered the typical heat emission pattern observed in control seeds. During first 12 hr, sharp increases in metabolic heat emission were detected, being twofold higher than in the control and reaching values of 165 and 80 $\mu\text{W } 0.1 \text{ g}^{-1} \text{ DW}$, respectively. After 12 hr, heat production decreased and stabilized at about 110 $\mu\text{W } 0.1 \text{ g}^{-1} \text{ DW}$, remaining constant until the end of experiment.

Discussion

One common effect of phytotoxic compounds is the inhibition of seed germination and the resulting abnormalities in seedling development. Standard germination processes consist of several key phases that include imbibition, catabolism, anabolism, and finally radicle protrusion. Therefore, radicle emergence is considered the culmination of the germination process and the starting point for seedling growth. Until this point, growth of the radicle is a result of cell expansion, which is a turgor-driven process (Cosgrove, 1997). It was demonstrated that mustard seed germination and viability were more depressed by sunflower foliar extracts than by the osmotic stress induced by treatment with PEG 8000 solution, characterized by equal CEC ($\Psi = -1.1 \text{ MPa}$) (Table 1). Although the germination process was delayed by low Ψ , almost all seeds remained viable. Thus, the lack of seed germination, observed in the presence of sunflower extracts, appeared to be a consequence of extract phytotoxicity rather than water availability.

During the catabolic phase, storage materials are hydrolyzed and provide substrates for biosynthetic processes and respiration. Phytotoxins produced by different plants are known to lower the total respiration rate in higher plants (Cruz-Ortega et al., 1988; Abraham et al., 2000). In our experiments, O_2 uptake increased sharply in control seeds, representing typical dynamics encountered during catabolic phases of germination, whereas in treated seeds, oxygen uptake was only 40% higher than in dry seeds. The biochemical reactions taking place in living cells are the source of heat emission. Heat is produced particularly during these processes of energy metabolism such as respiration. The microcalorimetric measurements of heat efflux from cells are useful in the estimation of their metabolic activity as well as in the analysis of response to stress conditions (Smith et al., 2000, 2001). The amount of heat emitted by germinating seeds reflects their viability and their reactions to stressors such as toxins or cold temperatures (Edelstein et al., 2001). In control seeds, heat emission was correlated with an increased respiration rate, whereas in seeds imbibing sunflower extracts this correlation was not detected. Maximum heat emission after 12 hr was neither accompanied by high oxygen uptake nor by any increase in ATP concentration. In our previous experiments, increased ROS (H_2O_2) production was detected in mustard seeds after 18 hr of imbibition of sunflower leaves extract (Bogatek et al., 2005). Therefore, we can not exclude the possibility that the transient increase in heat production may be a result of oxidative reactions taking place in seeds exposed to sunflower phytotoxins.

ATP is used in anabolic processes such as RNA and protein synthesis. In dry seeds, the adenylate pool is mainly composed of AMP and ADP (Bewley and Black, 1994). The increased respiration rate during early phases of germination generally results in increased ATP production. Earlier attempts to show a correlation between seed ATP content and germination have yielded conflicting results (Spoelstra et al., 2002), suggesting that the absolute level of ATP observed does not necessarily correlate well with germination. Moreover, ATP concentration is the result of both ATP synthesis and utilization, and depends on turnover of ATP pool. Monoterpenes and quinones present in sunflower extract may act as uncouplers of oxidative phosphorylation leading to a decrease in ATP synthesis. Moreover, inhibition of

cytochrome oxidase and the activation of an alternative respiration pathway has been induced in the past by other allelochemicals (Peñuelas et al., 1996; Abraham et al., 2000).

Reduced respiration rates in treated seeds and the likely inhibition of oxidative phosphorylation resulted in the observed reductions of the adenylate pools. In our experiments, although ATP concentration in seeds treated with sunflower extracts remained lower than in the control (Table 3), concentrations were sufficient to support germination, but perhaps not enough to maintain postgermination root growth. In addition, root growth in the postgermination phase is dependent on the supply of substrates for synthesis derived from reserve mobilization. Sugar content in mustard seeds was relatively low, and it appears that carbohydrates do not play a major role during mustard seed germination. Sucrose concentration in dry seed was also low (11 mg g⁻¹ DW) and decreased rapidly during the first 18 hr of germination to undetectable levels in both control and treated seeds (data not shown).

The initiation of protein mobilization is mediated by proteases, stored in the embryonic axes (Schlereth et al., 2001), so protein reserves are generally degraded early during the germination processes. An increase in total endopeptidase activity was observed in winter *Triticale* grains (Bielawski et al., 1994) and *Vigna mungo* (Hosokawa et al., 1999) seeds during 48–72 hr of germination. In seeds of Brassicaceae, breakdown of storage proteins starts earlier and proceeds faster than mobilization of storage oils. Digestion of proteins in *Brassica napus* is detectable after 24 hr, and is completed by 5 d after initiation. At this time, only 50% of the oil bodies appear broken (Murphy et al., 1989). Our results are consistent with these findings. A significant increase in total endopeptidase activity was detected after 48 hr of germination (data not shown), whereas activity of ICL increased 48 hr after this time. Digestion of protein storage material also was detected at an ultrastructural level. The first signs of proteolysis were visible in protein bodies after 18 hr of imbibition. Similar time courses of protein mobilization have been previously noted in germinating seeds of white mustard (Werker and Vaughan, 1974), where structural changes in protein bodies were visible between 12 and 36 hr. During undisturbed germination, hydrolysis of storage reserves, followed by accumulation of solutes, lowers the osmotic potential of the radicle cells, leading to a marked increase in water uptake and vacuolar volumetric growth (Bewley and Black, 1994). Inhibition of storage protein mobilization results in the lack of a vacuolar system, ensuring that the radicle cells remain in a state unable to support expansive growth.

Inhibition in storage lipid mobilization due to the presence of phytotoxins has been observed in germinating canola (*B. napus*) seeds (Baleroni et al., 2000). Although lipid content decreased slowly in seeds treated with ferulic and *p*-coumaric acids, it was always 50–80% higher than in control seeds. Baleroni et al. suggested that inhibition of seed germination and primary root elongation observed in the presence of these allelochemicals might be attributable to a reduction in lipid mobilization, probably caused by decreased activity of lipolytic enzymes and ICL. Both phenolic allelochemicals strongly inhibited radicle growth during germination of weed species (Reigosa et al., 1999). Data obtained by Maffei et al. (1999) suggest that benzoic acids affect not only ICL enzyme activity but also act at its transcriptional/translational level. The treatment of cucumber (*Cucumis sativus*) seedlings with 3,4-dimethoxybenzoic and vanillic acids resulted in lower ICL activity accompanied by the disappearance of ICL protein in seedlings extracts.

It has also been reported that the appearance of membranous so-called “sacculles” or “lipid-rich membranes” is typical in germinating seeds of Brassicaceae (Wanner and Theimer, 1978). They were originally thought to arise due to removal of the lipid contents faster than reduction of the surface area. Hence, the lipid body coat forms local invaginations, leading finally to collapsed rod-shaped structures with tripartite profiles (Huang, 1992). Localization of activity of alkaline lipase, the enzyme involved in TGA hydrolysis within these structures (Rosnitschek and Theimer, 1980; Lin and Huang, 1983) additionally confirmed their origin.

It has also been demonstrated that in developing *B. napus* seeds TAGs are synthesized within specialized subdomains of the ER and accumulated in the membrane's phospholipid bilayer. Then, nascent oil bodies are released by budding from terminal ends of tubular ER (Lacey and Hills, 1996). Similar models of biogenesis have been proposed for barley, supported by observation that oil bodies remain attached to the ER by long extensions of the outer leaflet of the ER membrane (Bethke et al., 1998). Considering that empty oleosomes "ghosts" were not observed after lipid mobilization, it seems that the rod-shaped structures were rather connections between oil bodies and ER than remnants of oil body coats. The fact that membranous extensions were visible in treated radicle cells after 8 d of extract exposure, when the activity of ICL was inhibited, confirms this interpretation. It seems likely therefore, that only cup-shaped oil bodies might be recognized as a result of partial digestion of storage oils. Both structural changes in oil bodies and initial activity of ICL indicate that breakdown of storage oils started in the embryos treated with sunflower phytotoxins. However, the total number of oil bodies did not decrease significantly, indicating that degradation of storage lipids was stopped. Similar results were described by Lewitt et al. (1984) with alkaloids from *Datura stramonium* in sunflower seedlings. Electron microscopy showed that treated cells contained greater amounts of lipid bodies than control cells.

Our data suggest that the inhibitory effect of phytotoxins in extracts of sunflower leaves on seed germination is mediated through the disruption of normal cellular metabolic processes rather than through direct organelle damage. Metabolism of seed storage reserves in germinating seeds is a process that usually takes place rapidly during early phases of germination. In the presence of sunflower aqueous extracts, metabolic processes are markedly reduced or even stopped in germinating seeds, providing evidence for the rapid impact of certain allelochemicals upon seed germination and ultimately the successful seedling growth of neighboring species.

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of both plants and insects, with the resulting induced plant volatiles and/or insect pheromones serving as important infochemicals (Turlings et al., 1990; Vet and Dicke, 1992; Landolt and Phillips, 1997; Reinecke et al., 2002). For example, some chrysomelid beetles are attracted to host plants that are infested or wounded by conspecifics (Bolter et al., 1997; Landolt et al., 1999; Kalberer et al., 2001; Tansey et al., 2005), and increase pheromone production when feeding on the host plant (Smyth and Hoffman, 2003). Once identified, semiochemicals can have useful pest management applications, such as improved monitoring and sampling of natural enemies (Burkholder and Ma, 1985; Howse 1998; Tinzaara et al., 2002).

The cottonwood leaf beetle, *Chrysomela scripta* (Fabricius) (Coleoptera: Chrysomelidae), is the most important arthropod pest affecting *Populus* plantations in North America (Coyle et al., 2005). Both larval and adult feeding can cause growth loss and destruction of leaders and shoots (Caldbeck et al., 1978; Bassman et al., 1982; Coyle et al., 2002). Current management strategies rely primarily on synthetic pesticides, largely because no simple and reliable sampling schemes are available. An ability to attract beetles to traps would greatly enhance development of sampling schemes for integrated pest management. To date, responses to volatiles have not been demonstrated for this insect. However, adult *C. scripta* have been observed to form feeding and mating aggregations on trees, suggesting the potential use of semiochemicals for location of conspecifics.

The first objective of this study was to identify the sources of attraction to *C. scripta*, with particular emphasis on whether these sources are likely to be a pheromone, host plant volatiles, induced plant volatiles, or a combination of herbivore and plant volatiles. The second objective was to evaluate the effectiveness of attractive odor sources in field trials.

Materials and Methods

Plants and Insects

Two-yr-old potted trees of clone DN34 (*Populus deltoides* × *nigra*) were obtained from KF Evergreens (Osseo, WI, USA). Clone NM6 (*P. nigra* × *maximowiczii*) was obtained as dormant cuttings from the USDA Forest Service in Rhinelander, WI, USA, and International Paper Company (Alexandria, MN, USA). Trees were maintained in a greenhouse (18L:6D photoperiod) with an average temperature of 20°C in the winter and 30°C in the summer, and were cultured for a period of 9 mo before being replaced by fresh material from the same sources. Plants were potted in commercial potting mix (Metro-Mix 300; Sungro, Bellvue, WA, USA), watered daily, and supplied with fertilizer via slow release pellets (Osmocote Plus; Scotts-Sierra Horticultural Products Company, Marysville, OH, USA). An additional aqueous fertilizer solution was delivered weekly at 300 ppm (Sunshine Technigro; Sungro, Bellvue, WA, USA). Foliage used for experiments and feeding was collected from actively growing branch terminals, washed in a mild dish-soap solution, rinsed with cold water, and placed in 22 -ml plastic water vials (#53–77; Syndicate Sales, Inc., Kokomo, IN, USA). Processed foliage was housed upright in an ice chest lined with a large plastic bag and stored in a walk-in cooler at 6°C.

A colony was established in the fall of 2004 from *C. scripta* collected in a hybrid poplar plantation near Carlos, MN, USA (45°N 59' 30"W, 95°W 06' 00"W). Beetles were housed in plastic boxes lined with a paper towel, and fed foliage from both DN34 and NM6 trees.

Foliage was supported above the box floor by metal screening. Beetle diet was occasionally supplemented with washed, field-collected foliage during the summer. Rearing boxes were housed in a growth chamber at 23°C and an 18L:6D photoperiod.

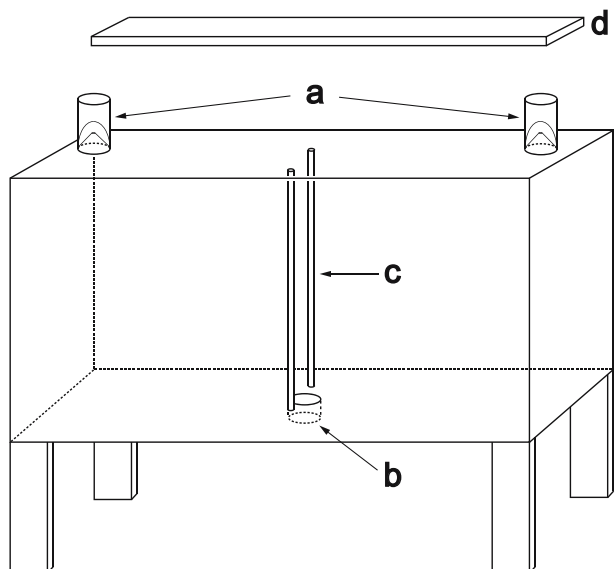
Bioassays

Behavioral choice tests were conducted in an olfactometer (122×62×62 cm) housed in a climate controlled room at approximately 23°C with no outside light (Fig. 1). The frame was constructed with wood, the floor was made of fiberboard, and the walls and ceiling consisted of fiberglass window screening. A release port consisting of a hole (7.6 cm diam) fitted with a wide-mouth jar ring was located in the center of the floor, allowing attachment of a 0.24 -l glass jar (Ball Corporation, Bloomfield, CO, USA). Two lucite plates (each 12.7 cm²) were mounted at either end of the ceiling, each having a 7.6 -cm diam. hole fitted with a wide-mouth jar ring. These two plates served as external attachment points for the 0.95 -l glass odor source jars (Ball Corporation), which were inverted above the ceiling.

The plates were roughened with a 40 -grit electric sander to facilitate beetle traction. Each odor source jar was fitted with a cone of aluminum window screening with an entrance hole (1 cm diam.) at the apex that allowed movement into the jar. A second aluminum screen prevented access to the odor source, and the space between the two screens functioned as a trap. Two PVC tubes (1.3 cm inner diam) eter) mounted on lucite bases (15 cm²) on each side of the release chamber facilitated climbing from floor to ceiling. Lighting consisted of two 34-W fluorescent tubes suspended 40 cm above the olfactometer. The ceiling of the olfactometer was hinged to provide access for cleaning.

For each replicate, twenty 1- to -4-wk-old beetles of mixed sex were placed in the release jar. Release and odor source jars containing living specimens contained a folded

Fig. 1 Olfactometer arena used for behavioral choice tests. The floor is solid; all other sides are screened. Insects are released from a central chamber in the floor and allowed to respond to odor sources in jars attached to the ceiling. Each odor source jar is fitted with a conical screen with a 1-cm hole serving as an insect entry. A second screen prevents access to the odor source and functions as a trap. Two vertically spanning posts focus insect activity in the center of the arena between the odor sources. (a) Odor source jars with trap screens, (b) release chamber, (c) beetle ladders, (d) light source



tissue (Kimwipe EX-L; Kimberly-Clark Corp., Roswell, GA, USA) to absorb extra moisture. Clone DN34 was used for all odor sources involving a plant, with sprigs trimmed as needed to standardize them to the meristematic tip through the first eight leaves, or through leaf plastochron index (LPI) 7 (Larson and Isebrands, 1971). Water-filled plastic vials preserved plants during experiments.

The location of odor sources was determined randomly. After 24 hr, non responding beetles were removed, and the response of trapped beetles was recorded. The arena was wiped down with ethanol and then hexane between trials. Odor source jars, release chamber jars, and screen traps were cleaned with a noninterfering cleansing solution (Powdered Precision Cleaner; Alconox, Inc., New York, NY, USA), air-dried, rinsed with ethanol, and then rinsed with hexane between trials. Each test consisted of 10 replicates unless otherwise noted. Insects and plants were used only once.

Test of Odor Source Components

In the first series of experiments, the full complement of odors emanating from plants, beetles, and the plant–beetle interaction was tested against a blank. After this, several putatively attractive components were tested vs. a blank to identify active components. The odor sources were prepared as follows.

- 1) Plant–*C. scripta* complex (beetles feeding on foliage): one sprig of foliage with 20 beetles of mixed sex. The blank control consisted of a water vial and a tissue.
- 2) Unwounded foliage: one sprig of foliage. The blank control consisted of a water vial and a tissue. $N=16$.
- 3) Beetles: 20 beetles of mixed sex. The blank control consisted of a tissue. All responding beetles were dissected to determine sex.
- 4) Frass: frass from 20 beetles that had been feeding on one sprig of foliage for 24 hr. Frass was collected from the wax paper lining of a rearing chamber using a moistened tissue. The blank control consisted of a moistened tissue that had been rubbed across clean wax paper.
- 5) Visual stimulus (green or yellow paper): two colors of laminated paper rectangles (8×14 cm) were tested. The blank control consisted of laminate with no paper inside.

Comparison of Attractiveness of Odor Source Components to the Full Complement of Odors

To determine if any single attractive component was as attractive as the full complement of odors from beetles feeding on foliage, we conducted direct comparisons between sources that proved attractive in the first series of assays. Each component was tested against the plant–*C. scripta* complex as described above and was prepared as follows.

- 1) Unwounded foliage: prepared as described above.
- 2) Beetles: prepared as described above with the addition of a water vial control.
- 3) Frass: prepared as described above with the addition of a water vial control and a tissue. A moistened tissue that had been rubbed over clean wax paper was added to the comparison odor source jar (plant–*C. scripta* complex) as a control.
- 4) Beetle-wounded foliage: one sprig of foliage was placed into a small plastic box and exposed to 20 beetles for 24 hr before being placed in the odor source jar.

Effect of Plant Wounding

To determine whether wounding must be done specifically by feeding, or whether mechanical wounds alter attraction, two comparisons were made to unwounded foliage.

- 1) Beetle-wounded foliage: prepared as described above. $N=11$.
- 2) Artificially wounded foliage: immediately preceding the choice test, one sprig of foliage was treated with a 6 -mm diam hole-punch to simulate a level of defoliation comparable to 20 beetles feeding for 24 hr (100 punches).
- 3) Washed-beetle-wounded foliage: foliage was prepared as for “beetle-wounded,” then hand-washed, and rinsed $\times 3$ in water. The unwounded comparison foliage was also hand-washed and rinsed $\times 3$ in water.

Comparison of Attractiveness of Volatiles Released from Starved vs. Fed Beetles

To discern whether frass plays a role in the attractiveness of volatiles from *C. scripta*, we made a direct comparison between the attractiveness of volatiles released from adults that had been starved for a period of 24 hr and volatiles from adults that had recently been fed.

Field Trapping

We tested the effects of two odors and two colors on trap catches of adult beetles in the field. This study was located in a heavily infested 28.1 -ha plantation in the location reported above. The planting was 4 yrs old and composed of clone NM6. A 1 -ha plot was established and 120 standard boll weevil traps (Nebeker et al., 2002) were deployed on a 10×12 grid with 10 m spacing. Traps were placed between rows on wooden posts at a height of 1 m from the ground.

There were two odor treatments: a blank control and three sprigs of NM6 foliage+20 beetles; and two color treatments: black and yellow. Bait treatments were prepared using washed greenhouse-grown NM6 foliage trimmed to include the meristematic tip through the first five leaves (LPI 4) and placed in plastic water vials as described above. The sprigs were then placed in a 12 -oz paper coffee cup with 20 beetles collected from a hybrid poplar nursery near Arlington, WI, USA ($43^{\circ} 17' 30''\text{N}$, $89^{\circ} 23' 00''\text{W}$) and then covered with mesh netting held in place with a rubber band. Controls consisted of a paper coffee cup containing three water vials covered with mesh netting. Bait cups were clipped to the inside of boll weevil traps using 0.95-cm metal binder clips.

Color treatments were prepared by spray painting traps with yellow or black plastic-bonding spray paint (Krylon Fusion Sunbeam and Black Gloss; Sherwin-Williams Co., Cleveland, OH, USA). The treatments were deployed in a completely randomized, 2×2 two factorial design. Due to loss of data from the first four rows of traps, the final replication is as follows: black control, $N=16$; black baited, $N=26$; yellow control, $N=18$; yellow baited, $N=20$. Trap catches were tallied and removed daily between 6 and 7 P.M. for the duration of the 4-d trial to prevent the build-up of potentially attractive adult *C. scripta* within traps.

Statistics

Statistical analyses were performed using SAS for Unix (v.8, SAS Institute Inc., Cary, NC, USA). Beetle responses to the two different odor sources were subjected to an ANOVA

with beetle response in a given trial as the experimental unit. ANOVA was chosen for the analysis instead of chi square in order to allow for a more conservative approach that included blocking factors of odor source location, arena used, and trial period. The sex ratio of beetles responding to beetle volatiles was subjected to chi square analysis to determine if it deviated from the expected 1:1 ratio.

Statistical analysis of trap data was performed using SAS software as described above. Trap catch totals were $\log(y+1)$ transformed and subjected to ANOVA for color, bait, and interaction effects. The least significant difference (LSD) method was used to determine mean separations between the four treatment combinations, with $\alpha=0.01$ to insure a conservative interpretation.

Results

Laboratory Assays

None of the blocking factors of odor source location, arena used, or trial period were significant except in one instance noted below. Odors released from the plant–*C. scripta* complex ($F_{1,8}=34.85$, $P<0.001$) as well as those from unwounded foliage ($F_{1,21}=10.59$, $P=0.004$), beetles ($F_{1,12}=47.15$, $P<0.001$), and frass ($F_{1,12}=9.91$, $P=0.008$), were more attractive than a blank control (Fig. 2). Responses to green ($F_{1,12}=2.69$, $P=0.127$) or yellow ($F_{1,12}=1.44$, $P=0.253$) visual stimuli did not differ from those to a blank. The sex ratio of responders to volatiles from beetles alone was 32:35 (M/F) and was not statistically different from 1:1 ($\chi^2_1, P=0.8$).

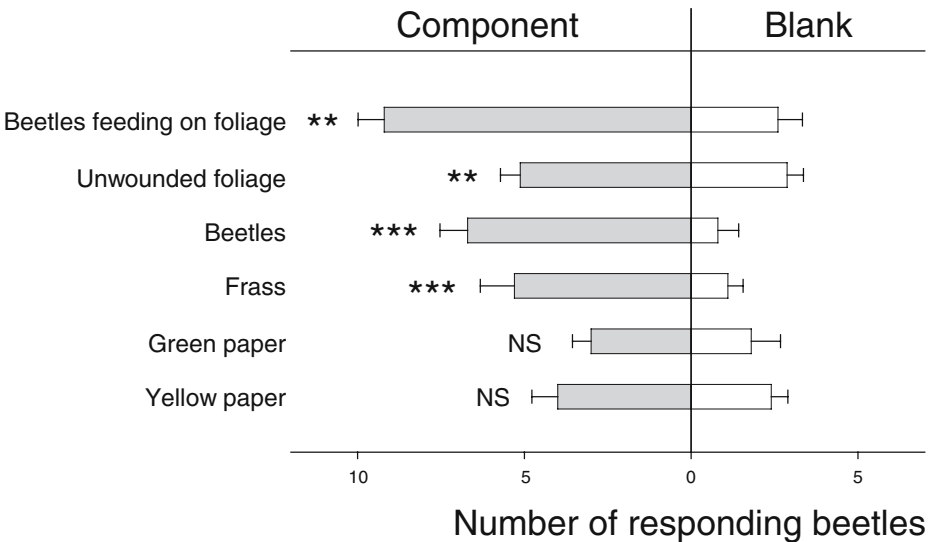


Fig. 2 Number of adult *C. scripta* responding to potentially attractive odor sources and visual cues vs. blank controls in two-way behavioral choice tests. Beetles feeding on foliage = plant–*C. scripta* complex; Unwounded foliage = plant alone; Beetles = *C. scripta* alone; Frass = frass alone; Green paper = green visual cue; Yellow paper = yellow visual cue; NS = not significant. An asterisk indicates significant attraction to a given odor source (* $P<0.05$, ** $P<0.01$, *** $P<0.001$)

In direct comparisons, only volatiles from beetle-wounded foliage were as attractive as the full complement of odor from the plant–*C. scripta* complex ($F_{1,12}=1.62, P=0.228$) (Fig. 3). Although attractive when compared to a blank, the odor sources of unwounded foliage ($F_{1,11}=17.79, P=0.001$), beetles ($F_{1,12}=122.93, P<0.001$), and frass ($F_{1,12}=7.11, P=0.020$) were not as attractive as the plant–*C. scripta* complex in a direct comparison.

Beetle-wounded foliage was more attractive than unwounded foliage ($F_{1,13}=13.97, P=0.002$) (Fig. 4). In contrast, attraction to artificially wounded foliage was not different from attraction to unwounded foliage ($F_{1,12}=0.01, P=0.922$). Even with frass removed by washing, beetle-wounded foliage remained more attractive than unwounded foliage ($F_{1,18}=8.17, P=0.010$). In this comparison only, the blocking factor of odor source location was also significant ($F_{1,18}=6.44, P=0.021$).

Attraction to adult beetles appears to exist independently of frass, with no difference in response to starved vs. fed beetles ($F_{1,12}=0.02, P<0.891$).

Field Trapping

Both color ($F_{1,76}=9.00, P=0.004$) and bait ($F_{1,76}=23.47, P<0.001$) affected trap catch (Fig. 5). There was no significant interaction between color and bait ($F_{1,76}=0.63, P=0.429$). Baited yellow traps caught more than any other trap. Unbaited yellow trap catches did not differ from either of the black treatments, but among black traps, baited traps caught more beetles than unbaited ones (LSD, $\alpha=0.01$).

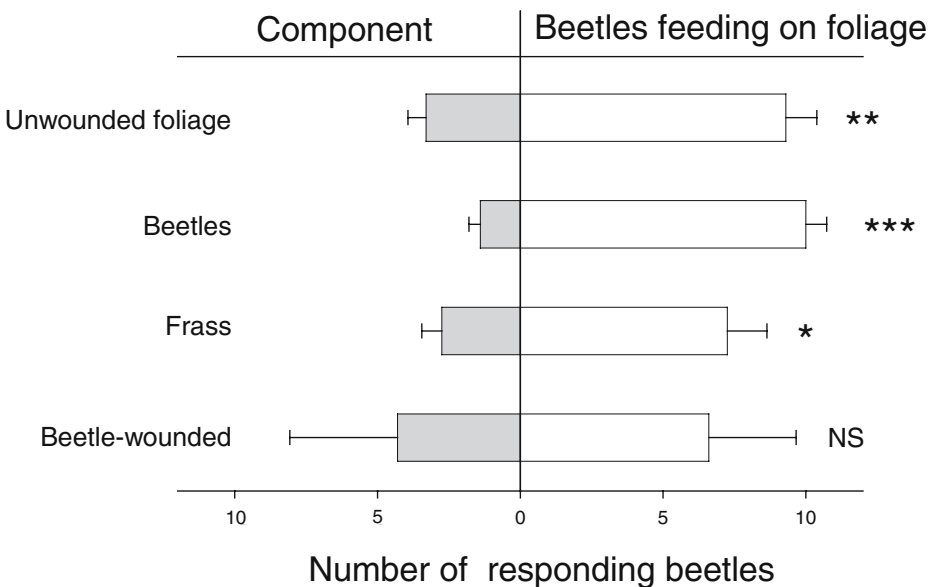


Fig. 3 Number of adult *C. scripta* responding to attractive odor source components when compared to the full complement of odors. Unwounded foliage = plant alone; Beetles = *C. scripta* alone; Frass = frass alone; Beetle-wounded = wounded plant with *C. scripta* by-products; Beetles feeding on foliage = plant–*C. scripta* complex; NS = not significant. An asterisk indicates significant attraction to a given odor source (* $P<0.05$, ** $P<0.01$, *** $P<0.001$)

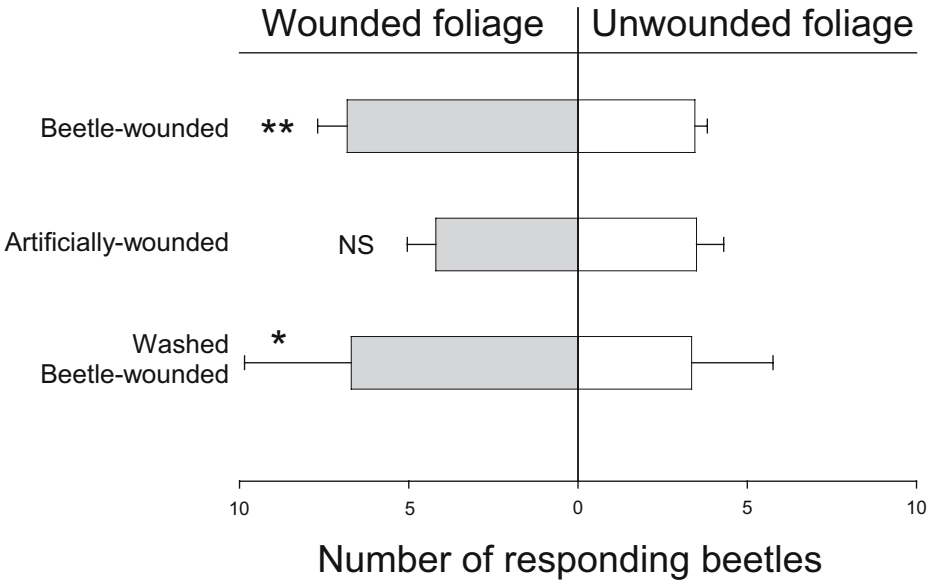


Fig. 4 Effect of wounding by beetle feeding and artificial means on attraction of *C. scripta* to foliage. Beetle-wounded = wounded plant with *C. scripta* by-products; artificially wounded = hole-punched plant; unwounded foliage = plant alone. Washed beetle-wounded = wounded plant with visible *C. scripta* by-products removed; NS = not significant. An asterisk indicates significant attraction to a given odor source (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

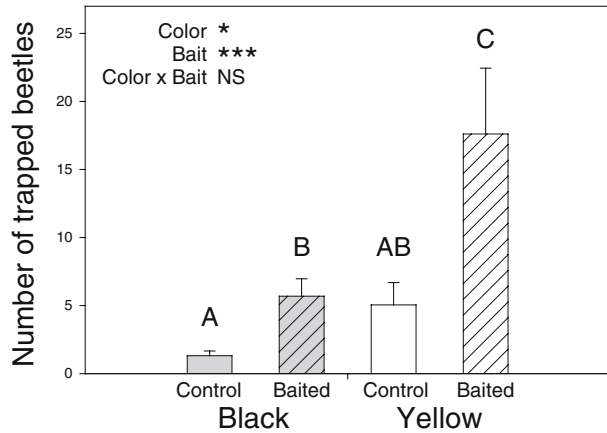
Discussion

These results suggest that aggregation by *C. scripta* is mediated by the presence of beetles and their feeding on host plants. Similar results have been found for other chrysomelid beetles, such as the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), and two flea beetles, *Phyllotreta cruciferae* (Goeze) and *Aphthona nigricutis* (Foudras), each of which are attracted to volatiles from feeding conspecifics (Peng and Weiss, 1992; Landolt et al., 1999; Tansey et al., 2005). Moreover, attraction by other beetle species to wound-induced foliage has been demonstrated (Loughrin et al., 1995; Bolter et al., 1997; Kalberer et al., 2001).

The data indicate that both *C. scripta* and their host plants, especially wounded plants, emit attractive signals. The behavioral responses of *C. scripta* are consistent with the presence of a pheromone for two reasons: 1) beetles are attracted either to conspecifics that have recently fed or to conspecifics that had been starved for 24 hr; and 2) frass alone is attractive (Fig. 2). The release of pheromones through frass is common in other Coleoptera (Wood 1982). One or both sexes may produce a pheromone, and examples of each are known in the Coleoptera (Landolt and Phillips, 1997). In this study, the odor source beetles were of both sexes, so it is not yet known if the attractive compound functions primarily as a sex pheromone. However, both sexes respond equally to odors emitted by conspecifics.

Although beetle-produced volatiles are attractive by themselves, the host plant, particularly a wounded host plant, is critical in eliciting the strongest response by *C. scripta*. Because wounding of the plant by *C. scripta* but not by artificial means increases the attractiveness of the foliage, and beetle-wounded foliage remains more attractive than unwounded foliage even after removal of insect by-products (Fig. 4), our results suggest

Fig. 5 Total number of adult *C. scripta* trapped in response to various odor and visual stimuli. Standard boll weevil traps were deployed for 4 d and assigned one of two color treatments: black or yellow; and one of two odor treatments: control or baited. Sampling was for 4 d. Letters A–C above the bars indicate significant differences in trap catches between treatments, with means sharing the same letter not significantly different (LSD, $\alpha=0.01$). An asterisk indicates a significant treatment effect (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). NS = not significant



wound induction plays an important role in attraction. This is consistent with reports on the more widely-studied *L. decemlineata* (Landolt et al., 1999). Attraction to beetle-wounded foliage is not mutually exclusive with the presence of a pheromone, as pheromone production may be stimulated by feeding or require the co-occurrence of host volatiles to elicit a full response (Wood 1982; Landolt and Philips, 1997; Reinecke et al., 2002). Although we have demonstrated that beetle-wounded foliage from which frass has been removed retains its attractiveness when compared to unwounded foliage, we did not directly compare this treatment with beetles feeding on foliage. Thus, it remains to be determined whether the attractiveness of beetles feeding on plants is due solely to the emission of damage-induced volatiles, or also includes compounds of beetle origin.

Field evaluations confirmed attraction to volatiles from beetles feeding on foliage. Although laboratory assays did not provide evidence for attraction to color alone, visual cues influenced beetle responses to attractive volatiles in the field. Color-based differences in trap catches have been previously documented for other chrysomelid adults, such as *L. decemlineata* and two *Diabrotica* species (Zehnder and Speese, 1987; Hesler and Sutter, 1993). The order in which *C. scripta* responds to cues is not known, but based on these results, it seems likely that initial landings are based on visual (Fig. 5, A and AB) and olfactory (Fig. 2, Unwounded foliage) attraction to the host plant, with volatile cues from conspecifics subsequently increasing arrestment by *C. scripta* in a particular location (Figs. 3, 4, and 5).

With more knowledge of the chemical ecology of *C. scripta*, efficient sampling schemes could be developed for improved monitoring. A close correlation between trap catch and population density would aid plantation managers in making threshold-based pesticide applications, which are the cornerstone of integrated pest management. In summary, our results demonstrate orientation by *C. scripta* adults to volatile cues, and indicate that these interactions are of sufficient strength to function in commercial plantations.

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Introduction

Bark residue from tree debarking is a byproduct of wood production. In Quebec, Canada, about 1.2 million tons of residues are produced each year (Anonymous, 2000) and left to degrade for decades. In northern Quebec, away from potential users, approximately 10% of bark residues are piled up as landfill in industrial bark wastelands. Bark contains many chemical constituents in varying concentrations (USDA, 1971); most residues are rich in cellulose and lignin, but also contain waxes, cutin, suberin, resins, and tannins. They have a low pH, and hydrolyzable phenolics are often released during degradation from the C6–C3 phenylpropanoid acid subunit of lignin (USDA, 1971), cutin, suberin, and tannins (Marchand et al., 2005).

Bark residue wastelands have increased in volume (i.e., to a height of several meters spread over several ha) and are aesthetically unpleasant. There is limited research on how to revegetate this industrial wasteland. Some residue sites have been covered with mineral soil and seeded; this has had some success, but in general it is unsustainable and not environmentally sound because it requires tons of soil to be transported.

The characteristics of industrial bark residue change over time with environmental exposure. Fresh bark wastelands are light brown and woody without vegetation. This inhibitory effect on the surrounding vegetation is identified as allelopathic and patterning vegetation effects have been noted (Rice, 1984). The oldest wastelands resemble black soil, and rhizobium–legume (*Vicia cracca* and *Trifolium* spp.) symbioses have been found growing on them. This phenomenon raises the possibility of a new organic soil cover being developed with bark residues so that these sites can be revegetated by legume species. To explore the potential for this approach, we needed to identify the soluble phytotoxic compounds from barks of various ages and to evaluate their potential toxicity.

Phenolic compounds are the most widely distributed allelopathic compounds, followed by terpenoids, flavonoids, alkaloids, and cyanogenic glycosides (Rice, 1984). One hypothesis for the absence of plant growth on fresh bark is the presence of phenolics that are soluble and known to affect biodiversity and the growth, development, and nutrition of plant species (Rice, 1984; Siqueira et al., 1991). Water extracts from bark have been reported to inhibit plant growth mainly due to their phenolic compounds (Rice, 1984).

The objectives of this study were to: (1) identify and quantify the phenolic compounds from bark of different ages; and (2) determine which barks could be used to develop a novel organic soil cover. We used simulated solutions (mixtures of standard phenolic compounds) to evaluate the potential toxic effects of phenolic compounds on rhizobial growth, the germination index, plant growth, nodule number, and the nitrogen fixation activity of two legume species under laboratory conditions.

Methods and Materials

Bark Materials

White spruce bark, provided by Tembec Inc. (Taschereau, QC, Canada), was collected from an 18-ha, 10-m high bark wasteland that was established in 1950. Samples were taken from

Table 1 Selected physicochemical characteristics of bark residues of five ages

Bark age (years)	Moisture (%)	Bulk density (gm/cm ³)		pH
		Fresh	Dry	
Fresh	60.2	0.33	0.12	4.1
<1	59.8	0.42	0.17	4.7
1–5	70.1	0.37	0.16	4.5
5–10	70.1	0.51	0.22	5.0
10–20	67.0	0.48	0.24	5.5

several locations within the top 30 cm of the pile. Bark was divided into five categories based on age: fresh bark coming directly from the industry, <1-yr-old bark, light brown 1- to 5-yr-old bark, brown 5- to 10-yr-old bark, and black 10- to 20-yr-old bark. Bark from these five categories had different physicochemical characteristics (Table 1). After collection, barks were transported on ice to the laboratory and analyzed as soon as possible.

Phenolic Compounds Extraction

Four extractant solutions were used: cold water (Whitehead et al., 1983; Blum et al., 1991), considered a weak extractant; hot water at 85°C (Chantigny et al., 2000) and 0.1 M NaOH (Whitehead et al., 1981), both considered to be mild extractants; and 2 M NaOH (Whitehead et al., 1983), considered to be a strong extractant. Unless stated otherwise, extractions were performed at room temperature. Each treatment combination (bark residue and extractant solution) was replicated $\times 3$.

The extraction method was a modification of Whitehead et al. (1981). Briefly, for the cold water, 0.1 M NaOH, and 2 M NaOH solutions, 20 g of bark (wet weight; w.w.) were added to 100 ml of extractant solution and agitated for 16 hr with a reciprocal shaker (Eberbach Corp., Ann Arbor, MI, USA). The hot water solution was agitated in a water bath set at 85°C. Each suspension was centrifuged at $23,435 \times g$ for 15 min (rotor Sorvall GSA, Sorvall RC-5B centrifuge). Supernatants were recovered and filtered through 5 layers of cheesecloth and then through filter paper (Whatman no. 1). Filtrates were adjusted to pH 2.5 by using HCl 1 N and maintained at 4°C overnight. The acidification of bark extracts resulted in the precipitation of humic acids (Charest et al., 2005) that were separated by centrifugation ($23,435 \times g$) and filtrated through Whatman no. 42 filter paper. Supernatants were washed $\times 3$ with 10 ml ethyl acetate. The resulting 30-ml pooled solution was evaporated to dryness under N₂ atmosphere at 40°C, and the solids were dissolved in 2 ml of 50% methanol and kept in darkness at -4°C before undergoing chromatography.

Table 2 Mobile-phase gradient program used to separate phenolic compounds in bark extracts^a

Time (min)	Mobile phase A (%)	Mobile phase B (%)
Initial	100	0
0	100	0
0.01	100	0
15	70	30
50	22	78
60	100	0

^a Flow rate was 0.7 mL/min. All changes were asymptotically done over time (small initial change followed by more rapid change) by using curve shape 5 except for the change from 0.01 to 15 min, which was asymptotically changed by using curve shape 7. (Peaknet software release 4.30 Dionex, Sunnyvale, Ca, USA)

Table 3 Phenolic compounds^a of bark residues of various ages extracted with different solvents

Phenolic compounds/extractant	Bark age (years)				
	Fresh	<1	1–5	5–10	10–20
Galic [µg/g (dry weight)]					
Cold water	1.56±0.04 ^b	ND ^c	0.09±0.16	ND	ND
Hot water	13.14±1.74	4.93±4.74	19.08±4.76	4.9±3.88	1.23±2.12
0.1 N NaOH	ND	0.23±0.4	ND	ND	ND
2 N NaOH	9.76±10.39	4.47±2.53	0.69±1.2	ND	ND
Protocatechuic [µg/g (dry weight)]					
Cold water	10.76±2.02	1.74±1.57	ND	ND	ND
Hot water	108.1±17.43	59.51±68.87	105.27±12.62	62.61±26.37	63.67±1.73
0.1 N NaOH	23.51±14.27	35.53±17.49	5.33±4.01	4.46±1.930	22.76±13.53
2 N NaOH	199.91±50.43	195.66±31.64	171.26±55.78	38.89±46.73	144.84±109.55
Catechol [µg/g (dry weight)]					
Cold water	60.46±14.29	ND	ND	ND	ND
Hot water	81.65±73.67	27.07±46.89	18.19±22.41	52.03±29.81	36.76±31.84
0.1 N NaOH	53.56±4.2	77.77±15.01	41.91±7.21	45.23±5.50	30.16±10.63
2 N NaOH	145.79±27.64	276.69±16.95	395.31±100.64	241.96±56.99	209.58±92.21
(<i>p</i>)-Hydroxybenzoic [µg/g (dry weight)]					
Cold water	12.19±2.79	0.23±0.20	ND	ND	0.01±0.01
Hot water	24.93±6.40	3.11±1.67	4.06±0.35	3.41±1.24	4.85±0.23
0.1 N NaOH	23.61±2.59	7.87±2.28	12.48±4.85	15.56±2.46	16.58±1.35
2 N NaOH	34.21±3.73	21.32±0.64	23.59±2.30	28.01±8.24	47.26±13.03
Vanillic [µg/g (dry weight)]					
Cold water	15.71±2.19	1.25±1.51	ND	0.05±0.08	ND
Hot water	27.78±2.12	9.46±8.79	18.94±7.2	13.17±7.94	22.73±3.02
0.1 N NaOH	31.31±1.88	15.1±1.77	23.28±5.94	24.69±2.95	43.88±3.95
2 N NaOH	57.3±2.11	41.69±8.45	66.51±7.13	70.65±48.17	187.31±67.37
Caffeic [µg/g (dry weight)]					
Cold water	12.18±1.70	ND	ND	ND	ND
Hot water	18.65±1.50	5.18±4.14	4.41±3.83	4.77±2.76	1.11±0.79

0.1 N NaOH	7.39±0.81	6.39±1.56	10.98±3.81	16.62±2.66	26.27±29.08
2 N NaOH	15.38±2.78	29.32±11.38	50.48±1.66	50.66±27.97	50.96±10.15
Vanillin [$\mu\text{g/g}$ (dry weight)]					
Cold water	131.95±9.04	3.95±4.98	1.11±1.93	0.36±0.63	1.13±1.95
Hot water	97.92±1.50	7.79±4.19	10.75±2.86	6.08±1.87	3.75±0.4
0.1 N NaOH	39.53±8.30	15.51±2.34	21.63±6.15	36.62±6.13	17.92±10.45
2 N NaOH	83.31±22.80	57.52±4.92	94.84±14.64	115.83±21.32	105.48±11.93
(<i>P</i>)-Coumaric [$\mu\text{g/g}$ (dry weight)]					
Cold water	52.15±0.69	3.84±5.13	ND	ND	ND
Hot water	39.23±19.69	9.66±11.18	4.72±3.25	4.02±2.17	3.69±3.34
0.1 N NaOH	80.10±66.72	15.32±11.02	36.98±18.35	10.26±3.9	7.62±0.45
2 N NaOH	96.14±31.50	241.64±286.17	78.48±14.41	82.17±52.47	74.44±35.9
Ferulic [$\mu\text{g/g}$ (dry weight)]					
Cold water	76.69±18.48	3.19±4.97	ND	ND	ND
Hot water	113.38±17.48	8.36±6.91	3.48±0.25	5.01±4.14	0.24±0.21
0.1 N NaOH	90.51±35.41	8.01±0.83	6.1±2.28	5.24±0.64	2.56±0.22
2 N NaOH	349.43±65.67	188.58±77.76	231.67±74.29	173.62±35.23	78.32±18.54
3-Hydroxy-4-methoxycinnamic [$\mu\text{g/g}$ (dry weight)]					
Cold water	10.17±1.85	2.37±3.78	ND	ND	ND
Hot water	12.64±8.68	1.87±2.54	1.03±0.68	0.84±0.72	ND
0.1 N NaOH	13.75±22.13	1.39±1.42	0.71±0.47	0.54±0.07	0.14±0.25
2 N NaOH	46.21±28.02	2.12±3.04	8.37±2.09	6.69±2.23	0.15±0.26
Phenylacetic [$\mu\text{g/g}$ (dry weight)]					
Cold water	383.72±115.35	5.87±10.17	ND	ND	ND
Hot water	277.38±53.25	61.96±63.17	43.33±5.41	4.82±5.41	3.26±2.84
0.1 N NaOH	428.98±214.95	45.26±60.17	27.79±21	14.25±3.61	ND
2 N NaOH	233.84±127.12	125.91±112.6	97.44±30.05	27.33±47.34	21.94±38.01
Benzoic [$\mu\text{g/g}$ (dry weight)]					
Cold water	3.33±1.77	0.27±0.46	2.31±4.00	6.24±10.80	4.02±6.96
Hot water	10.8±11.5	0.3±0.53	0.37±0.64	ND	ND
0.1 N NaOH	13.26±11.71	ND	ND	ND	ND
2 N NaOH	ND	ND	ND	3.75±6.49	20.81±13.03

Table 3 (continued)

Phenolic compounds/extractant	Bark age (years)				
	Fresh	<1	1–5	5–10	10–20
Salicylic [$\mu\text{g/g}$ (dry weight)]					
Cold water	19.21 \pm 19.3	1.26 \pm 1.10	0.66 \pm 1.14	0.83 \pm 1.43	0.95 \pm 0.95
Hot water	ND	3.05 \pm 5.29	3.03 \pm 1.63	3.89 \pm 5.21	4.14 \pm 4.71
0.1 N NaOH	ND	ND	ND	ND	ND
2 N NaOH	ND	19.16 \pm 33.18	6.87 \pm 11.89	ND	ND
<i>trans</i> -Cinnamic [$\mu\text{g/g}$ (dry weight)]					
Cold water	0.82 \pm 0.68	0.07 \pm 0.13	ND	ND	0.08 \pm 0.14
Hot water	2.69 \pm 1.28	0.14 \pm 0.12	0.29 \pm 0.27	0.04 \pm 0.08	ND
0.1 N NaOH	12.51 \pm 2.11	0.43 \pm 0.46	2.59 \pm 1	2.13 \pm 0.38	1.52 \pm 1.19
2 N NaOH	7.32 \pm 1.32	5.85 \pm 3.35	5.61 \pm 1.46	4.38 \pm 0.5	4.99 \pm 2.72
Total [$\mu\text{g/g}$ (dry weight)]					
Cold water	788.71 \pm 109.12	24.00 \pm 18.90	4.18 \pm 5.30	7.47 \pm 10.00	6.182 \pm 5.70
Hot water	828.29 \pm 139.34	202.39 \pm 122.00	236.95 \pm 26.30	165.59 \pm 64.80	145.43 \pm 38.00
0.1 N NaOH	818.02 \pm 218.37	228.8 \pm 59.75	189.77 \pm 58.60	175.59 \pm 21.47	169.41 \pm 31.85
2 N NaOH	1278.6 \pm 154.07	1209.92 \pm 99.40	1231.09 \pm 274.44	843.92 \pm 161.2	946.07 \pm 352.78

^a The order of the phenolic compounds in this table followed their order of elution

^b Mean of three replicates \pm standard deviation

^c ND = Not detected

Liquid Chromatography

Methanol-dissolved samples were filtered through 0.45- μm nylon filters (Supelco, Bellefonte, PA, USA). Phenolics were identified by injecting a 10- μl aliquot into a Dionex DX 500 chromatograph (Dionex Corp., Sunnyvale, CA, USA) equipped with an AS40 Automated Sampler and a 10- μl valve loop injector, a Dionex GP40 Gradient Pump, and an AD20 absorbance detector. Chromatography was performed with a DuPont Zorbax ODS (4.6 mm \times 25 cm) column and a Zorbax guard column. Peaknet software release 4.30 (Dionex) was used for data acquisition and processing.

Chromatographic Conditions

The eluting solvents used were as follows: mobile phase A, water–methanol–acetic acid–ethyl acetate (93+2+4+1, v/v/v/v); mobile phase B, water–methanol–acetic acid–ethyl acetate (35+60+4+1, v/v/v/v) with a variable gradient at a flow rate of 0.7 ml/min (Table 2; adapted from Kelley et al., 1994). Phenolic compounds were characterized by their UV absorbance at 254 nm. Peaks were identified by comparing retention times with pure standards.

Standards

Preliminary analyses by gas chromatography-mass spectrometry identified 14 phenolic compounds in the bark residue samples. These compounds were gallic acid, protocatechuic acid, catechol, caffeic acid, vanillic acid, vanillin, (*P*)-coumaric acid, (*P*)-hydroxy-benzoic acid, ferulic acid, 3-hydroxy-4-metoxycinnamic acid, (*O*)-hydroxy-phenylacetic acid, benzoic acid, salicylic acid, and *trans*-cinnamic acid. Samples were obtained from Sigma (St. Louis, MO, USA). Standards were of a high-purity grade. Each was dissolved in high-performance liquid chromatography (HPLC)-grade methanol/deionized water (1:1 v/v) and injected, without prepurification, alone or in mixture, to calibrate the chromatograph.

Quantification was based on peak areas as determined by Peaknet software release 4.30 (Dionex) using external standards. These values (in $\mu\text{g/g}$) were transformed by using the following formula: Value ($\mu\text{mol/l}$)=(Value ($\mu\text{g/g}$) \times Bark dry bulk density (g/cm^3) $\times 10^3$)/Molecular weight.

The mean and standard deviation of each phenolic compound was determined (Table 3). The total phenolic content represents the sum of the mean quantities of the phenolic compounds detected.

Solutions for Toxicity Tests

Pure phenolic compounds were used for the toxicity tests to avoid possible interactions with other chemicals during extraction. First, concentrated solutions in 50% methanol were prepared for each compound. Second, dilutions from 150- to 4500-fold were made (in water) to obtain a mean for each compound (Table 3). These high dilutions allowed us to minimize the amount of methanol in our simulated solutions. A total of 20 solutions was made, i.e., five for each bark age category for the four extractants. These solutions contained, on average, 12 phenolics in mixture. They were kept at 4°C, in darkness, and used within 24 hr. Since bark pH varies with bark age, no pH adjustments were made to reflect the natural conditions of bark wastelands.

Toxicity Test on Rhizobial Growth

Four rhizobial species were selected (known as the microsymbionts of legume species that are used in plant toxicity evaluations and described below): *Mesorhizobium loti* for birdsfoot trefoil (*Lotus corniculatus*) and *Rhizobium leguminosarum* bv. *viciae* for nodulation of common vetch (*Vicia sativa*). Three strains of *M. loti* were used: NZP 2213 (also designated USDA 3471 or ATCC 33669), NZP 2234, and L3 (Agriculture and Agri-Food Canada, Sainte-Foy, Quebec, Canada). The three strains of *R. leguminosarum* bv. *viciae* were: USDA 2370 (also designated ATCC 10004), USDA 2489, and 175P1 (Nitragin, Milwaukee, WI, USA). *Mesorhizobium* sp., strain N28, and *Rhizobium* sp., strain SM2, respectively, sensitive and resistant to sainfoin seed diffusates (phenolics), were also used (Prévost et al., 1990). Each strain was grown in 50 ml of yeast mannitol broth (YMB; Vincent, 1970) contained in 125-ml Erlenmeyer flasks placed on a rotary shaker at 28°C. This medium was buffered with CaCO₃ and had a pH of about 7. As soon as the optical density reached 0.6–0.7 (630 nm), 20 ml of the rhizobial culture were incorporated per liter of sterile YMA, maintained as a liquid at 45°C. Then, 10 ml of inoculated YMA were distributed on Petri plates. After cooling, 4 disks of sterile paper (10 mm diam) were placed on the surface of the YMA for each plate. Each disk was impregnated with 100 µl of one of 20 different phenolic solutions. Petri plates were kept at 4°C for 18 hr to allow the compounds to diffuse, and then were incubated at 28°C for 4 d. Finally, the diameters of the zones of inhibition were recorded. This test was repeated ×3. A factorial experiment, using the microbial strains as the main factor and the solvents and bark ages as subfactors, was set up according to a split-plot design (Snedecor and Cochran, 1967; Steel and Torrie, 1980).

Phytotoxicity Test on Seed Germination

We determined the effects of phenolic compounds on seed germination and rootlet growth in combination with a germination index (Zucconi et al., 1981). Cress (*Lepidium sativum*) was used as a reference (Zucconi et al., 1981); it is an acid-tolerant species and its root growth is not affected by pH (Bonanomi et al., 2006). Vetch (*V. sativa*), a close relative of the weed *V. cracca* that has been observed under pioneer vegetation conditions, was also selected because this species prefers sandy soils and has a pH ranging from 5.5 to 8.5. The third species was birdsfoot trefoil (*L. corniculatus*). This species is tolerant of acidic pH (about 4.5) and is often used for revegetation (Hardy BBT Ltd., 1989). Preliminary tests with water, adjusted to pH levels of 3.5–5.5 to correspond to the pH of the barks and the simulated solutions (4.1±0.4), yielded 100% germination after 24 hr (data not shown). These tests indicate that germination could be evaluated with 15 seeds per Petri plate for cress and birdsfoot trefoil and with eight seeds for vetch.

Three sterile filter papers were placed at the bottom of a Petri plate and impregnated with 4 ml of a phenol acid solution or acidic water (control). Petri plates were placed in darkness at 27°C; the cress seeds were kept for 36 hr and the vetch and birdsfoot trefoil seeds for 3 d. Percentage of seed germinated and the rootlet lengths were recorded. The germination index was calculated by multiplying the % of germination by the average length of the rootlets (Zucconi et al., 1981). All tests included a water control, and each was repeated ×4. A factorial experiment, using the bark ages and extractant solutions as the main factors, was set up in a completely randomized block design (Snedecor and Cochran, 1967; Steel and Torrie, 1980). Analyses of variance included a least significant difference (LSD) treatment comparison. Pearson correlation coefficients between the germination index and the total

phenolic compounds, and between the germination index and each single compound, were calculated.

Phytotoxicity Test on Plant Growth, Nodulation, and Nitrogen Fixation Activity

Plants were grown in transparent plastic growth pouches to allow visualization of nodulation according to a standard method for testing legume inoculants (Anonymous, 2005). Two nitrogen forms were studied: nitrogen obtained through dinitrogen fixation (i.e., in the presence of rhizobia) and mineral nitrogen (in the presence of mineral nitrogen in the nutrient solution). A split-plot design with four blocks was set up with the two forms of nitrogen as the main factor and the 20 phenolics solution as the subfactor. The analyses of variance included an LSD treatment comparison (Snedecor and Cochran, 1967; Steel and Torrie, 1980).

Seeds were surface sterilized for 10 min in a 20-ml solution of Javex (5%), 4 ml of Tween 20 (50% V/V), and a 4-ml buffer [8 ml concentrated HCl mixed with 32 ml KH_2PO_4 (1 M)]. Seeds were washed several times in sterile distilled water and dried under sterile conditions. Plant nutrient solutions contained 0 or 15 mol m^{-3} NO_3^- -N as KNO_3 and Ca $(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (Chalifour and Nelson, 1988), and solutions were balanced for their K and Ca concentrations. Eight seeds were placed in each growth pouch (Anonymous, 2005) that was filled with the appropriate nutrient solution (pH 4.2 ± 0.1) without the phenolic compounds. Pouches containing the N-free nutrient solution were inoculated with about 10^9 cells of the appropriate rhizobial strain, i.e., 175P1 for vetch and NZP 2234 for birdsfoot trefoil. These growth pouches were wrapped in aluminum foil and kept in the dark for 3 d. Thereafter, growth pouches containing 8 seedlings were selected, and the nutrient solution was removed and replaced with 30 ml of nutrient solution containing the phenolic compounds or the control. These solutions had pH values of 4.1 ± 0.4 .

Plants were kept under light for 16 hr at a temperature of 20°C, and then placed in the dark for 8 hr at 15°C. They were watered with bidistilled water every second day. After 10 d, nutrient solutions containing the phenolics were removed and replaced with fresh solutions. At 40 d of age, the dry weight of the aerial and root parts was measured for each growth pouch. All results are expressed on a dry weight basis relative to the control, where % biomass = [(treatment (d.w.)/control (d.w.))] \times 100. The number of nodules per root was also determined and is expressed as: % nodule number = (nodule number per root system for one treatment/nodule number per root system for the control) \times 100. The aerial parts and the nodules were ground and their total N content was determined by dry combustion (CNS-1000 Analyser, Leco Co., St. Joseph, MI, USA).

Results

To identify and quantify the phenolic compounds from bark of different ages, several extractants were compared. For most extractants, the highest yield of total phenolic compounds was obtained from the youngest bark (Table 3). With the exception of young bark residues, more phenolics tended to be extracted by hot water, 0.1 M NaOH, and 2 M NaOH than with cold water (Table 3). However, certain compounds, such as salicylic acid, were better extracted with cold water than with 2 M NaOH. Also, others, such as proto-catechuic acid, catechol, caffeic acid, vanillin, and ferulic acid, were better extracted with hot water than with 0.1 M NaOH. These results were used to reproduce solutions of various concentrations to simulate the different phenolic concentrations of the bark residues so as to avoid possible interactions from other bark compounds.

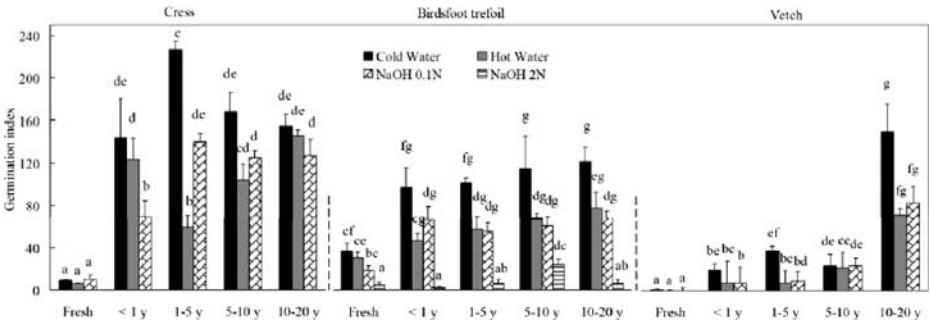


Fig. 1 Germination index of cress, birdsfoot trefoil, and vetch in the presence of phenolic simulated solutions of bark of various ages extracted with cold water, hot water, NaOH 0.1 N, or NaOH 2.0 N

Growth inhibition of *Mesorhizobium* sp. and *Rhizobium* sp. strains was not detected from treatments involving the 20 solutions that simulated extracts from bark of different ages from the four solvents.

To determine phytotoxic effects on seed germination, we used the 20 simulated bark extract solutions. Cress and vetch did not germinate in the solution that simulated the 2 M NaOH extract; this treatment was excluded from the statistical analyses. In general, stronger extractants, and younger bark residues, lowered the germination index for all species (Fig. 1). However, the germination index differed among species according to extractant strength and age of the bark residue; the highest germination index was with cress, followed by birdsfoot trefoil, and then vetch. However, only birdsfoot trefoil germinated in the solutions that simulated the 2 M NaOH solvent and resulted in a germination index higher than that in vetch.

For cress, on average, the germination index decreased from the cold to the hot water extract, but increased from the hot water extract to the 0.1 M NaOH extract of the fresh bark, 1- to 5-yr-old bark, and 5- to 10-yr-old bark (Fig. 1). Correlation analyses shows a negative relationship between the germination index and the total phenolic content, and the 11 phenolic compounds, i.e., the germination index decreased with concentration of phenolics (Tables 3 and 4).

Table 4 Pearson correlation coefficients (*R*) between the germination index tests and the phenolic compounds

Phenolic compounds ^a	Cress	Birdsfoot trefoil	Vetch
Protocatechuic	-0.76** ^b	-0.84**	-0.58**
Catechol	-0.84**	-0.77**	-0.654**
(<i>P</i>)-Hydroxybenzoic	-0.75**	-0.69**	-0.62**
Vanillic	-0.60**	-0.55**	NS
Caffeic	-0.72**	-0.61**	-0.52**
Vanillin	-0.83**	-0.64**	-0.77**
(<i>P</i>)-Coumaric	-0.82**	-0.88**	-0.71**
Ferullic	-0.89**	-0.85**	-0.75**
3-Hydroxy-4-methoxycinnamic	-0.57**	-0.54**	-0.57**
Phenylacetic	-0.43**	NS	-0.65**
<i>trans</i> -Cinnamic	-0.74**	-0.781**	-0.71**
Total	-0.98**	-0.93**	-0.88**

^a The order of the phenolic compounds in this table followed their order of elution

^b Levels of significance: ***P* < 0.01, NS = not significant

For birdsfoot trefoil, seeds germinated even in the simulated solution of 2 M NaOH solvent (Fig. 1). The germination index decreased from the cold to the hot water extract, and from the 0.1 M NaOH to the 2 M NaOH extract of most barks, but increased with the ages of bark extracted with 0.1 M NaOH. Finally, the total phenolic content and 10 individual phenolics were correlated with the germination index of birdsfoot trefoil all, except for phenylacetic acid (Table 4).

For vetch, the germination index decreased from the cold to the hot water extract or 0.1 M NaOH, but increased with the age of the bark (Fig. 1). The total phenolic content and all individual phenolic were correlated with the germination index, except for vanillic acid (Table 4).

To determine the phytotoxicity of the 20 simulated bark extracts, we tested their effects on the rhizobium–legume symbiosis. For birdsfoot trefoil, the control had a mean aerial biomass of 22 ± 3 mg in the presence of rhizobia, but 16 ± 4 mg in the presence of mineral nitrogen. Its mean root biomass was 9 ± 1 mg in the presence of rhizobia and 13 ± 2 mg in the presence of mineral N. For vetch, the control had a mean aerial biomass of 87 ± 2 mg in the presence of rhizobium and 69 ± 4 mg in the presence of the mineral N. Its mean root biomass was 24 ± 3 mg in the presence of rhizobium and 31 ± 1 mg in the presence of mineral N.

For the aerial and root biomasses of birdsfoot trefoil and for the aerial biomass of vetch, there were significant interactions between the nitrogen source and the simulated phenolic solutions; the LSD test was applied to the 40 treatments. For the root biomass of vetch, the nitrogen source and simulated phenolic solutions factors were significant but their interaction

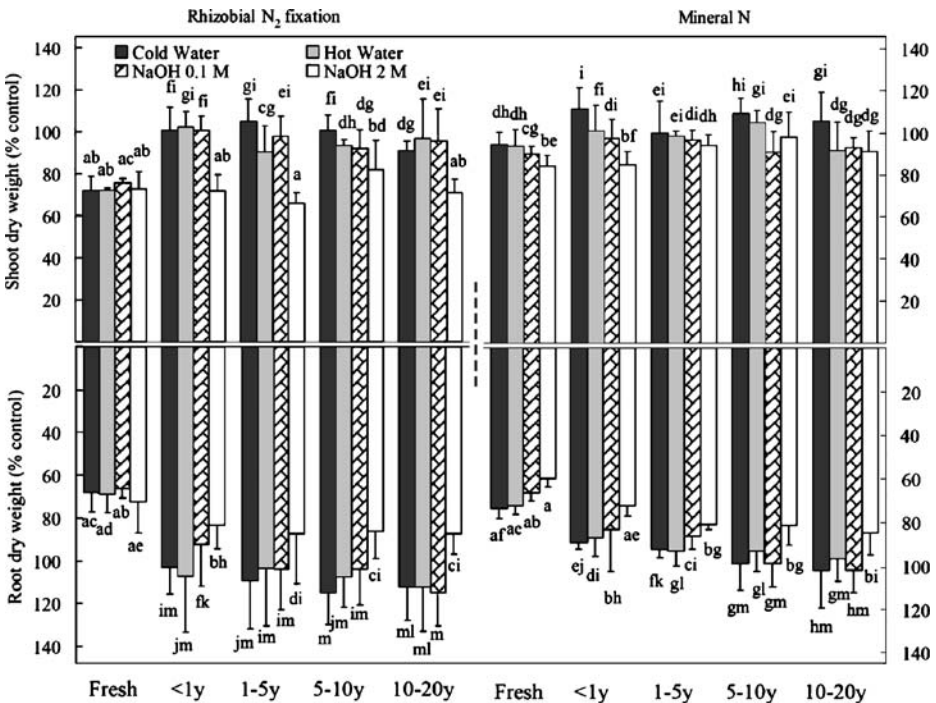


Fig. 2 Percent control of shoot and root biomasses of vetch grown in the presence of phenolic simulated solutions of bark of various ages with fixed N (rhizobial N₂ fixation) or mineral N as N source

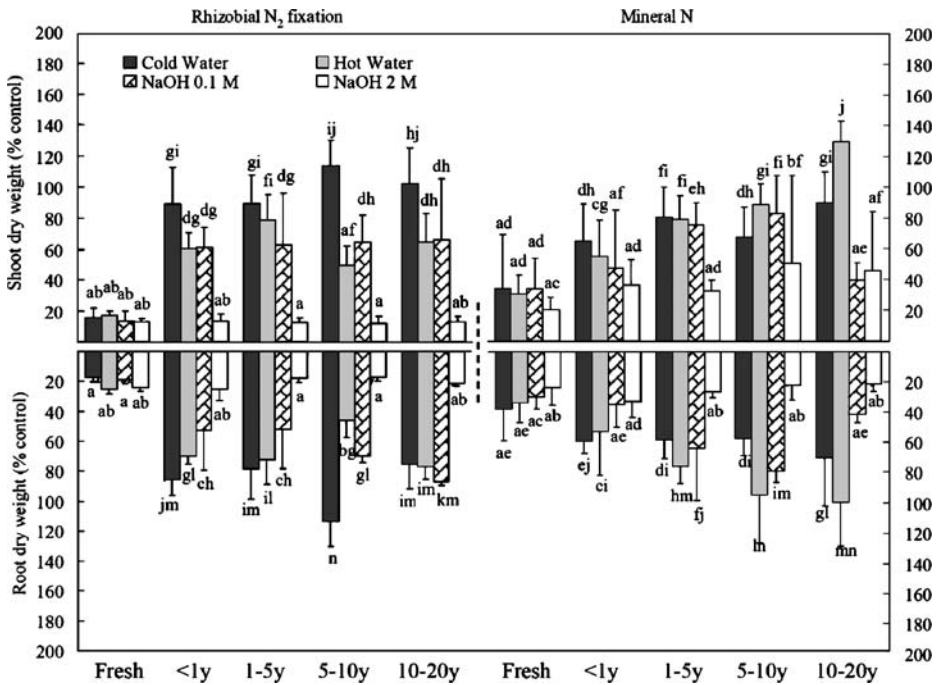


Fig. 3 Percent control of shoot and root biomasses of birdsfoot trefoil grown in the presence of phenolic simulated solutions of bark of various ages with fixed N (rhizobial N₂ fixation) or mineral N as N source

Table 5 Pearson correlation coefficients (*R*) between the aerial and root biomasses and the nitrogen content of the aerial biomass of birdsfoot trefoil grown in the presence or absence of rhizobium, and the simulated phenolic compounds of bark

Phenolic compounds ^a	Aerial biomass (d.w.)	Aerial biomass N content	Root biomass (d.w.)	Aerial biomass (d.w.)	Aerial biomass N content	Root biomass (d.w.)
	In the presence of rhizobia			In the presence of mineral nitrogen		
Catechol	-0.97 ^b	NS	-0.97*	NS	-0.97*	NS
(<i>P</i>)-Hydroxybenzoic	-0.98*	NS	-0.98*	-0.94*	-0.94*	NS
Vanillic	-0.99**	NS	-0.99**	-0.94*	-0.96*	NS
Caffeic	-0.98**	NS	-0.98**	-0.98*	-0.97*	NS
Vanillin	NS	-0.94*	NS	NS	-0.97*	NS
(<i>P</i>)-Coumaric	-0.96*	NS	-0.95*	-0.96*	-0.99**	NS
Ferullic	-0.94*	-0.94*	-0.97*	NS	-0.96*	NS
3-Hydroxy-4-methoxycinnamic	-0.96*	NS	-0.96*	-0.96*	-0.97*	0.98**
Benzoic	NS	NS	NS	-0.96*	-0.97*	NS
Total	-0.98*	NS	-0.97*	-0.96	-0.96*	-0.96*

^a The order of the phenolic compounds in this table followed their order of elution

^b Levels of significance: ***P*<0.01; **P*<0.05, NS = not significant

was not. However, for the purposes of data presentation, the LSD test was performed over the 40 treatments. For birdsfoot trefoil and vetch, in the presence of rhizobia, the least growth (aerial and root biomasses) was with the fresh bark regardless of the strength of the extractants and with the 2 M NaOH extractant regardless of bark age (Figs. 2 and 3). Often, plant growth decreased with an increase in total phenolic content and with some specific phenolic compounds (Tables 5 and 6). The greatest growth was generally measured in the cold water extracts of <1-yr-old bark. In the presence of mineral N, the least growth followed the same trends as that in the presence of rhizobia for both plant species. For birdsfoot trefoil, the greatest growth was measured from the solution that simulated bark older than 1 to 5 yr extracted with hot water or 0.1 M NaOH. For vetch, the greatest aerial biomass accrued from the solution that simulated the 10- to 20-yr-old bark extracted with hot water; the greatest root biomass accrued with solutions that simulated bark older than 1 to 5 yr extracted with hot water.

For birdsfoot trefoil, the control had a shoot N content of $1.00 \pm 0.18\%$ in the presence of rhizobia, and $2.00 \pm 0.11\%$ in the presence of mineral N. Also, in the presence of rhizobia, the number of nodules per control plant was 25 ± 1 with a nodule N content of 8.64%. For vetch, the control had a shoot N content of $3.00 \pm 0.26\%$ in the presence of rhizobia, but $2.4 \pm 0.18\%$ in the presence of N in the nutrient solution. Also, in the presence of rhizobia, the number of nodules per control plant was 16 ± 1 with a nodule N content of $6.43 \pm 0.94\%$.

For both plant species, the interaction between the nitrogen source and the extractant solutions was significant for shoot N content; again, the LSD test was applied to the 40 treatments. For the number of nodules and the nodule N content, only the rhizobia treatments were further analyzed. For the nodule N contents of birdsfoot trefoil, nodule weights were too low to perform the N analyses. In the presence of rhizobia for vetch, the shoot N contents increased with the solutions that simulated the oldest barks (Fig. 4a); these

Table 6 Pearson correlation coefficients (*R*) between the root biomass, the aerial, root, and nodule nitrogen contents of vetch grown in the presence or absence of rhizobium, and the simulated phenolic compounds of bark

Phenolic compounds ^a	Aerial biomass N content	Root biomass (d.w.)	Nodule N content	Aerial biomass N content	Root biomass (d.w.)
	In the presence of rhizobia			In the presence of mineral nitrogen	
Catechol	NS ^b	-0.98*	NS	NS	-0.99**
(<i>P</i>)-Hydroxybenzoic	-0.99*	-0.98*	-0.95*	-0.97*	-0.97*
Vanillic	-0.99**	-0.99	NS	-0.97*	-0.99**
Caffeic	NS	-0.99*	NS	-0.96*	-0.99**
Vanillin	-0.97*	-0.96*	NS	NS	-0.95*
(<i>P</i>)-Coumaric	-0.99**	-0.99**	NS	NS	-0.98*
Ferullic	-0.97*	-0.96*	NS	NS	-0.97*
3-Hydroxy-4-methoxycinnamic	-0.98*	-0.97*	NS	NS	-0.98*
<i>trans</i> -Cinnamic	NS	NS	-0.96*	NS	NS
Total	-0.98**	-0.98**	NS	-0.99*	-0.99**

^a The order of the phenolic compounds in this table followed their order of elution

^b Levels of significance: ** $P < 0.01$; * $P < 0.05$, NS = not significant

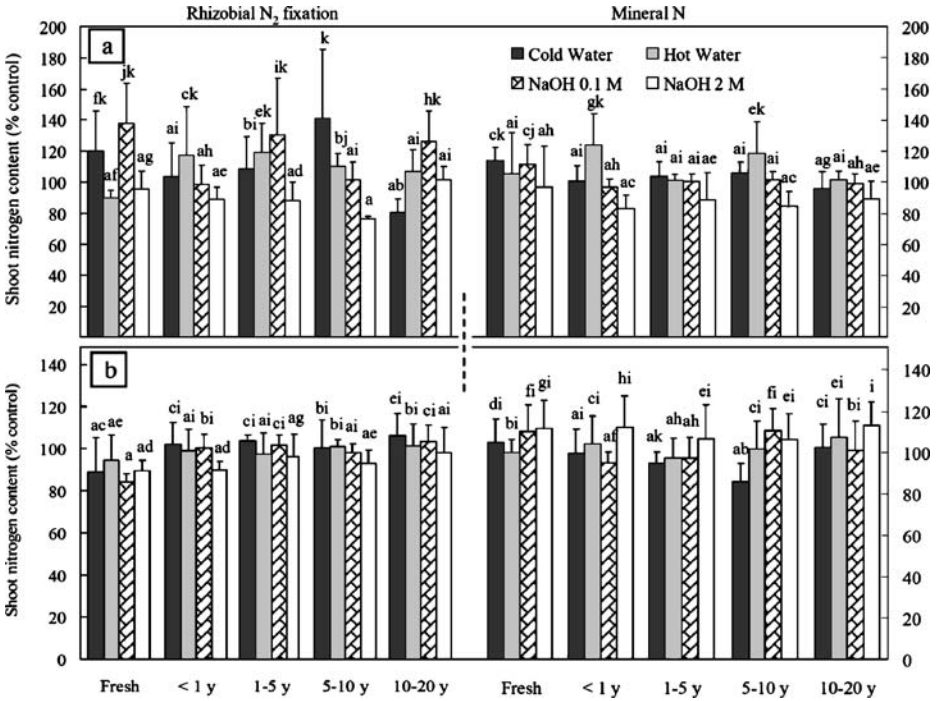


Fig. 4 Percent control of the shoot nitrogen content of birdsfoot trefoil (a) and vetch (b) grown in the presence of phenolic compound simulated solutions of bark of various ages with fixed N (rhizobial N₂ fixation) or mineral N as N source

increases were generally greater when plants were grown in the presence of mineral N with strong extractants. For birdsfoot trefoil in the presence of rhizobia, shoot N content was greatest in the fresh or 5- to 10-yr-old barks extracted with cold water or 0.1 M NaOH, the <1-yr-old barks extracted with hot water, the 1- to 5-yr-old bark extracted with hot water or 0.1 M NaOH, and the 10- to 20-yr-old bark extracted with 0.1 M NaOH (Fig. 4b). However, in the presence of mineral N, the shoot N content was greater for <1-yr-old barks or 5- to 10-yr-old barks extracted with hot water. The correlation analyses showed a decrease in aerial biomass N content with vanillin and ferulic acids in the presence of rhizobium, but in the absence of rhizobium there was a decrease for most phenolic compounds and for the total phenolic content (Tables 5 and 6).

For birdsfoot trefoil, the number of nodules tended to increase when the solutions simulated 1- to 5-yr-old bark extracted with cold water, 5- to 10-yr-old bark extracted with 0.1 M NaOH, and the 10- to 20-yr-old bark extracted with hot water as compared with the other extracted bark residues (Fig. 5a). However, for vetch, the number of nodules tended to increase when the solutions simulated 1- to 5-yr-old bark extracted with cold water compared with the other extracted bark residues (Fig. 5b). Vetch nodule N content was higher when the solutions simulated barks older than 1 yr extracted with cold and hot water compared with the other extracted bark residues (Fig. 6). Again, the analyses of correlation showed a decrease in nodule N content with an increase in the (*P*)-hydroxybenzoic acid and *trans*-cinnamic acids (Table 5).

Fig. 5 Percent nodule number compared to control of birdsfoot trefoil (a) and vetch (b) grown in the presence of phenolic simulated solutions of bark of various ages in the presence of rhizobia

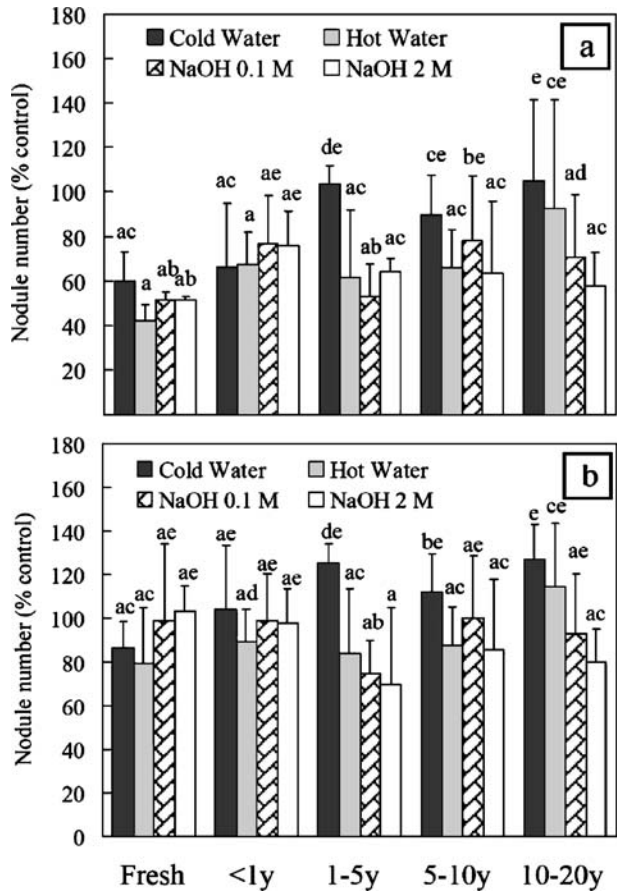
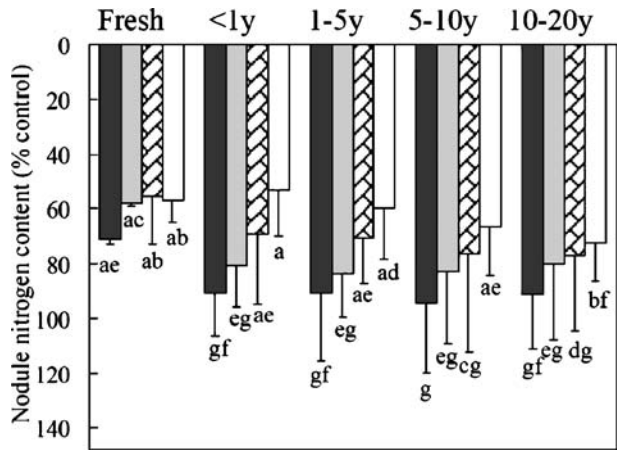


Fig. 6 Percent nodule nitrogen content compared to control of vetch grown in the presence of phenolic simulated solutions of bark of various ages in the presence of rhizobia



Discussion

Detection of water-soluble phenolics has been reported from several plants, debris, and litter (Dalton, 1999), and now from bark residues. Various methods have been used to extract phenolic compounds, including water (Whitehead et al., 1983; Blum et al., 1991), chelating agents, methyl alcohol, and NaOH (Rice, 1984; Dalton et al., 1987; Blum et al., 1997; Dalton, 1999). A link between the concentration of the compound extracted and biological activity has not previously been established; this information will be important for developing a soil cover medium using bark residues.

This study showed that the total content of phenolics and the content of most individual compounds increased with extractant strength; cold water = hot water = 0.1 N NaOH < 2 M NaOH for fresh bark, and cold water < hot water = 0.1 N NaOH < 2 M NaOH for bark less than 1 yr old. In general, these results agree with those of Dalton (1999) in the ability of various extractants to recover ferulic acid from soil, i.e., water = methanol < sodium acetate < EDTA = DTPA < NaOH. However, fresh bark appears to carry substantial amounts of soluble phenolics and, in this particular case, the extracting abilities of cold water, hot water, or 0.1 N NaOH were similar. Under our conditions, extensive sorptive and complex reactions, and degradation of phenolics by abiotic or biotic factors, such as microbes (Inderjit et al., 1999; Schmidt and Ley, 1999), were possible but unlikely. Fresh bark results using cold and hot water and 0.1 N NaOH were often similar in the extraction of individual phenolics, even though pH, hydrolysis, and oxidative conditions differed. Also, the hot temperature (85°C) and alkaline pH (NaOH) conditions inhibited microbial growth. For the cold water extractant, there was probably no growth of microorganisms because a N source was not available and the conditions were acidic. These results are in contrast with those of a cold water soil incubation, where the addition of ferulic acid, a carbon source, created an environment that was favorable for the growth of microbes (Dalton, 1999).

In general, the concentration of each phenolic decreased with bark age. For fresh bark, the phenolic content varied from 789 to 1279 $\mu\text{g/g}$ (d.w.) (9 to 12×10^{-4} M), regardless of the strength of the extractant. Other bark ages had a phenolic acid content ranging from 5 to 19 $\mu\text{g/g}$ (d.w.) (5 to 26×10^{-6} M) when extracted with cold water, 145 to 236 $\mu\text{g/g}$ (d.w.) (21 to 29×10^{-5} M) when extracted with hot water and 0.1 M NaOH, and 843 to 1231 $\mu\text{g/g}$ (d.w.) (13×10^{-4} to 16×10^{-4} M) when extracted with 2 M NaOH. These results show that more than 60% of the total phenolic contents in fresh bark were soluble in cold water, and thus, they would inhibit biological activity more than older residues.

The lower yield of cold water extraction for bark less than 1 yr old, and older residues, was probably attributable to the water failing to penetrate the bark, washing only its surface where phenolic compounds exist in a free state. Also, during the first year of exposure in the bark wasteland, there would have been substantial water leaching as a result of environmental conditions (rain and snow), and the microbes would have biodegraded over time. When the strength of the solvent increased, from hot water or 0.1 M NaOH to 2 M NaOH, more internal tissues were probably reached, resulting in a more complete hydrolysis. Also, the bound forms of phenolics are normally released by 2 M NaOH (Rice, 1984; Dalton et al., 1987).

The predominant phenolics identified in bark residues are phenylacetic, vanillin, ferulic, catechol, and coumaric and protocatechuic acids. The C6–C2 phenylacetic acid is a deaminated product of phenylalanine, the precursor of cinnamic acid, coumaric acid, coniferaldehyde, ferulic acid, or conyferyl alcohol in the lignin biosynthetic pathway (Sibout et al., 2005). The chromatographic peak for phenylacetic acid has to be optimized to be detected by UV. Its presence in bark could be linked to the lignin biosynthetic or degradation

pathway, involving at least the decarboxylation of 3-phenyl-propionic acid or the oxidation under acidic conditions of the etherified coniferylaldehyde to phenylacetic acid (Pan et al., 2000). In addition, the presence of phenylacetic acid could originate from plant material during degradation (Rice, 1984). Some of the other compounds belong to the vanillyl phenols (vanillin and vanillic acids) that are typical of the gymnosperm lignin signature (Hedges and Mann, 1979). The cinnamyl phenols (coumaric and ferulic acids) were present in our bark residue extracts. These compounds have been reported in the lignocellulose complex, and ferulic acid can be derived from holly tree bark (Hedges and Mann, 1979), suberin-associated waxes, and cutin (Otto et al., 2005). The bark residues may also have contained needles that would explain the presence of the cinnamyl phenols. The coumaryl and synapyl alcohol-derived units have been reported in minor amounts in spruce lignin (Kögel, 1986). The origin of the cinnamyl phenols may also be related to the degradation of the coumaryl and synapyl alcohol-derived units present in the lignocellulose complex, suberin, or cutin.

The decrease in concentration of phenolics over time, under prevailing environmental conditions, could be attributable to abiotic factors (such as rain and melting snow), photo-oxidation (Siqueira et al., 1991; Chaves et al., 2002), or to biotic phenomena that occur during the decomposition of organic matter (An et al., 2000), mainly the progressive degradation by microorganisms (Siqueira et al., 1991; Blum, 2004). For example, we found that the content of phenylacetic and ferulic acids decreased with bark age. The catabolism of ferulic acid by microorganisms produces vanillin, vanillic acid, protocatechuic acids (Peng et al., 2003), and catechol (Bocks, 1967), and they increased in older barks, especially the latter compounds. The dominance of vanillyl phenols over time in our study agrees with the results of the incubated needle litter experiment of Sjöberg et al. (2004), whereas the decrease in cinnamyl phenols agrees with results of Hedges and Weliky (1989), cited in Sjöberg et al. (2004).

The biological activities of the bark extracts were determined with biological tests, especially from fresh bark residues. The phenolic solutions were made by simulating bark composition over various ages and extractants in order to minimize the interference from other compounds that might be present in bark residues. No pH correction was made, and the effects of hydrogen ion concentration and the phenolic mixtures are combined, but they are still within the range occurring in the bark residues. Use of these simulated phenolic solutions increased the relevance of our results for the development of a new soil cover, in agreement with Blum's (1999) reflections on bioassays.

Based on biological activity, we made the following conclusions. First, simulated phenolic solutions did not inhibit rhizobial growth on YMA, even though total phenolics were between 5×10^{-6} and 1.6×10^{-3} M. Inhibition of rhizobial growth was expected because concentrations above 10^{-4} (Rice et al., 1981) to 10^{-5} M have been reported to inhibit growth (Gauv and Pareek, 1976). Rice (1964), using a YMA medium at pH 8.2, demonstrated the inhibitory activity of plant extracts on *Rhizobium*. The synergistic effects of a 10^{-3} M mixture of (*P*)-coumaric and ferulic acids, (*P*)-hydroxybenzoic and (*O*)-hydroxyphenylacetic acids, or ferulic and vanillic acids, were found to inhibit rhizobial growth on YMA (Rice et al., 1981). Gauv and Pareek (1976) attributed the decreased growth of *Rhizobium* to (*P*)-hydroxybenzoic and salicylic acids at concentrations of 10^{-3} M. In our study, these phenolics were present in all bark extracts, but possibly in combinations and concentrations too low to inhibit rhizobia. Also, the presence of mannitol and CaCO_3 , which buffered the pH to about 7 in the culturing media, have been shown to prevent toxicity effects of phenolics on *Rhizobium* growth (Blum et al., 2000; Seneviratne and Jayasinghearachchi, 2003). Under our experimental conditions, mixtures were not toxic to rhizobial growth on

YMA; therefore, this test was not useful in relating the rhizobial growth to phenolic toxicity.

Second, the simulated phenolic solutions had inconsistent effects on germination and root elongation of cress, vetch, and birdsfoot trefoil. Cress had the highest germination index, followed by birdsfoot trefoil; both are acidic-resistant species. Vetch, a species that prefers a soil pH above 5.5, had the lowest germination index. There was an improvement in index and plant growth with the aging of the residues, and with the decreasing strength of the extractant solutions. Allison et al. (1963) and Rice (1984) report that phytotoxic compounds are usually present in large quantities in bark. The germination of cress and vetch was completely inhibited with the 2 M NaOH simulated solution, with a total phenolic content of about 1.2 to 1.6×10^{-3} M, whereas the germination of birdsfoot trefoil was inhibited by 90%. These results are similar to those of Siqueira et al. (1991), i.e., a concentration of 1 to 7×10^{-3} M was the minimal concentration needed to inhibit germination by 50%. Our germination test was useful in linking the biological activity to the phenolic toxicity of fresh barks with that of barks older than <1 year for all plant species. Three major toxicity groups were detected for vetch: fresh bark, <1- to 10-yr-old bark, and 10- to 20-yr-old bark.

Third, the phenolic extracts had inconsistent effects on vetch and birdsfoot trefoil growth, nodule number, and N_2 fixation activity. In the presence of rhizobia or mineral N, the simulated solutions at 8.8×10^{-4} to 1.6×10^{-3} M of total phenolics decreased the aerial and root dry weights and the nodule number. For vetch and birdsfoot trefoil, N content decreased at the highest concentration of phenolics. The shoot and root weights of birdsfoot trefoil were more affected than those of vetch, especially with fresh bark residues and 2 N NaOH extracts. High phenolic concentrations have been reported to decrease plant growth, to be phytotoxic, and to alter N_2 fixation activity (Rice et al., 1981; Rice, 1984; An et al., 2000; Seneviratne and Jayasinghearachchi, 2003). Our results support this concept of phytotoxicity on rhizobium–legume symbioses for high concentrations of total phenolics. In addition, the presence of various phenolics in a mixture could have antagonistic, neutral, or synergistic effects on seed germination, plant growth and N fixation. For example, Li et al. (1993) suggests that mixtures of phenolics, including caffeic, ferulic, and *trans*-cinnamic acids, enhance their inhibitory effects. In our simulated solutions, the analyses of correlation shows a negative relationship with six other phenolic compounds and with the germination index for cress, vetch, and birdsfoot trefoil, i.e., protocatechuic acid, catechol, (*P*)-hydroxybenzoic acid, vanillin, (*P*)-coumaric, and 3-hydroxy-4-methoxycinnamic acids. Also, the shoot and root dry weights, N content of the aerial part, nodule number and nodule N content were negatively correlated with the total phenolics content, and catechol, (*P*)-hydroxybenzoic acid, vanillic acid, caffeic acid, vanillin, (*P*)-coumaric, ferulic acid, 3-hydroxy-4-methoxycinnamic acid, benzoic acid, and *trans*-cinnamic acid. (*P*)-Coumaric, ferulic, (*P*)-hydroxybenzoic, and vanillic acids at 10^{-3} M have been reported to reduce the number of nodules, and ferulic and (*O*)-hydroxyphenylacetic acids have been shown to reduce the N_2 fixation on bush black beans (Rice et al., 1981).

On the other hand, in the presence of rhizobia, when the simulated phenolic solutions content was between 5×10^{-6} and 1.3×10^{-5} M of total phenolics, then the aerial and root dry weights of birdsfoot trefoil and the root dry weight of vetch increased, generally resulting in a greater shoot N content, nodule number, and nodule N content than in the presence of mineral N. Rice et al. (1981) and Seneviratne and Jayasinghearachchi (2003) also report that low concentrations of phenolic compounds stimulated nodule formation or N_2 fixation activity. Rice et al. (1981) reports that (*P*)-coumaric, (*P*)-hydroxybenzoic, and vanillic acids could stimulate N_2 fixation. At low concentrations, our results suggest that phenolics modified the rhizobium–legume symbiosis positively. In the case of the cold

water extraction of the 1- to 5-yr-old bark, 5- to 10-yr-old bark, and 10- to 20-yr-old bark, only three phenolics were present, namely, vanillin, benzoic and salicylic acids; their effects alone or in mixture could be investigated further. For the other simulated solutions that improved the symbioses, the presence of more than 14 compounds in the mixture does not allow us to identify any individual phenolic that was beneficial to the symbiosis. More attention should be given to understanding the beneficial effects of older bark residues on legume symbiosis.

Plant growth and shoot N content were greater in the presence of rhizobia than in the presence of mineral N at $15 \text{ mol m}^{-3} \text{ NO}_3^- \text{-N}$. This suggests that N_2 fixation was providing more nitrogen to the plants than the added mineral N or that the rhizobia could detoxify the phenolic compounds.

The effect of the extracts on vetch and birdsfoot trefoil is of interest because such decreases in biological activity can be related to the toxicity of the phenolics extracted from fresh bark and 2 N NaOH 2N extractant, particularly in terms of plant growth, N_2 fixation, and nodule number for both plant species in the presence of *Rhizobium*. For both plant species, the solutions simulating the cold water extract from bark residues older than <1 year and the hot water extractant for the 10- to 20-yr-old bark residues increased the nodule number. From a practical point of view, the <1-yr-old bark residues should be selected in combination with legume species for revegetation purposes. Finally, the inoculated birdsfoot trefoil seems to be more sensitive to fresh bark and to the 2 N NaOH extractant that decreased the nodule number. Thus, again, from a practical point of view, fresh bark should not be used as an organic soil cover.

In summary, rhizobial growth on YMA was not affected by solutions that simulate the composition of the phenolic compounds of bark residues. On the other hand, the germination index of cress, birdsfoot trefoil, and vetch, and the growth, nodule number, and N_2 fixation activity of birdsfoot trefoil and vetch, were strongly inhibited by the simulated solutions from the young bark and the 2 N NaOH extractant. Germination inhibition varied with the species; vetch appears to be the most sensitive, and cress and birdsfoot trefoil the most resistant, which is related to the pH sensitivity of these species. However, plant growth, nodule number, and the N_2 fixation activity of vetch were less negatively affected by the high concentration of phenolics than birdsfoot trefoil, whereas a low concentration of phenolics affected birdsfoot trefoil more than vetch. In this case the pH sensitivity of these species was not related to the plant response. Vetch and birdsfoot trefoil need to be further investigated under greenhouse or field conditions. For revegetation purposes involving a novel organic soil cover, <1-year-old bark and older bark residues should be used in combination with legume species.

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Stapley, 2003; Labra and Niemeyer, 2004; Amo et al., 2004; reviewed in Kats and Dill, 1998; Apfelbach et al., 2005). Since prey species also respond to unknown predator odors, some have suggested that recognition of predator odors as a sign of potential danger is innate (Epple et al., 1993; Apfelbach et al., 2005). Such an innate recognition of predator odor would be an enormous advantage. After perceiving a predator odor, the prey animal can avoid the encounter with the predator. This is a highly critical advantage because the very first encounter with a predator usually ends fatally for the prey (Edut and Eilam, 2003). However, another possible explanation for the observed effects of unknown predator odors is that they contain components of a known predator. This would mean that recognition of the unknown predator odor is not innate but is rather based on previous experience with other predators.

A better way to test for innate recognition of predator odors is to use prey animals that have had no previous experience of predator odors, i.e., laboratory-reared animals, and to observe their behavior during exposure to different predator and nonpredator odors. A number of studies have shown that the odors of certain predators (usually cats or foxes) induce autonomic and behavioral signs of fear in laboratory animals (reviewed in Blanchard et al., 1990a; Dielenberg and McGregor, 2001; Apfelbach et al., 2005; Fendt et al., 2005; Takahashi et al., 2005). Since these laboratory animals (rats, mice) were totally naive to predators and predator odors, this is evidence that rats and mice innately recognize predator odors as signs of potential danger. Major drawbacks to these studies, however, are that in each case they have invariably used only one particular predator odor and that they have carried out control experiments either with synthetic odors instead of nonpredator odors or simply by eliminating all odors. As a result, the responses observed in these studies were perhaps general responses to unfamiliar animal odors.

The aim of the present study was to collect further data supporting the hypothesis that predator odor recognition is innate. To this end, the behavioral effects of different rodent odors were tested on male laboratory rats that were all naive to the odors used in the study. Urine samples from different canids and felids (wolf, coyote, bobcat, fox, and cat) were used as test stimuli, and urine samples of herbivore nonpredators (cow and horse) or of female rats were used as control stimuli, along with saline (i.e., no odor). Each rat was exposed in a test arena to all odors in a randomized order, and the behavior of the rat was observed. If laboratory rats are innately able to recognize predator odors, defensive behavior is expected during exposure to predator urine, but not during exposure to the control stimuli. The following defensive behaviors of rats are described in laboratory but also in field studies (Blanchard and Blanchard, 1971, 1990; Dielenberg and McGregor, 2001; Carobrez et al., 2001; McNaughton and Corr, 2004; Apfelbach et al., 2005): flight, hide and avoidance behavior, freezing behavior, potentiation of defensive reflexes, and “risk assessment behavior” such as flat back approaches, prolonged approach to and short direct contacts with the potentially dangerous stimuli.

Methods and Materials

Animals

Fourteen male Sprague–Dawley rats (Charles River GmbH, Sulzfeld, Germany) weighing 220–260 g (age, 3–4 mo) at the beginning of the experiments were used. Animals were maintained on a 12:12 hr light/dark cycle, and food and water were available *ad libitum*.

All experiments were performed in accordance with ethical guidelines for the use of animals in experiments and were approved by the local animal care committee (Regierungspräsidium Tübingen, ZP 4/02).

Odor Probes

Urine probes of bobcats, cougars, foxes, coyotes, and wolves were purchased from PredatorPee Inc. (Bangor, ME, USA). Urine probes of female cows and horses (females, males, geldings) were self-collected at two ecological farms in Waldhausen, a village close to Tübingen. Urine probes of female rats ($N=6$) were self-collected by using a metabolic cage at the animal facility of the Department of Animal Physiology of the University of Tübingen. Lastly, urine probes of healthy female and male cats were drawn by a local veterinarian by means of bladder catheterization. All samples were collected in the week before the experiments and were stored in a refrigerator at -18°C until they were used.

Samples were presented as follows: 3 ml of predator or nonpredator urine or saline (control) were administered in a Petri dish (5 cm diam.). The Petri dish was put in one corner of the arena (see below) at a distance of 5 cm from each wall of the arena.

Apparatus

All behavioral tests were conducted in a square arena with walls and a cover made from transparent Plexiglass (92×92 cm; ActiMot, TSE Systems, Bad Homburg, Germany). Illumination of the arena was between 200 and 250 lx. There was no ventilation of the arena during the tests. Rat movements were monitored by infrared detectors (distance between two detectors: 2.5 cm, height: 2.5 cm), and rearings were detected by a second arrangement of infrared detectors (height: 12.5 cm). The ActiMot software automatically calculated the motor activity [distance traveled (m)], the number of rearings, the duration of “resting behavior” (activity <1 cm/sec), the total time spent in the middle of the arena (size of the middle: 46×46 cm), and the total time spent in the corner with the urine probe (size of the “corner”: 23×23 cm).

In addition, the behavior of the animal was videotaped. The videotapes were analyzed by an observer who was unaware of the test conditions. The duration and frequency of the following types of behavior were recorded: flat back approach, contact with the sample, grooming, and freezing. The time elapsed before first contact with the sample and the number of fecal boli were recorded.

Behavioral Procedures

To familiarize the rats with the arena, each animal was placed together with odorless saline solution in a Petri dish (3 ml saline) into the arena for 10 min once per day on 3 consecutive d. On the following 10 d, a urine sample or saline control solution was put in one corner of the arena, and the rat was put in the opposite corner. The rat’s behavior was monitored for the next 10 min by the infrared detectors and videotaped as described above.

Each rat was tested daily between 8:00 AM and 4:00 PM with one of the samples. Samples were presented in a pseudorandomized order, and the corner with the urine samples (and thereby also the animal’s “start” corner) was changed in pseudorandomized order. The arena was cleaned with soapy water and 70% ethanol after each test, and then ventilated with clean air for 45 min (ca. 15 l/min). Control studies in our laboratory with

trimethylthiazoline, an intensive synthetic predator odor inducing strong fear behavior, showed that the cleaning procedure removed all odors that might affect the animal's behavior.

Statistical Analysis

Data are presented as mean values with standard error of the mean. The data were also grouped for each animal according to sample type (i.e., predator urine, nonpredator urine, female rat urine, and saline controls). All data were normally distributed except for the number of rearings. Normally distributed data were analyzed by repeated-measures analyses of variance (ANOVAs) with odor or odor group as a within-subject factor. Student's *t*-test (with Bonferroni correction) was used for pairwise comparisons with the saline control. Nonnormally distributed data were analyzed by a Kruskal–Wallis test; pairwise comparisons were done by the nonparametric multiple comparison according to Zar (1999, p. 224).

Results

The type of urine used strongly affected the time the animals spent in the corner with the urine probe (ANOVA: $F_{9,108}=11.59$, $P<0.001$; Figure 1a). Pairwise comparisons with the saline control showed that animals spent less time in the sample corner in the case of urine from wolves ($t=5.01$, $P<0.001$), coyotes ($t=4.7$, $P=0.001$), bobcats ($t=3.75$, $P=0.003$), cougars ($t=3.79$, $P=0.003$), and foxes ($t=2.42$, $P=0.03$), but more time in this corner in the case of urine from female rats ($t=2.66$, $P=0.02$). The urine of cows, horses, and cats did not affect the time spent in the sample corner ($t<1.09$, $P>0.30$). After the data had been grouped according to sample type, ANOVA revealed significant effects of the different sample types ($F_{3,39}=14.78$, $P<0.001$). Pairwise comparison with the saline controls showed significant effects for predator urine ($t=2.6$, $P=0.02$) and female rat urine ($t=3.04$, $P=0.009$), but not for nonpredator urine ($t=1.21$, $P=0.25$). Furthermore, the effects of exposure to predator urine and nonpredator urine differed ($t=5.26$, $P<0.001$). Typical traces of an individual rat during exposure to different urine samples illustrate avoidance behavior indicated by reduced time spent in the corner with the predator urine samples (Figure 2).

Flat back approaches are not direct and fast approaches to the samples; rather, they are more of a hesitant, sneaking approach to the threatening stimulus, with many stops. Nonparametric analysis with the Kruskal–Wallis test revealed an effect of urine samples on the number of flat back approaches ($H=29.87$, $P<0.001$; Figure 1b). *Post-hoc* pairwise comparisons showed an increase in the number of flat back approaches during exposure to the urine of coyotes, bobcats, cougars, foxes, and wolves ($Q>10.56$, $P<0.001$), but not during exposure to the urine of cats, cows, horses, and female rats ($Q=0$). The results were confirmed by an analysis of the grouped data ($H=19.72$, $P<0.001$): compared with the saline controls, predator urine led to an increase in the number of flat back approaches ($Q=6.35$, $P<0.001$), whereas the urine of nonpredators or female rats did not ($Q=0$). In addition, a difference was found between the effects of predator urine and nonpredator urine ($Q=56$, $P=0.007$).

The type of urine used influenced the time elapsed before the animal's initial contact with the samples (ANOVA: $F_{9,108}=2.41$, $P<0.02$; Figure 1c). On the other hand, pairwise comparisons showed no significant differences in this elapsed time before contact with the saline sample and other samples ($t<2.1$). However, after the data were grouped (ANOVA:

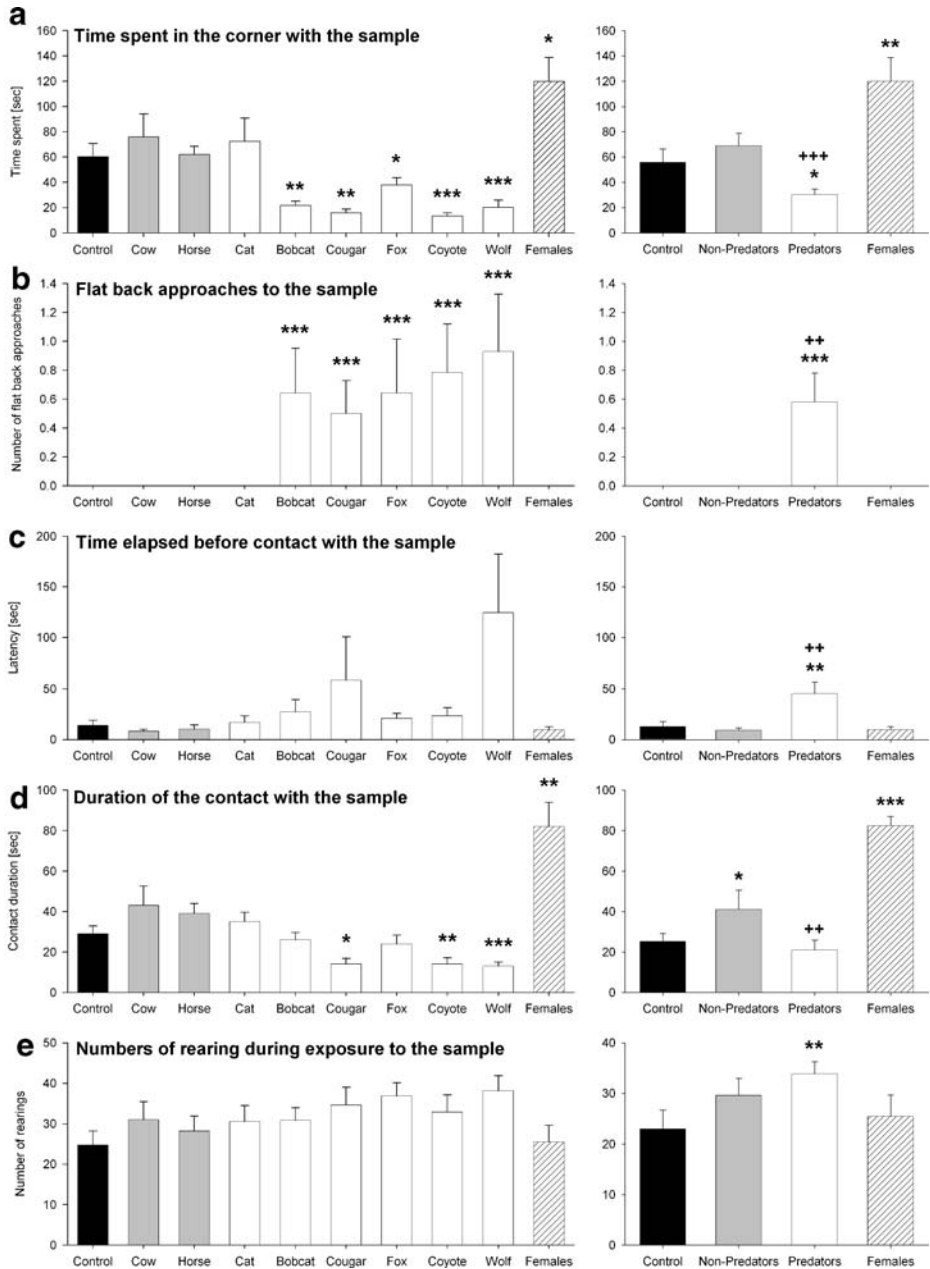
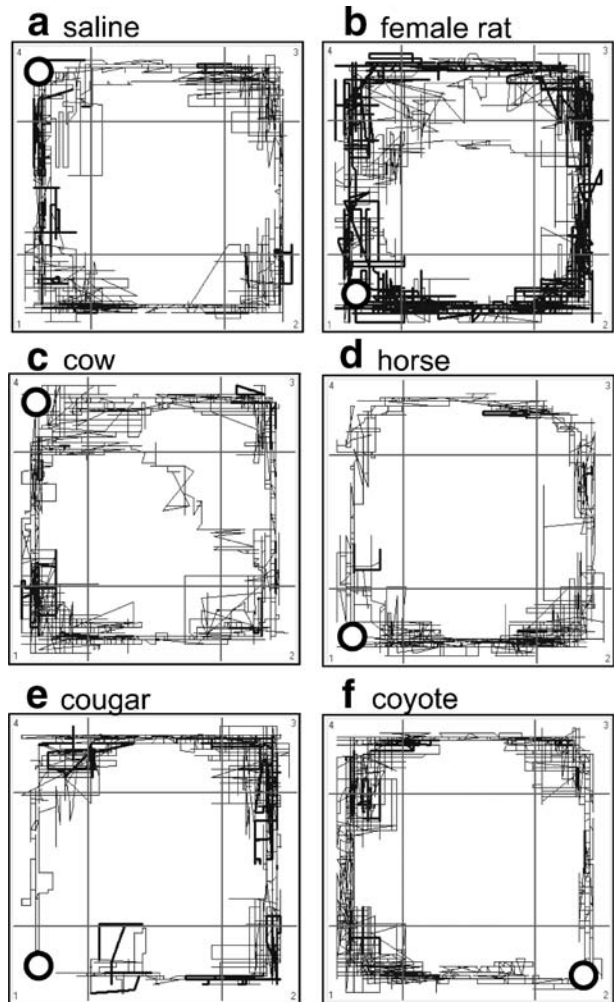


Fig. 1 Behavioral effects of exposure to different urine samples. (a) Time spent in the corner with the sample; (b) numbers of flat back approaches to the sample; (c) time elapsed before contact with the sample; (d) duration of the contact with the sample; (e) numbers of rearing during exposure to the different samples. Left panel illustrates the mean behavioral effects to each urine sample (\pm SE); right panel illustrates the mean behavioral effects to the different odor groups (\pm SE). *** P <0.001, ** P <0.01, * P <0.05, comparison with the saline control (after an ANOVA). +++ P <0.001, ++ P <0.01, comparison with exposure to nonpredator urine (after an ANOVA)

Fig. 2 Typical examples of traces of one rat during exposure to the saline control (a), to urine of a female rat (b), to urine of the nonpredator cow (c) and horse (d), and to urine of the predator cougar (e) and coyote (f). Dark ring represents the placement of the urine sample



$F_{3,39}=8.34$, $P<0.001$), an increase was found in the time elapsed before contact with predator urine samples compared with that before contact with the saline controls ($t=3.60$, $P=0.003$) and the nonpredator urine samples ($t=3.29$, $P=0.006$).

For contact duration, ANOVA showed an influence of the urine samples ($F_{9,108}=11.41$, $P<0.001$; Figure 1d). Pairwise comparison with the saline controls revealed an increase in the duration of contact with the urine of female rats ($t=3.65$, $P=0.004$) and a reduced duration of contact with the urines of coyotes, wolves, and cougars ($t>3.07$, $P<0.01$). However, after grouping the data (ANOVA: $F_{3,39}=14.45$, $P<0.001$), the effects of female rat urine were still found ($t=4.21$, $P=0.001$), but longer contact with nonpredator urine was found ($t=2.59$, $P=0.02$). No effect of predator urine was found on contact duration relative to the saline control ($t=0.88$, $P=0.38$), but there was a difference with nonpredator urine ($t=3.42$, $P=0.005$).

There were no effects of individual urine samples on the rat's rearing behavior (ANOVA: $F_{9,108}=1.63$, $P=0.12$; Figure 1e). However, after grouping the data (ANOVA:

$F_{3,39}=3.01$, $P<0.041$), a significant increase was found in rearing during exposure to predator urine ($t=3.32$, $P=0.006$). There was also a trend to increased rearing behavior with nonpredator urine ($t=1.99$, $P=0.07$). No significant difference was found in rearing behavior during exposure to predator urine and exposure to nonpredator urine ($t=1.44$, $P=0.17$).

Analysis of the data on urine samples both singly and as group showed no statistical effects of the predator urine samples on resting behavior, time spent in the middle of the arena, general motor activity (distance traveled), freezing, grooming, and numbers of fecal boli (data not shown).

Discussion

Our expectation was that defensive behavior is only observable during exposure to predator urine, but not during exposure to other samples. It should be noted that the rats used in the study were totally naive to all odors tested, i.e., that observed differences cannot be a consequence of some odors being unknown, with others being known. Exposure to urine samples of canid and feline predators induced different defensive behaviors in the animals. Behavioral responses to the different predator urine samples, with exception of cat urine samples, were similar. Possible reasons for this exception are discussed below.

Animals spent less time in corners with urine samples of canid and feline predators than in corners with saline controls and nonpredator urine, indicating avoidance behavior. Furthermore, flat back approaches, a form of risk assessment (Blanchard et al., 1990b), were only observed only in the presence of predator urine samples. In addition, there was a longer delay in making contact with predator urine samples, and the duration of contact with some of the predator odors (coyote, cougar, and wolf) was significantly decreased. Interestingly, rearing behavior—an indicator of exploratory behavior and vigilance—was increased in frequency during exposure to both predator and nonpredator urine. Other behavior indicators such as resting behavior, freezing, general motor activity (distance traveled), and grooming were not affected by exposure to predator urine. The first two indicators often increase and the latter two often decrease in frequency during exposure to aversive stimuli (Yamada and Nabeshima, 1995; Blanchard et al., 2003a; Misslin, 2003; Apfelbach et al., 2005). Female rat urine increased both the time spent in the sample corner and the duration of contact with the sample (cf. Markham et al., 2004), clearly showing that our experiment was sensitive not only to aversive, but also to attractive odors.

All types of behaviors analyzed in the present study are potentially modulated by danger. Freezing behavior is a species-specific defensive response in rats and is often used to measure fear (Fendt and Fanselow, 1999; Wiltgen and Fanselow, 2003; Sullivan et al., 2004). Surprisingly, freezing behavior was infrequent in the present study and was not exclusive to predator urine, ostensibly indicating that the canid and feline predator odors used here were unable to induce defensive behavior. However, this conclusion must be rejected for several reasons. Freezing behavior is observed only when the threatening stimulus is very intense, i.e., danger is acute (e.g., Blanchard and Blanchard, 1990; McNaughton and Corr, 2004). Indeed, intense stimuli such as foot shocks or high concentrations of trimethylthiazoline (a component of the fox odor) were able to induce freezing behavior in the experimental setup used in the present study (Fendt, unpublished observations). Results of the present study, therefore, indicate that the canid and feline predator urine samples used in this study are not a strong indicator of acute danger and do not induce freezing behavior. It is also questionable whether freezing would be an adequate

response to predator odor. While predator odor indicates the presence of a predator in the area, it is not implicitly an indicator of acute and direct predatory threat. Therefore, it is more expedient for the prey animal to intensify its risk assessment behavior or to avoid an area with predator odor in order to prevent an encounter with the predator (cf., McNaughton and Corr, 2004). On the other hand, freezing responses and flight or fight behavior are more relevant when there is acute and direct danger of encountering a predator. Another possible explanation for the lack of freezing behavior in experiments involving the presence of predator odors is that the occurrence of freezing behavior depends upon the test context. It has been shown repeatedly that a threatened animal responds by freezing only if it has no way to avoid the threatening stimulus (e.g., Blanchard and Blanchard, 1971). Otherwise, it responds with flight or avoidance behavior rather than freezing behavior.

Consequently, either the quality or intensity of the stimuli in the present study or the testing context itself was such that freezing behavior and the related inhibition of spontaneous activity and of grooming were not an appropriate response for our test animals during exposure to the samples of predator urine. As discussed above, a more appropriate response when the threatening stimulus is lower in intensity might be risk assessment behavior (cf., McNaughton and Corr, 2004) such as flat back approaches (or “stretch approaches”; e.g., Markham et al., 2004). In the present study, flat back approaches were observed only in response to the urine probes of canid and feline predators and were much more frequent as the animals approached these samples.

Direct exploration of stimuli occurs much faster when the stimuli are potentially dangerous (cf., Markham et al., 2004). For example, I found that the duration of contact with the urine probes of canid and feline predators was much shorter than with other stimuli. In contrast, the urine of female rats, which is highly attractive to male rats, was explored much longer (cf., Markham et al., 2004).

Potentially dangerous stimuli are approached not only more slowly and for a shorter period of time, but they are also avoided more thereafter (reviewed in McGregor et al., 2002; Apfelbach et al., 2005; Takahashi et al., 2005). In the present study, animals spent less time in corners with urine samples of canid and feline predators than those containing urine of nonpredators and female rats or a saline control solution. Quantitatively, in fact, this type of behavior, i.e., time spent in the urine sample corner or avoidance of that corner, was the most pronounced of all the behavioral responses assessed.

In summary, different defensive behaviors were induced by urine samples of canid and feline predators, but not by the other nonpredator samples. Although the behavioral effects of the predator urine were by and large similar, there was one major exception: the cat urine did not induce defensive behavior. This has also been demonstrated by Blanchard et al. (2003b). In that study, avoidance behavior, decreased duration of contact, and increased frequency of risk assessment behavior and freezing were induced by exposure to cat feces and cat fur/skin, but not by exposure to cat urine. This may be explained as follows. First, predator urine in general appears to be less fear-inducing than the odor of feces or skin/fur (cf., Blanchard et al., 2003b; only cat odor was tested in this study). Second, cat odor may change during domestication so that its fear-inducing properties are weakened or lost altogether. Third, cat urine was acquired in the present study by bladder catheterization. Urine acquired in this way is likely to induce less fear, as the urine used for scent marking is usually enriched by secretions of the anal gland. Fourth, domesticated cats may have a vegetarian or mixed diet changing the properties of the cat odor especially of the urine odor (cf., Berton et al., 1998).

In contrast to cat urine, urine of the other canid and feline predators used in the present study induced defensive behavior. Similar effects have been shown in a number of studies

with other prey species: beavers, black-tailed deer, snowshoe hares, deer mice, guinea pigs, house mice, gray squirrels, meadow voles, and mountain voles during exposure to coyote, wolf, fox, and bobcat urine (Sullivan et al., 1985a,b, 1988; Epple et al., 1993; Nolte et al., 1994; Swihart et al., 1999; Rosell, 2001), and, most relevantly, in rats during exposure to fox urine (Burwash et al., 1998; Campbell et al., 2003; Farmer-Dougan et al., 2005). The results of all these studies were confirmed here. In contrast to the previous work, however, the present study used (1) both predator and nonpredator urine samples and (2) laboratory animals with no experience whatever of predators or their odors. In other words, this present experiment is the first to conclusively demonstrate that several predator odors (here: canid and feline urine), but not other natural odors (here: urine of conspecifics and herbivores) induce innate defensive behavior in inexperienced rats.

Taken as a whole, the study showed that exposure to canid and feline predator urine induces defensive behaviors, particularly avoidance and risk assessment behavior, in predator-odor naive rats. It should be mentioned that this study is limited in that only canid and feline urine was used as predator odors and only herbivore urine was used as nonpredator odor. However, it is the first to test *different* predator and nonpredator probes in laboratory-reared animals. The results support the hypothesis that the effect of predator odors on the behavior of many species observed in numerous studies (reviewed in Kats and Dill, 1998; Apfelbach et al., 2005) is based on an innate recognition of predator odors. Such an innate “recognition” of predator odor is a survival advantage. In parallel, an innate recognition of visual and acoustic predator cues has also been discussed in other studies (e.g., Hirsch and Bolles, 1980; Hendrie, 1991; Hendrie et al., 1996; Eilam et al., 1999).

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Introduction

Acoustic and chemical signals can play important roles during mate choice in many insects, including *Drosophila* species (Gerhardt and Huber, 2002). Pheromonal signaling systems can influence both reproductive isolation and speciation, in addition to influencing proximate decisions during courtship (Greenfield, 2002). It is of considerable interest to identify particular mate–mate signals that influence mating success in local populations and those that may determine isolation between populations or species. In some cases, mate recognition systems can influence both sexual selection and sexual isolation (Boake et al., 1997; Carson, 2000). Understanding both the genetic and environmental influences on expression of these signaling systems should help to reveal the nature of genetic differences in mate recognition systems and their evolution (Etges, 2002).

In many *Drosophila* species, epicuticular hydrocarbons (EHC) are thought to serve as contact pheromones as well as to enhance desiccation resistance (Blomquist et al., 1987). Although the genetic basis of some hydrocarbon components is partially understood in some drosophilids (reviewed in Gleason et al., 2005), genetic and environmental influences on production and deposition (Wigglesworth, 1988) are poorly known for most species. Once revealed, the structure and function of individual hydrocarbon genes alone will be insufficient without knowledge of how EHCs are expressed in natural or experimental populations subject to potential environmental modification. We seek to gain a further understanding of the role of lifetime environmental influences on genetic variation in EHC production in insects and how this EHC variation influences behavior, mate choice, sexual selection, and sexual isolation in nature (e.g., Toolson and Kuper-Simbron, 1989; Howard et al., 1995; Howard, 1998; Liang and Silverman, 2000; Woodrow et al., 2000; Savarit and Ferveur, 2002; Buczkowski et al., 2005).

Here, we investigate the role of dietary triacylglycerols in larval *Drosophila mojavensis* on subsequent adult EHC profiles. Differences in natural larval feeding substrates vs. synthetic laboratory media influence EHC abundance in this species (Stennett and Etges, 1997). These differences also influence adult mating preferences (Etges, 1992, 1998; Brazner and Etges, 1993). In *D. mojavensis*, the function of cuticular lipids seems unrelated to desiccation resistance by reducing water loss (Gibbs et al., 1998), underscoring their role, and probable maintenance, as contact pheromones (Etges and Ahrens, 2001). We hypothesize that identifying substrate-specific precursors that influence adult EHC profiles will help to resolve host plant influences on mating behavior, as well as identify ecological sources of variation on patterns of reproductive isolation in nature.

Fatty acid metabolism and EHC biosynthesis have been well studied in *D. melanogaster* and should help in understanding lipid metabolism in *D. mojavensis*. Adult EHC production involves modification of fatty acid precursors, but little is known about the influences of fatty acids in larval diets on adult EHCs. Production of adult *D. melanogaster* sex pheromone (*Z,Z*)-7,11-heptacosadiene starts from either acetate or from medium-sized fatty acids (Chan Yong and Jallon, 1986; Jallon et al., 1986). From preformed fatty acids produced by fatty acid synthetase (de Renobales and Blomquist, 1984), palmitate is a better substrate than stearate and is desaturated into palmitoleate (ω -7) (Ferveur et al., 1989) by the *desat1* gene product (Wicker-Thomas et al., 1997; Dallerac et al., 2000; Labeur et al., 2002). From palmitoleate there is elongation to *cis*-vaccenate and subsequently several elongations, a second desaturation (the substrate of which has not yet been characterized) by another desaturase (Wicker-Thomas and Jallon, 2001; Jallon and Wicker-Thomas, 2003), and a subsequent decarboxylation (Jallon, 1984; Pennanec'h et al., 1997). Fatty acid

analysis of sexually mature *D. melanogaster* indicates high percentages of palmitoleate, but lesser quantities of oleate and *cis*-vaccenate, with oleate and linoleate decreasing as (*Z,Z*)-7,11-heptacosadiene synthesis occurs (Pennanec'h et al., 1997). Very long chain (C_{20} , C_{22} , C_{24} , and C_{26}) fatty acids with apparent ω -7 unsaturation and female dienoic acids with 22 and 24 carbons have also been detected (Pennanec'h et al., 1997). Together, these experiments and those of other groups (Keith, 1967a,b; Pennanec'h et al., 1991) suggest that fatty acids, either from biosynthesis or the diet, impact the biosynthesis of pheromones and other dipteran EHCs.

We hypothesize that different fatty acids in the cactus diets of *D. mojavensis* can change pheromone and other adult EHC profiles. A number of different hosts are used by *D. mojavensis*, including agria cactus, *Stenocereus gummosus*, in Baja California and organ pipe cactus, *S. thurberi*, in mainland Mexico and Arizona (Heed and Mangan, 1986). Fatty acid profiles of both agria and organ pipe cactus are mainly characterized by caproic (C_{10}) and lauric (C_{12}) acids in concentrations of 34–45%, with much lower concentrations of caprylic acid (C_8) and others in sizes up to C_{18} (Fogleman and Kircher, 1986). There are no overall significant differences between cactus species with respect to fatty acid composition (Fogleman and Kircher, 1986). However, cactus fatty acid analysis shows greater quantities of unsaturated fatty acids (12:1, 12:2, and 18:1) in agria vs. organ pipe cactus, but the location of double bonds has not been determined. It may be that minor quantities of unsaturated fatty acids in the diet of *D. mojavensis* influence biosynthesis of unsaturated EHCs, so we employed both saturated and unsaturated triacylglycerols to determine whether they influence EHC profiles of adult *D. mojavensis*.

EHCs of adult *D. mojavensis* are composed of C_{28} to C_{40} alkanes, 2-methylalkanes, alkenes, methyl-branched alkenes, and multimethylene interrupted alkadienes, with the largest fraction of adult hydrocarbons composed of C_{35} alkadienes (Toolson et al., 1990; Stennett and Etges, 1997; Etges and Jackson, 2001). We hypothesize that the EHCs of *D. mojavensis* are derived from three fatty acid pools: ω -9, ω -7, and saturated fatty acids. The ω -9 fatty acids are precursors of the 9-alkenes and the 8, *X*-alkadienes (e.g., $C_8=C_{16}=C_9$, $C_8=C_{18}=C_9$, $C_8=C_{20}=C_9$, etc.) since they are really 9, *X*-alkadienes counting from the other end. The ω -7 fatty acids are precursors to the 7-alkenes and the 7, *X*-alkadienes (e.g., $C_7=C_{16}=C_{10}$, $C_7=C_{18}=C_{10}$, $C_7=C_{20}=C_{10}$, etc.). The saturated fatty acids are precursors to the alkanes, and the 10-, 12-, and 8-alkenes also result from saturated fatty acids when desaturation occurs late in the biosynthetic pathway.

Given the previously described fatty acid profiles in agria and organ pipe cactus, and the substrates known to be involved in EHC biosynthesis, we used tripalmitolein ($C_{16}:1$, ω -7), tristearin (C_{18}), and triolein ($C_{18}:1$, ω -9) in differing concentrations in larval diets to assess their influences on egg to adult viability and on adult EHC composition in two populations of *D. mojavensis*.

Methods and Materials

Fly Stocks

Populations of *D. mojavensis* were collected in 1996 from south of Santa Rosalia, Baja California Sur (stock no. A975, 15 adults), and El Fuerte, Sinaloa (stock no. A991, 185 adults), over baits or by collecting adults emerging from fermenting cactus tissues, “rots,” returned to the laboratory. All flies were maintained on banana–malt–yeast–agar food at room temperature in mass cultures (Brazner and Etges, 1993). Several hundred adults from

each population were placed in separate oviposition chambers and kept in an incubator programmed for a 14:10 L/D photoperiod and a temperature cycle of 27:17°C. Eggs were collected daily on oviposition media from 8:00 AM to 6:00 PM in removable Petri dishes attached to each chamber. Eggs were washed with deionized water, 70% ethanol, and surface sterilized for 10 min in 70% ethanol. Eggs were then counted in sterile, deionized water in groups of 100 onto 1-cm² pieces of sterile filter paper and placed into vials containing *Drosophila* medium (see below). Filter paper was removed after 24–48 hr, and numbers of unhatched eggs were counted in order to calculate egg to adult viability.

Food vials were prepared with standard Instant *Drosophila* Medium[®] formula 4–24 (Carolina Biological Supply Co., Burlington, NC, USA); or with “defatted” medium (both controls), as well as by adding different concentrations of triacylglycerols (TAGS) to the dry, homogeneous defatted diet. For the defatted medium, lipids were removed from the standard medium by Soxhlet extraction with hexane. We prepared 9% stock solutions of each triacylglycerol. Triolein (ω -9), tripalmitolein (ω -7), and tristearin in pentane were added to 2 g of dry defatted diet in final concentrations of 1%, 3%, and 9%. Each 2-g aliquot of food was completely dried under a fume hood, added to a sterile 8 dram shell vial, and rehydrated with an equal volume of sterile, deionized water. Combinations of equal amounts of all three TAGs were also prepared so that the final concentrations of the three combined would be 1%, 3%, and 9%. These latter treatments were prepared to test the hypothesis that all three TAGS should produce EHC profiles more like the standard or defatted diets, but at comparable concentrations to the single TAG treatments. Two replicate cultures of each TAG concentration were created for each population of *D. mojavensis*, and six replicates were included for both the standard and defatted diets. Live baker’s yeast was added to each vial *ad libitum* to insure larval survivorship.

Epicuticular Hydrocarbon Analyses

All eclosed adults were aspirated from each culture vial daily, sexed under light CO₂, and placed into separate vials containing banana–yeast–malt–agar food for at least 14 d and then frozen in hexane-rinsed glass vials at –20°C. EHCs were extracted from groups of males and females in Biosil minicolumns. Each column consisted of a Pasteur pipette containing packed glass wool and Biosil (silica gel, Sigma[™] S-4133, Sigma-Aldrich, St. Louis, MO, USA) flushed several times with HPLC-grade hexane. Flies were added to the top of the column, washed in 8 ml of hexane, and the hydrocarbons were collected in hexane-rinsed vials. After the hexane was evaporated with nitrogen, each sample was sealed and stored at –20°C. When only one or two flies were available from some replicates, we used hydrocarbon extraction procedures described by Savarit et al. (1999). Flies were placed in conical glass microvials and immersed in 50 μ l hexane per fly. The vials were agitated, and after 20 min, flies were removed, the hexane was evaporated off, and each vial was frozen.

Each hydrocarbon sample was redissolved in hexane (2 μ l/fly) containing 382 ng of docosane (C₂₂) per μ l as an internal standard with the exception of samples containing only one or two flies, which were redissolved in 5 μ l hexane with the same concentration of internal standard. One μ l of each sample was analyzed by capillary gas–liquid chromatography using a Shimadzu GC14 (Shimadzu Scientific Instruments, Columbia, MD, USA) fitted with a 30 m DB-1 fused silica column (Agilent Technologies, Palo Alto, CA, USA). Injector and detector temperatures were set at 345°C with the injector port in split mode (split ratio=100:1) and the column was heated from 200°C to 345°C at 10°C/min holding at 345°C for 5 min (Stennett and Etges, 1997).

Statistical Analyses

Egg to adult viability was calculated as the number of eclosed adults divided by the number of hatched eggs. Variation in viability due to substrate and TAG concentration was assessed with analysis of variance (ANOVA; SAS Institute, 2004). Viability data were arcsin transformed prior to analysis.

EHC amounts were estimated by analysis of peak integrations using EZCHROM software (ver. 2.1) provided by Shimadzu. Each sample amount was normalized by the amount of internal standard. Replicate groups of flies were analyzed together. All data were expressed as ng/fly of EHCs and were analyzed by ANOVA and multivariate analysis of variance (MANOVA) using PROC GLM in SAS (SAS Institute, 2004) with population, TAG, TAG concentration, and sex as main effects and all interactions between main effects. Population and all interactions with population were considered random effects.

Post-hoc multiple comparisons were evaluated, and canonical correlation analysis in PROC GLM and CANDISC was employed to reveal EHC responses to different TAGs. Contrasts among diet treatments were also carried out using EHCs grouped by chemical structure according to Etges and Jackson (2001).

Results

Effects of Triacylglycerols on Egg to Adult Viability

Type of TAG, concentration of TAG, and their interactions had significant effects on egg to adult viability in these two populations of *D. mojavensis* (Fig. 1, Table 1). Overall, flies reared on standard medium, defatted medium, or defatted medium with tristearin had equivalent viabilities that were significantly higher than those from flies reared on defatted medium with triolein, tripalmitolein, or combinations of all three TAGs (Tukey's multiple range test, $P < 0.05$). Except for tristearin, concentrations of TAGs exceeding 1% drastically reduced viability: egg to adult viability of controls (mean \pm 1 SD on standard diet = 0.47 ± 0.21 ; on defatted diet = 0.57 ± 0.30) and of defatted diet with all three TAGs at 1% (0.24 ± 0.10) were higher than viabilities for diets with TAGs at 3% and 9% (both 0.18; Tukey's multiple range test, $P < 0.05$). Egg to adult viability of laboratory populations of *D. mojavensis* reared on fermenting cactus or banana food tend to be much higher (> 0.80 , see Etges and Ahrens, 2001) than on standard diet, suggesting that the latter medium reduced preadult fitness in these experiments. Similar viabilities of flies reared on standard and defatted diets were surprising given the removal of lipids in the latter (Fig. 1), but may have been mediated by the addition of live baker's yeast.

At triolein and tripalmitolein concentrations of 3% and 9%, very few flies survived to eclosion (Fig. 1), with many never making it to the pupal stage. At these higher concentrations, flies that did eclose seemed to be unable to initiate cuticle hardening and died before wing expansion.

Effects of Triacylglycerols on Adult Epicuticular Hydrocarbons

Differences in EHC amounts due to TAG type were apparent for all but six of the 25 EHC components assayed (Table 2 for quantities and Appendix for ANOVA of the 20 most abundant EHCs). The ANOVA results were based on all TAGs employed, plus combinations of them at each concentration for which flies eclosed. The two control media were not included in these ANOVAs so we could focus on TAG differences. In most cases,

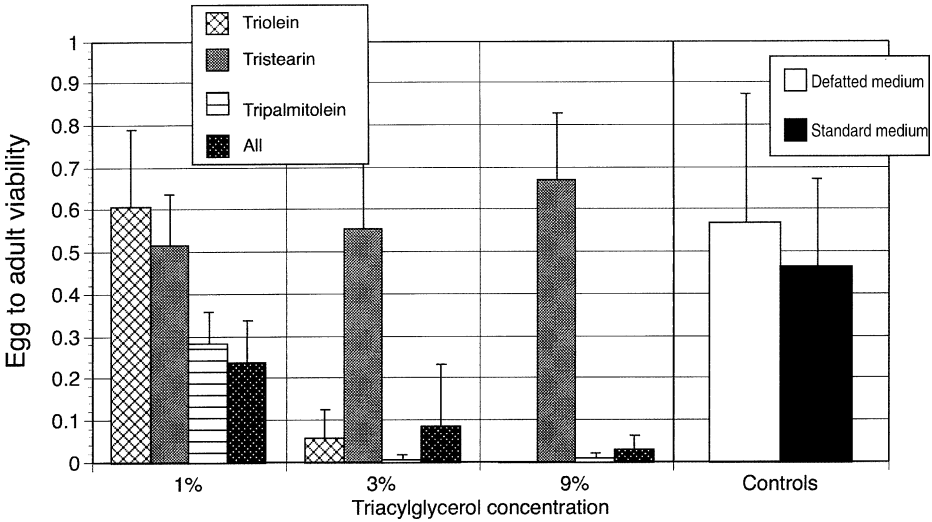


Fig. 1 Egg to adult viability (± 1 SD) of two populations of *Drosophila mojavensis* cultured on defatted media with 1%, 3%, and 9% triacylglycerols alone and in combination. Live Baker's yeast was added to all cultures when initiated. See the text for details

EHC amounts were lowest for flies reared on defatted media, but similar among flies reared on standard medium and defatted media supplemented with TAGs (Table 2). Missing data due to high or total egg to adult mortality, particularly in the 3% and 9% tripalmitolein-containing cultures, meant that TAG type and concentration were somewhat confounded in the ANOVAs. This may partially explain the lack of a concentration effect in these analyses, except in a few cases.

EHC differences due to TAG type were overlaid on the known population differences that in some cases resulted in Population \times TAG interactions (Appendix). Consistent with most previous studies, 13 of the EHC components differed between the Baja California and Sinaloa (mainland) populations, especially 2-methyloctacosane ($C_{28.65}$), 8,24-tricontadiene ($C_{33.63}$), 9,25-pentatricosadiene ($C_{34.59}$), and 9,27-heptatricosadiene ($C_{36.5}$). The latter three alkenes showed characteristic regional differentiation in amounts, where mainland populations all expressed much greater amounts of these EHCs than Baja California populations (Stennett and Etges, 1997; Etges and Ahrens, 2001). Two of these EHCs, $C_{33.63}$ and $C_{34.59}$, exhibited Population \times TAG interactions suggesting that even though amounts

Table 1 ANOVA results for the effects of triacylglycerol type and concentration on egg-to-adult viability in two populations of *Drosophila mojavensis*^a

Source	<i>df</i>	Type III SS	<i>F</i>	<i>P</i>
Population	1	0.118	2.28	0.140
Triacylglycerol (TAG)	4	2.375	11.42	<0.001
Concentration	2	0.641	6.19	0.005
Population \times TAG	5	0.247	0.95	0.461
TAG \times Concentration	6	0.866	2.78	0.025
Population \times TAG \times Concentration	8	0.084	0.20	0.989
Error	36	1.871		

^a Both control substrates were included in this analysis and all data were arcsin transformed prior to analysis.

Table 2 Quantities of the major epicuticular hydrocarbons in *Drosophila mojavensis* (ng/fly)^a

Hydrocarbon (ECL) ^c	Diet ^b												
	Group ^d	Standard	N	Defatted	N	Triolein	N	Tristearin	N	Tripalmitolein	N	Combination	N
<i>n</i> -Octacosane (C ₂₈)	M fem	6.93	6	9.37	2	10.33	3	5.35	6	11.90	4	0.61	1
	M mal	3.62	5	9.88	2	9.30	3	3.41	4	0.0	2	16.91	2
2-Methyloctacosane (C _{28:65})	B fem	2.24	5	2.80	2	3.36	3	6.38	6	0.89	2	2.84	6
	B mal	2.44	6	4.24	2	2.97	3	4.54	6	1.04	2	0.58	5
2-Methyltricosane (C _{30:65})	M fem	65.73	6	40.64	2	81.53	3	64.79	6	102.87	4	89.96	1
	M mal	84.10	5	57.92	2	92.22	3	58.73	4	107.93	2	109.96	2
2-Methyltricosane (C _{30:78})	B fem	52.22	5	59.56	2	81.63	3	46.96	6	57.54	2	74.29	6
	B mal	37.69	6	28.81	2	42.03	3	26.08	6	59.81	2	47.66	5
7- and 9-Hentricontene (C _{30:78})	M fem	74.05	6	55.49	2	83.09	3	77.55	6	136.06	4	79.76	1
	M mal	104.75	5	83.68	2	116.44	3	82.35	4	135.11	2	117.03	2
Unknown (C _{33:br1})	B fem	114.65	5	107.78	2	147.18	3	98.59	6	116.39	2	116.54	6
	B mal	104.48	6	69.45	2	77.57	3	58.10	6	148.58	2	89.83	5
11- and 13-Methyldotricontane (C _{33:br2})	M fem	11.17	6	9.81	2	12.19	3	8.72	6	10.21	4	14.45	1
	M mal	12.21	5	5.72	2	11.27	3	7.07	4	14.81	2	19.45	2
31-Methyldotricont-8-ene (C _{32:47})	B fem	8.50	5	11.81	2	20.00	3	11.77	6	8.54	2	12.91	6
	B mal	7.55	6	6.00	2	10.46	3	5.67	6	8.24	2	8.10	5
Unknown (C _{33:br1})	M fem	11.89	6	9.75	2	10.38	3	8.71	6	15.07	4	15.42	1
	M mal	11.72	5	12.02	2	11.87	3	7.90	4	15.96	2	12.29	2
11- and 13-Methyldotricontane (C _{33:br2})	B fem	5.99	5	6.18	2	5.92	3	6.29	6	3.77	2	6.74	6
	B mal	4.24	6	4.71	2	5.58	3	3.96	6	4.96	2	5.79	5
31-Methyldotricont-8-ene (C _{32:47})	M fem	13.62	6	10.35	2	13.32	3	15.04	6	15.12	4	17.4	1
	M mal	13.72	5	11.21	2	13.12	3	7.82	4	21.19	2	14.27	2
Unknown (C _{33:br1})	B fem	6.26	5	8.18	2	6.50	3	6.82	6	5.29	2	7.86	6
	B mal	4.13	6	4.37	2	5.98	3	4.00	6	5.79	2	5.42	5
31-Methyldotricont-8-ene (C _{32:47})	M fem	51.93	6	33.66	2	47.94	3	56.32	6	63.64	4	62.59	2
	M mal	66.81	5	51.19	2	58.92	3	37.01	4	74.78	2	73.43	6
Unknown (C _{33:br1})	B fem	42.53	5	52.67	2	67.37	3	44.86	6	43.79	2	51.55	6
	B mal	34.13	6	26.61	2	43.63	3	27.34	6	50.93	2	40.83	5

Table 2 (continued)

		Diet ^b											
Hydrocarbon (ECL) ^c		Standard	N	Defatted	N	Triolein	N	Tristearin	N	Tripalmitolein	N	Combination	N
Group ^d													
8,24-Tricontadiene (C _{32.63})	M fem	23.89	6	14.89	2	26.93	3	23.13	6	29.67	4	50.56	1
	M mal	45.91	5	30.05	2	32.95	3	27.95	4	38.87	2	63.86	2
	B fem	9.29	5	13.80	2	17.39	3	10.17	6	9.78	2	14.34	6
7,25-Tricontadiene (C _{32.70})	B mal	8.57	6	6.89	2	13.41	3	6.84	6	13.25	2	11.60	5
	M fem	44.30	6	29.83	2	38.01	3	44.91	6	65.43	4	60.78	1
	M mal	67.41	5	44.83	2	40.96	3	37.42	4	74.63	2	87.85	2
10-, 12-, and 14-Tritricontene (C _{32.79})	B fem	36.35	5	46.33	2	56.33	3	38.48	6	42.40	2	44.89	6
	B mal	29.68	6	23.62	2	26.69	3	22.85	6	51.06	2	29.23	5
	M fem	28.95	6	18.23	2	24.28	3	25.30	6	25.24	4	37.39	1
8,26-Tetracontadiene (C _{34.ene1})	M mal	26.59	5	12.24	2	19.11	3	12.45	4	32.05	2	45.68	2
	B fem	12.27	5	17.53	2	23.57	3	12.66	6	12.40	2	17.44	6
	B mal	7.79	6	6.95	2	9.17	3	6.22	6	14.09	2	9.43	5
6,26- and 6,24-Tetracontadiene (C _{34.ene2})	M fem	4.29	6	1.71	2	4.16	3	3.49	6	6.55	3	5.35	1
	M mal	7.91	5	8.35	2	7.72	3	4.71	4	6.48	2	12.32	2
	B fem	5.78	5	2.16	2	5.58	3	2.46	6	3.77	2	2.58	6
10-, 12-, and 14-Tetratritcontene (C _{34.ene})	B mal	5.02	6	3.66	2	4.92	2	3.29	6	4.19	2	3.55	5
	M fem	16.52	6	7.40	2	11.50	3	11.24	6	18.96	3	16.87	1
	M mal	26.97	5	18.32	2	18.89	3	16.78	4	28.57	2	43.75	2
33-Methyltetracont-10-ene (C _{35.ene1})	B fem	10.23	5	12.57	2	19.36	3	8.37	6	9.69	2	12.93	6
	B mal	14.45	6	11.35	2	20.54	2	10.55	6	21.65	2	17.45	5
	M fem	5.02	6	3.71	2	3.66	3	3.22	6	8.43	3	4.69	1
33-Methyltetracont-10-ene (C _{35.ene1})	M mal	15.08	5	6.41	2	8.72	3	5.59	4	7.89	2	12.99	2
	B fem	6.47	5	12.22	2	23.19	3	8.68	6	8.04	2	11.64	6
	B mal	21.14	6	7.11	2	12.72	2	8.43	6	20.64	2	13.95	5
	M fem	13.94	6	6.66	2	11.44	3	11.18	6	15.08	4	14.08	1
	M mal	14.99	5	10.26	2	12.28	3	8.34	4	21.23	2	13.00	2
	B fem	12.49	5	8.42	2	12.15	3	8.24	6	9.28	2	10.15	5

33-Methyltetracont-8-ene (C _{35ene2})	B mal	10.12	6	4.23	2	5.29	2	5.50	6	15.80	2	9.31	4
	M fem	12.83	6	7.22	2	9.91	3	10.59	6	16.65	4	11.77	1
	M mal	16.17	5	10.88	2	15.25	3	7.97	4	19.59	2	13.67	2
	B fem	19.35	5	15.52	2	19.36	3	15.52	6	19.46	2	15.70	5
	B mal	23.41	6	10.85	2	12.05	2	11.42	6	30.64	2	19.63	4
9,25-Pentatriacontadiene (C _{34.59})	M fem	119.17	6	53.13	2	128.50	3	118.84	6	196.25	4	192.53	1
	M mal	251.47	5	131.32	2	194.74	3	129.08	4	174.05	2	276.47	1
	B fem	48.79	5	62.75	2	104.00	3	35.65	6	39.46	2	65.30	6
	B mal	40.47	6	42.14	2	89.88	3	36.26	6	50.14	2	45.67	5
	M fem	235.63	6	150.38	2	211.04	3	196.62	6	370.85	4	264.95	1
8,26- and 7,27-Pentatriacontadiene (C _{34.66})	M mal	360.07	5	273.21	2	325.89	3	195.64	4	335.62	2	410.35	2
	B fem	285.24	5	379.67	2	621.31	3	299.10	6	241.24	2	386.02	6
	B mal	312.52	6	245.37	2	409.28	3	227.41	6	448.76	2	287.34	5
	M fem	27.11	6	9.45	2	28.94	3	18.84	4	27.96	4	25.21	1
	M mal	26.29	5	14.02	2	21.75	3	12.68	4	26.94	2	29.46	2
8,28-Heptatriacontadiene (C _{36.6})	B fem	7.38	5	11.40	2	18.67	3	8.70	6	6.88	2	11.45	6
	B mal	10.62	6	7.60	2	12.01	3	5.99	6	14.26	2	6.91	5
	M fem	48.30	6	22.37	2	30.96	3	33.98	6	60.23	4	37.61	1
	M mal	31.00	5	21.48	2	27.59	3	16.08	4	42.04	2	28.71	2
	B fem	22.32	5	26.55	2	59.59	3	17.94	6	26.04	2	30.55	6
14-, 16-, and 12-Hexatriacontene (C _{36.7})	B mal	24.05	6	11.33	2	34.33	3	11.91	6	32.25	2	21.01	5
	M fem	29.71	6	19.06	2	24.71	3	23.49	6	32.42	4	22.70	1
	M mal	22.76	5	15.78	2	21.48	3	11.41	4	31.78	2	26.89	2
	B fem	33.59	5	39.39	2	82.67	3	34.30	6	27.44	2	43.09	6
	B mal	31.30	6	20.69	2	34.66	3	18.82	6	33.85	2	20.56	5

^a See Toolson et al. (1990) and Erges and Jackson (2001) for details of the GC-MS analyses and determination of the double bond locations.

^b Diet refers to the two control diet treatments in this study and the three triacylglycerols (concentrations combined) added to the defatted diet. Combination refers to the combination of all three triacylglycerols. *N*=number of replicate cultures.

^c ECL is the equivalent chain length of each hydrocarbon component, if known.

^d Group definitions are M fem=mainland females; M mal=mainland males; B fem=Baja females; B mal=Baja males.

differed between populations, Sinaloa EHCs responded to the three TAGs far differently than Baja California EHCs (Fig. 2a). In both cases, combinations of the three TAGs increased EHC amounts in the Sinaloa population far more than in the Baja population. For the large $C_{34.59}$ diene component, 9% TAG concentrations vastly reduced the amounts of this EHC as compared to 1% and 3% concentrations (Tukey's multiple range test, $P < 0.05$).

Multivariate ANOVA of EHC differences revealed significant variation due to all treatments except for the Population \times Sex interaction (Table 3). Here, Roy's Greatest Root is the largest characteristic root or eigenvalue that explains the greatest proportion of variation (percent) in each factor of the MANOVA. The largest eigenvalues for population and sex accounted for 100% of the total variation in EHC abundance, with more complex sources of variation explaining TAG differences given that the first eigenvalue accounted for approximately half of the variation (Table 3). Males had more EHCs than females (likelihood ratio = 0.3331, $F = 4.76$, $df = 24/57$, $P < 0.001$). Because the data were unbalanced by the absence of flies in the 3% and 9% triolein and tripalmitolein treatments (Fig. 1), concentration effects were confounded with TAG type, and may have spuriously resulted in a significant TAG \times concentration interaction term.

The overall pattern of EHC variation due to control and TAG treatments was assessed by calculating Mahalanobis distances between groups in PROC CANDISC (Table 4). Significance of these differences indicated that these dietary treatments almost always caused significantly different EHC profiles, except for a number of comparisons involving control diets (overall variation indicated by Roy's Greatest Root = 3.33, $F = 7.91$, $df = 24/57$, $P < 0.001$). EHC profiles were different between triolein and tripalmitolein treatments, and among these two diets and all others. Both control diets produced similar EHC profiles, but only the defatted control diet resulted in similar EHC profiles as the combination treatment ($P = 0.159$, Table 4).

Loadings for each diet on the first three canonical variables, accounting for 89.8% of TAG variation, showed clearer similarities between tristearin and control diet treatments (Fig. 2b). The overall effects of tripalmitolein and triolein strongly influenced the effects of the combination diets, i.e., EHC differences due to the three TAGs in combination were more "similar" to the tripalmitolein and triolein centroids than to the tristearin centroid (Fig. 2b). Removing the control diets from this analysis made this tripalmitolein-combination comparison somewhat more apparent (Fig. 2a). Here, population differences were included, revealing some overlap between EHC differences from tristearin-treated Baja California files and those due to the combination diet. Qualitatively, these results suggest that there may have been a greater influence of this unsaturated TAG in the combination diets for this Baja California population than for the Sinaloa population. However, replicate numbers were limited for the Sinaloa population.

Pairwise *post-hoc* comparisons revealed the effects of dietary treatments on EHC profiles (Table 5). Missing data due to the absence of flies in the 3% and 9% triolein and tripalmitolein treatments did not permit the use of the CONTRAST option in the MANOVA for all EHCs together except for one case, so pairwise contrasts between treatments were performed, despite a loss of power (Scheiner, 1993). Only one *post-hoc* comparison was possible, tristearin vs. TAG combination, revealing the nature of this unsaturated TAG on EHC profiles ($F = 3.62$, $df = 24/7$, $P = 0.035$). Least square means were greater for a majority of the EHCs assayed due to the TAG combination diets than tristearin, including $C_{28.65}$, $C_{30.78}$, $C_{32.63}$, $C_{32.70}$, $C_{32.79}$, all C_{34} components, $C_{34.59}$, $C_{34.66}$, and $C_{36.5}$. Thus, although not toxic even at higher concentrations (Fig. 1), tristearin is insufficient alone as a substrate for EHC biosynthesis vs. the combination of the unsaturated and saturated TAGs used here.

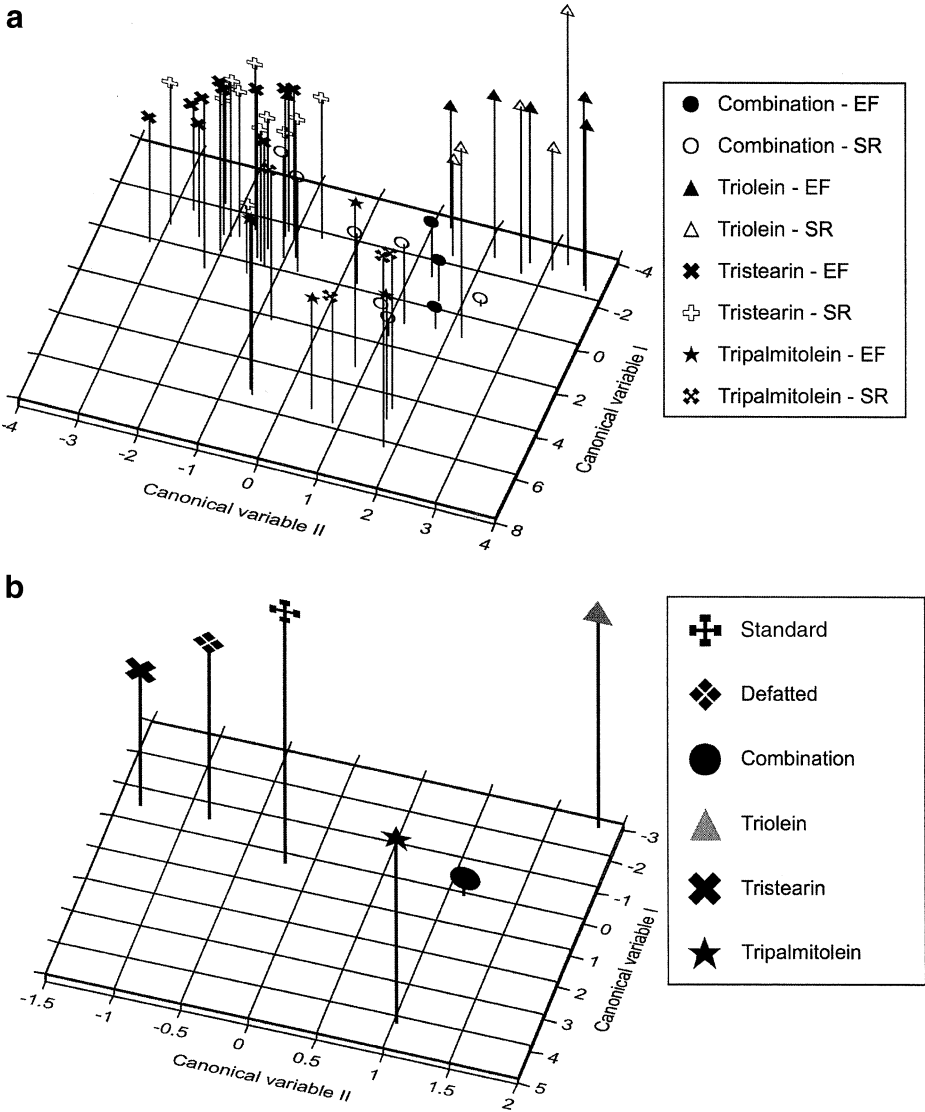


Fig. 2 Canonical discriminant function plot (a) showing the effects of triacylglycerols for each population (EF: El Fuerte, Sinaloa, mainland; SR: Santa Rosalia, Baja California) with the control diet data excluded to better illustrate the effects of the three triacylglycerols on epicuticular hydrocarbon variation. Canonical discriminant function plot (b) showing the effects of the three triacylglycerols alone and in combination, plus the two control diets on variation in epicuticular hydrocarbon profiles for all *Drosophila mojavensis* in this study grouped by population, sex, and triacylglycerol concentration

The remaining pairwise TAG comparisons for EHC profiles caused a reduction in the degrees of freedom for the error term required for significance testing, and this resulted in undefined eigenvalues. To increase degrees of freedom, EHC components were combined into hydrocarbon classes, i.e., alkanes, methylalkanes, alkenes, methylalkenes, and alkadienes (Etges and Jackson, 2001). Grouping the EHCs together by chemical structure

Table 3 MANOVA results for the effects of the experimental treatments on epicuticular hydrocarbon composition in two populations of *Drosophila mojavensis*^a

Source	Roy's Greatest Root				
	Percent	Root	<i>F</i>	<i>df</i>	<i>P</i>
Population	100.0	47.81	13.94	24/7	<0.001
Triacylglycerol (TAG)	52.1	24.17	9.06	24/9	<0.001
Concentration	77.5	16.92	5.64	24/8	0.008
Sex	100.0	18.17	5.30	24/7	0.015
Population × Sex	100.0	10.42	3.04	24/7	0.067
Sex × TAG	73.1	16.87	6.33	24/9	0.004
Population × TAG	61.1	11.93	4.47	24/9	0.012
TAG × Concentration	43.98	16.86	7.02	24/10	0.001
Population × TAG × Sex	75.54	15.39	5.77	24/9	0.005

^a Combinations of all three triacylglycerols (TAGs) were included, but control diets were not, in order to assess TAG effects in combination with the other factors.

reduced the dimensionality and perhaps some of the variation in the data (Table 5). Several of the comparisons were not significantly different despite the significance of the Mahalanobis' distances (Table 4). Pairwise *post-hoc* comparisons again showed that most EHC differences were caused by tristearin vs. the unsaturated TAGs (Fig. 2b), suggesting that TAG substrate-induced shifts in adult EHCs may not have necessarily influenced specific classes of hydrocarbons, but caused overall shifts in most types of hydrocarbons (Table 5). Triolein caused greater amounts of all EHCs than tristearin, particularly in 2-methyloctacosane (C_{28.65}), 2-methyltricontane (C_{30.65}), 7- and 9-hentricontene (C_{30.78}), 8,24-tricontadiene (C_{32.63}), 9,25-pentatricontadiene (C_{34.59}), 8,26- and 7,27-pentatricontadiene (C_{34.66}), and all three major C₃₇ components (Table 2 and Tukey's multiple range tests, all *P*<0.05 based on ANOVAs in Appendix). Similarly, tripalmitolein caused greater amounts of all EHCs than tristearin in many of the same EHCs including 2-methyloctacosane (C_{28.65}), 2-methyltricontane (C_{30.65}), 7, 25-tricontadiene (C_{32.70}), 10-, 12-, and 14-tritricontene (C_{32.79}), 10-, 12-, and 14 tetratricontene (C_{34.ene}), 9,25-pentatricontadiene (C_{34.59}), 8,26- and 7,27-pentatricontadiene (C_{34.66}), 9,27-heptatricontadiene (C_{36.5}), and 8,28-heptatricontadiene (C_{36.6}) (Table 2 and Appendix, Tukey's multiple range tests, *P*<

Table 4 Pairwise probabilities for squared mahalanobis distances among *Drosophila mojavensis* epicuticular hydrocarbon profiles due to standard and defatted control diets as well as to defatted diets supplemented with three triacylglycerols^a

	Combination ^b	Defatted ^c	Standard ^d	Triolein	Tripalmitolein	Tristearin
Combination	–	0.159	0.028	0.004	0.001	0.023
Defatted		–	0.609	0.036	0.001	0.995
Carolina			–	0.001	<0.001	0.070
Triolein				–	<0.001	<0.001
Tripalmitolein					–	<0.001

^a Overall, diets caused significant differences in hydrocarbon profiles, Roy's greatest root=3.33, *F*=7.91, *df*=24/57, *P*<0.001.

^b Defatted media plus all 1%, 3%, or 9% total concentration of all three fatty acids.

^c See text for details.

^d Instant *Drosophila* Medium[®] formula 4–24 (Carolina Biological Supply Co.).

Table 5 Pairwise *post-hoc* comparisons among the three individual and combination tag treatments based on class of epicuticular hydrocarbon^a of *Drosophila mojavensis*

	df	Alkane		Methylalkanes		Alkenes		Methylalkenes		Alkadienes	
		SS	F	SS	F	SS	F	SS	F	SS	F
All	3	19.65	0.75	72,263.6	5.46	12,939.3	2.85 ⁺	10,668.8	2.25	670,380.7	3.66*
TO vs. TS	1	11.64	0.38	35,263.6	9.86**	9702.4	5.99*	8644.3	5.23*	463,505.5	7.88**
TO vs. TP	1	43.90	1.23	10,111.2	2.27	18.3	0.01	91.4	0.07	68,134.7	0.85
TS vs. TP	1	7.73	0.26	52,260.4	12.85**	3942.5	4.37*	4895.7	2.86	268,444.1	5.91*
CB vs. TO	1	0.35	0.02	748.1	0.15	60.9	0.03	383.3	0.27	383.3	0.27
CB vs. TP	1	0.59	0.03	16,307.9	2.71	32.3	0.02	1064.1	0.74	6402.7	0.10
CB vs. TS	1	7.71	0.36	15,210.8	3.46 ⁺	6670.5	5.24*	2329.4	1.34	384,530.9	7.35*

^a Pairwise comparisons for each class of molecule. TO: triolein, TS: tristearin, TP: tripalmitolein, CB: combination.

+ : $0.10 < P < 0.05$; * : $P < 0.05$; ** : $P < 0.01$.

0.05). Thus, we tentatively rejected the hypothesis that particular unsaturated or saturated TAGs in larval diets induced variation in specific saturated or unsaturated EHCs in the adults. Both unsaturated TAGs caused higher levels of all adult EHCs, suggesting they are more easily incorporated into EHC biosynthesis than tristearin despite the higher toxicity of the unsaturated TAGs during larval development (Fig. 1).

Discussion

Low larval diet concentrations of some triacylglycerols present in the fermenting cactus tissues used by *D. mojavensis* as breeding sites caused significant shifts in amounts of EHCs expressed in mature adults. The hypothesis that different fatty acid pools from larval diets would be preferentially used as substrates for different saturated and unsaturated adult EHCs was rejected. Unsaturated triacylglycerols caused increases in a wide range of adult EHCs in *D. mojavensis* (Tables 1, 2, and 5), and although not toxic at higher concentrations, tristearin diets were consistently associated with lower quantities of most adult EHCs. Thus, tristearin is not likely a suitable substrate for lipid biosynthesis in *D. mojavensis*, consistent with Pennac'h et al. (1997), who found that stearic acid was not a preferred precursor for hydrocarbon production in adult *D. melanogaster*, yet palmitic acid was readily incorporated. The chemical cues that determine mate recognition and sexual isolation in *D. mojavensis* are therefore sensitive to some of the triacylglycerols present in natural rearing substrates.

The observed variation in TAG response shown by the Population \times TAG interactions (Appendix) in EHC abundance suggests these genetically differentiated populations differ in ability to synthesize adult EHCs from fatty acid precursors. The large geographic differences in EHC profiles, particularly due to differences between Baja California and mainland Mexico and Arizona populations (Stennett and Etges, 1997; Etges and Ahrens, 2001) in amounts of 8,24-tricontadiene ($C_{32.63}$), 9,25-pentatricontadiene ($C_{34.59}$), and 9,27-heptatricontadiene ($C_{36.5}$) are thus sensitive to TAG variation. Overall, variation in amounts of EHCs was more influenced by population differences than by the types of TAGs used in this study (Fig. 2a, Table 1), but the mainland population here (Sinaloa) was more sensitive to TAG amounts than the Baja California population. Such geographical variation is consistent with previous observations that mainland populations of *D. mojavensis* are more

sensitive to available nutrients in rearing substrates than Baja populations (Etges and Heed, 1987). At higher larval densities in cactus cultures where competition for food is increased, mainland populations express reduced viability and longer development times than Baja California populations, suggesting lower genetic homeostasis in mainland populations for these components of fitness (Etges, 1989).

Even though all larval cultures were supplemented with live baker's yeast and adults were aged to maturity on banana media, EHC profiles from triolein- and tripalmitolein-treated flies were significantly different among other treatment groups, with few differences due to the control diets (Table 4). Flies were aged at least 12–14 d on lab food in order to ensure that they were sexually mature (ca. 8–10 d for males at 25°C; Markow, 1982). This may have influenced adult EHC profiles despite the effects of these TAGs during larval stages, along with the effects of including live yeast to insure survivorship on the diet. Supplemental yeast provides larvae with nucleic acids, sterols, and essential vitamins and nutrients, including fatty acids. However, cultures from all treatment groups included the yeast, so if the additional yeast affected fatty acid metabolism in the flies, all treatment groups should have been influenced to the same degree. Because EHC amounts increase in abundance from eclosion to sexual maturity in *D. mojavensis* (Toolson et al., 1990), we wanted to be sure that our assays were performed with adults that had been reared in the same way as in previous experiments that had shown the consequences of preadult rearing environments on adult mating behavior.

EHC production has been intensively studied in adult insects (reviewed in Blomquist, 2003), but the influence of metabolism of lipids during larval stages on adult EHC profiles is not well understood. Biosynthesis of adult EHCs has been intensively studied, particularly in immature males and females in *D. melanogaster*, *D. simulans*, and *D. erecta*. EHC sexual dimorphism increases with age until sexual maturity is attained (Jallon and Wicker-Thomas, 2003). In *D. mojavensis*, rearing larvae on fermenting cactus lowers pre-mating isolation compared to laboratory media (Etges, 1992; Brazner and Etges, 1993) despite a maturation period of up to 2 wk on banana food. We used this protocol to directly compare the effects of preadult rearing conditions without changing adult rearing procedures used in previous experiments. Thus, the rearing substrates experienced by preadult *D. mojavensis* cause a “carryover” effect that influences adult EHC composition and mating behavior past the age of sexual maturity.

The effects of tristearin in larval diets on adult *D. mojavensis* EHCs were consistent with earlier observations of its role as a lesser used substrate for adult EHC biosynthesis (Pennanec'h et al., 1997). Tristearin's effects on larval viability suggest it is either not metabolized effectively in larvae, or once assimilated, it has negligible effects even at higher concentrations (Fig. 1). Since the effects of tristearin on EHC profiles were not significantly different from either of the control diets, but very different from both triolein and tripalmitolein (Table 4), it may not be assimilated into adult hydrocarbons. More data will be needed to confirm this. Tripalmitolein, tristearin, and triolein are present in much lower concentrations (0–2.3%) in agria and organ pipe cacti than the most abundant saturated fatty acids, capric (C₁₀) and lauric (C₁₂) acid, which together account for 30–45% of total fatty acids (Fogleman and Kircher, 1986). Capric and lauric acids have been shown to have negligible effects on larval viability in *D. mojavensis* at 0.5% concentrations, but lauric acid significantly depressed viability at 1%. Thus, saturated fatty acids *per se* are not poor substrates for lipid and hydrocarbon metabolism: some are assimilated during larval growth and development and have variable effects depending on concentration.

Direct chemical links between host substrates and the resulting pheromonal cues used as both mate recognition signals and part of species recognition systems have broad

significance to studies on ecological speciation (Funk, 1998; Nosil et al., 2002; Sandoval and Nosil, 2005). In general, host plant adaptation typically involves host chemistry to some degree (Harborne, 1982; Bernays and Chapman, 1994; Thompson, 1994; Becerra, 1997; Funk et al., 2002), so host plant fatty acid or TAG determinants of EHC abundance in adult *D. mojavensis* provide direct evidence for how differential host use might determine reproductive isolation among insects. Although future studies will be necessary to show that TAG-induced shifts in adult EHC abundance directly influence patterns of mate choice, these diet-induced phenomonal shifts need to be considered in studies on reproductive isolation, particularly in *Drosophila* species. Enough is known about cactus stem chemistry that we can now design further experiments to examine the effects of these secondary compounds on the behavior and physiology of the insects that use these host plants.

Perhaps half of the species in the large *D. repleta* group (ca. 100 species) use cacti to carry out their life cycles. Along with a few other drosophilids that are cactus specialists, e.g., species in the *nannoptera* group (Heed, 1982), there are likely other species in which adult physiology and behavior has been shaped by the host plants used for feeding and breeding. In the Sonoran Desert, cactophilic *Drosophila* use different species of columnar cacti as breeding sites (Heed and Mangan, 1986). Host chemistry has been studied in detail in order to understand why certain host cacti are used preferentially by particular *Drosophila* species (Fogleman and Abril, 1990; Fogleman and Danielson, 2001). For *D. mojavensis*, crude lipid extracts from its major host plants, agria and organ pipe cactus, are not toxic because fatty acids and sterol diols are esterified to either neutral triterpenes, betulin and calenduladiol, or di-hydroxysterols (Fogleman et al., 1986). These neutral triterpenes are not toxic, but low concentrations of medium chain fatty acids and sterol diols can significantly depress larval viability. However, *D. mojavensis* is more tolerant of free fatty acids during larval growth and development than other desert drosophilids (Fogleman and Kircher, 1986). Agria and organ pipe cacti contain higher concentration of these fatty acids than other host cacti, so this may help to explain why *D. mojavensis*, but not other *Drosophila* species, tend to specialize on these cacti.

Throughout its evolutionary history, *D. mojavensis* has expanded its range by switching to different host cacti. Originating in Baja California, *D. mojavensis* colonized mainland Mexico, Arizona, and southern California by switching host plants. Use of agria cactus in Baja California is considered ancestral, with derived mainland populations using organ pipe cactus in Sonora and Sinaloa, Mexico, as well as southern Arizona (Etges et al., 1999). Sina cactus, *S. alamosensis*, is occasionally used in Sonora and northern Sinaloa, and in southern California, California barrel cactus, *Ferocactus cylindraceous*, is a major host. The colonization of mainland Mexico from Baja California by switching to organ pipe cactus has caused widespread genetic differences in life history traits, chromosome and genic frequency shifts, and physiological adaptation to these secondary hosts that has also resulted in the evolution of altered mating preferences in mainland populations (Etges, 1998; Etges and Ahrens, 2001, 2002). The evolutionary relationships between *D. mojavensis* and its host cacti suggest that this is an ideal system with which to pursue the molecular and chemical causes of ecological speciation, particularly if the mechanisms underlying these evolutionary shifts are related to host plant chemistry. These host plants have shaped the development and physiology of the insects that use them, causing not only adaptive responses in terms of host use and specialization, but also potential reproductive isolation among species that use different hosts.

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different herbivores) and specificity of effect (the range of organisms affected by a given induced response) (Karban and Baldwin, 1997; Stout et al., 1998; Agrawal, 2000). Specificity of elicitation, the focus of our study, occurs when the induced response varies among herbivores, across chemical types, or if it varies in magnitude or location within the plant. For example, mechanical wounding can differ from herbivory (Hartley and Lawton, 1987; Turlings et al., 1990; Mattiacci et al., 1995; Alborn et al., 1997; McCloud and Baldwin, 1997), and different herbivores, species and/or developmental stages, can generate distinct responses (Stout et al., 1994; Takabayashi and Dicke, 1996; Inbar et al., 1999; Schittko et al., 2000). Furthermore, certain compounds within a class may be induced and not others (Clausen et al., 1989; Bodnaryk, 1994; Stout et al., 1994, 1998; Doughty et al., 1995; Ruuhola et al., 2001), or the response may only occur in certain tissues (Jones et al., 1993; Stout et al., 1996). Whereas these studies document specificity of induction, no single study simultaneously examines how contrasting herbivores differentially induce closely related chemicals and how these induced responses vary both locally (in the damaged tissue) and systemically (but see combined studies of Stout et al., 1994, 1996, 1998). This study focused on the specificity of phytochemical induction in *Salix sericea*.

Like many members of the Salicaceae, *S. sericea* is attacked by a diversity of herbivores and produces phenolic glycosides, a group of chemicals that deter feeding of many generalist herbivores (Tahvanainen et al., 1985; Lindroth et al., 1988). The two most common phenolic glycosides in *S. sericea* are salicortin and 2'-cinnamoylsalicortin, which can account for 11.6 and 1.5% of the dry leaf mass, respectively (Orians et al., 1996). Induction of phenolic glycosides has been previously reported in cuttings from adult plants of *S. myrsinifolia* but not *S. pentandra* (Julkunen-Tiitto et al., 1995; Ruuhola et al., 2001). More specifically, Ruuhola et al. (2001) only found significant induction of certain phenolic glycosides in undamaged immature leaves, but not in damaged mature leaves of *S. myrsinifolia*. Mature damaged leaves only showed increases in degradation products. Stevens and Lindroth (2005), using 2-yr-old *Populus tremuloides* obtained from root micropropagation, did not observe rapid induction of phenolic glycosides immediately after manual damage but found that levels were elevated later in the season (termed within-season-delayed systemic induction). Although these studies show that induction can occur, it is not known whether different herbivores have contrasting effects or if seedlings can be induced, locally or systemically.

Here, we examined whether *Plagioderia versicolora* adults, *P. versicolora* larvae, and *Calligrapha multipunctata bigsbyana* adults (Chrysomelidae, Coleoptera) induce phenolic glycosides both locally (damaged leaves) and systemically (in undamaged leaves above and below the damaged leaves). We hypothesized that the induction of phenolic glycosides would be pronounced in response to both species, as high concentrations of phenolic glycosides are known to inhibit their feeding and growth rates (Tahvanainen et al., 1985; Orians et al., 1997). We also expected induction to be greatest in the young damaged leaves, intermediate in the younger undamaged leaves, and minimal in mature undamaged leaves.

Methods and Materials

Plants and Insects

Salix sericea (Marshall), silky willow, is a 0.5- to 4-m high shrub that occurs in riparian and swampy areas in eastern Canada and northeastern United States. In New York, *S. sericea* produces leaves continuously from May until September. Plants flower in late April, and

the female plants set seed at the end of May. Seed germination occurs immediately. *S. sericea* is attacked by numerous specialist and generalist herbivores, including several species of coleopteran leaf chewers (Orians and Fritz, 1996; Orians et al., 1997).

S. sericea full-sibling progeny (S47×S16) was generated from a wild population of willows in Milford, NY. Crosses were made by transferring pollen from S47 male to catkins of S16 female. We used a single full-sib family to minimize possible genetic variation in constitutive chemistry and induced responses (Stevens and Lindroth, 2005). Catkins had been covered with mesh pollination bags to prevent visitation by insect pollinators, and, once pollinated, bags were replaced and left until seed maturation. On June 3, 2002, seeds were sown in separate trays, and the resulting seedlings were grown in a shaded open-end greenhouse (30% of full sun) located at our field station in Milford, NY. Following germination, each seedling was transferred to a 0.75-dl pot. Seedlings were watered daily and received a weekly standard solution of fertilizer (6 g/l of Peters Professional NPK 20:20:20). The experiments were initiated on July 28, when the seedlings were between 6 and 13 cm tall and had 5–11 fully expanded leaves.

Beetles (Coleoptera: Chrysomelidae) were collected from the immediate area surrounding the field station on the day before the experiments were initiated. *C. multipunctata bigsbyana* (Kirby) adults and *P. versicolora* (Laicharting) adults and larvae, hereafter referred to as *Calligrapha* adults and *Plagioder*a adults and larvae, respectively, were collected while feeding on *S. sericea* and *S. sericea* × *S. eriocephala* hybrids. While both species are generalist feeders on *Salix*, *Calligrapha* is native, whereas *Plagioder*a is not. It is, therefore, conceivable that the response to the native *Calligrapha* could be greater. Adults were held without food prior to induction assays; larvae were caged along with the leaf they were feeding on at the time of collection.

Plant Treatments

Treatments were initiated on July 28, 2002 (d 0). Each seedling was randomly assigned to one of four treatments: *Plagioder*a larvae (PVL; $N=28$), *Plagioder*a adults (PVA; $N=29$), *Calligrapha* adults (CMA; $N=30$), and control (CON; $N=26$). Each seedling was randomly placed within the greenhouse on d 0 and again on d 2.

For each plant, a clip cage (5.0-cm diameter, consisting of two plastic petri plate tops hinged together with a metal hair clip and ventilated with eight 1-mm holes) was attached to the third youngest fully expanded leaf on d 0, ensuring that the entire leaf was inside the cage. Preliminary experiments on *S. sericea* found that the clip cages had no effect on salicylate concentrations after 4 d of being attached to the plant (unpublished data). Plants in the PVL group received a clip cage containing two *Plagioder*a larvae, plants in the PVA group received a clip cage containing two *Plagioder*a adults, plants in the CMA group received a clip cage containing one *Calligrapha* adult, and plants in the CON group received an empty clip cage. Two *Plagioder*a were used because a single *Calligrapha* adult consumes more leaf material per unit time. On d 1, clip cages were transferred to the next youngest, fully expanded leaf. On d 2, all clip cages and herbivores were removed from the plants.

Based on previous experiments looking at induction strength over time, plants were harvested on d 4, the time of maximal induction (unpublished data). Foliage was divided three ways: local, systemic young (SY), and systemic mature (SM) leaves. The local sample consisted of the two leaves enclosed within the clip cage, the SY sample consisted of all leaves above (younger than) the locally damaged leaves, and the SM sample consisted of all the leaves below (older than) the local leaves. All leaves from each location (SY, local, and

SM) were vacuum-dried at room temperature for 2 d, weighed, and stored at -20°C until high-performance liquid chromatography (HPLC) analysis.

Chemical Analysis

Salicortin and 2'-cinnamoylsalicortin were analyzed with standard techniques (Orians 1995). Briefly, leaf samples were ground to a fine powder and mixed thoroughly using a ball-mill grinder (Kleco). For each sample, 10.0 ± 0.3 mg leaf powder, or as much tissue that was available, were added to a microcentrifuge tube. Each tube received 1.0 ml of 6.4 mM 1,3-dimethoxybenzene in methanol as an internal standard. Following 5 sec of vortexing, the samples were sonicated at $1-2^{\circ}\text{C}$ for 13 min, and centrifuged at 7,000 rpm/min for 3 min. The extract was poured off and filtered using 0.45- μm pore Acrodiscs. Samples were analyzed at 277 nm on an HPLC (Hewlett Packard Model 1100) equipped with a 3.9×150 mm Nova-Pak C18 column (Waters) and an UV detector, using a gradient of methanol and dH_2O . Peaks representing phenolic glycosides were quantified using purified laboratory standards.

Measurement of Leaf Damage

Because the amount of leaf area consumed varied among herbivory treatments, we tested whether the amount of leaf area consumed had an effect on chemical concentrations. To estimate leaf area, leaves were photocopied and their images scanned by using NIH Imaging Software version 1.62 (<http://rsb.info.nih.gov/nih-image/>). For those plants that received herbivory, this was a measurement of the postherbivory area. To estimate preherbivory leaf area, leaves were photocopied and the missing portions of the chewed leaves were filled in. The manipulated image was scanned and analyzed as before to estimate preherbivory leaf area. The amount of leaf area consumed was calculated as: (preherbivory area) – (postherbivory area). If a leaf was consumed entirely, its area was assumed to be equal to the area of the plant's other local leaf.

Data Evaluation and Statistics

The amount of leaf area consumed was compared among the treatments using one-way ANOVA, and correlations were performed to determine if the amount of leaf area consumed was related to phenolic glycoside concentrations. Because larger seedlings (as measured by total above-ground biomass) had higher concentrations of phenolic glycosides (data not shown), we also examined the correlation between the total mass of the leaves at each location (local, SY, or SM leaves) and 2'-cinnamoylsalicortin and salicortin concentrations. There was a strong positive correlation for all samples (see [Results](#)), further indicating that larger plants produced higher concentrations of phenolic glycosides. Total leaf mass at each location was, therefore, included as a covariate in subsequent analyses to control for the effects of seedling size.

To examine the effects of leaf age on baseline phenolic glycoside concentration, we compared the concentration of the two phenolic glycosides across the three leaf locations (SY, local, and SM) in CON plants using a one-way analysis of covariance (ANCOVA; with location as the main effect and total leaf mass as the covariate). To test for induction at each leaf location, the effect of herbivore treatment was analyzed by using a one-way ANCOVA (with herbivore treatment as the main effect and leaf tissue mass as the covariate). Significance of differences among leaf locations and treatments were determined

with a LSMeans Student's *t*-test (JMP statistical software version 5.0.1.2, SAS Institute Inc., Cary, NC, USA, 1989–2003).

Results

2'-Cinnamoylsalicortin and salicortin were detected in all samples and accounted for 1.30 ± 0.09 and $11.59 \pm 0.30\%$ (mean \pm SE), respectively, of the mean dry leaf mass of CON plants (similar to previous reports on this species [Orians et al., 1996]). There was a strong positive relationship between 2'-cinnamoylsalicortin and salicortin concentrations within SY, local, and SM leaves for all treatments ($r^2=0.366$, 0.414 , and 0.153 , respectively; $P < 0.001$). There were also strong positive relationships between total leaf mass and salicylate concentrations within the SY, local, and SM leaves for all treatments (for 2'-cinnamoylsalicortin, $r^2=0.249$, 0.260 , and 0.322 , respectively, and for salicortin, $r^2=0.283$, 0.264 , and 0.146 , respectively; $P < 0.001$). The same trend was observed in the local leaves of the CON treatment when analyzed separately, indicating that larger plants (more leaf biomass at each location) were capable of producing higher concentrations of phenolic glycosides.

In CON plants, 2'-cinnamoylsalicortin concentrations were highest in the SY leaves, whereas salicortin concentrations were highest in the local leaves (Fig. 1). Specifically, 2'-cinnamoylsalicortin concentrations were significantly higher in SY leaves compared to local leaves and in the local leaves compared to the SM leaves (Fig. 1a–c; $F=26.01$; $P < 0.001$). Salicortin concentrations were significantly higher in local leaves compared to SY and SM leaves, whereas SY and SM leaves had similar concentrations (Fig. 1d–f; $F=12.03$; $P < 0.001$).

Plants in the CMA treatment lost the greatest amount of leaf area to herbivory (2.490 ± 0.230 cm²), whereas plants in the PVL treatment lost the least amount of leaf area to herbivory (1.370 ± 0.200 cm²), significantly less than the plants in the CMA treatment ($P < 0.001$). The amount of leaf area lost to herbivory in the PVA plants (2.110 ± 0.200 cm²) was not significantly different from either of the other two treatments ($P > 0.05$). Leaf area removed by PVL, PVA, or CMA was not positively correlated with phenolic glycoside concentration. In fact, leaf area removed was correlated with lower phenolic glycoside concentration in three cases: for both 2'-cinnamoylsalicortin and salicortin concentration in the local leaves in response to *Plagioder*a larvae, and for salicortin concentration in the SY leaves (Table 1).

Of the two phenolic glycosides, only 2'-cinnamoylsalicortin was induced. There was a significant overall treatment effect of herbivory on 2'-cinnamoylsalicortin concentration in the SY leaves ($F=2.71$; $P=0.049$). Mean 2'-cinnamoylsalicortin concentration was higher in SY leaves of the three herbivore treatments than in CON treatment (Fig. 1a), whereas 2'-cinnamoylsalicortin concentration in local and SM leaves were similar among the treatments (Fig. 1b, c). Mean salicortin concentrations in SY and SM leaves were similar among all treatments (Fig. 1d, f). Mean salicortin concentrations were significantly lower in local leaves of the PVL plants than in CON plants (Fig. 1e).

Discussion

Overall, we found evidence for specificity of induction. All herbivores tested caused 2'-cinnamoylsalicortin concentrations to increase systemically, but only in the youngest,

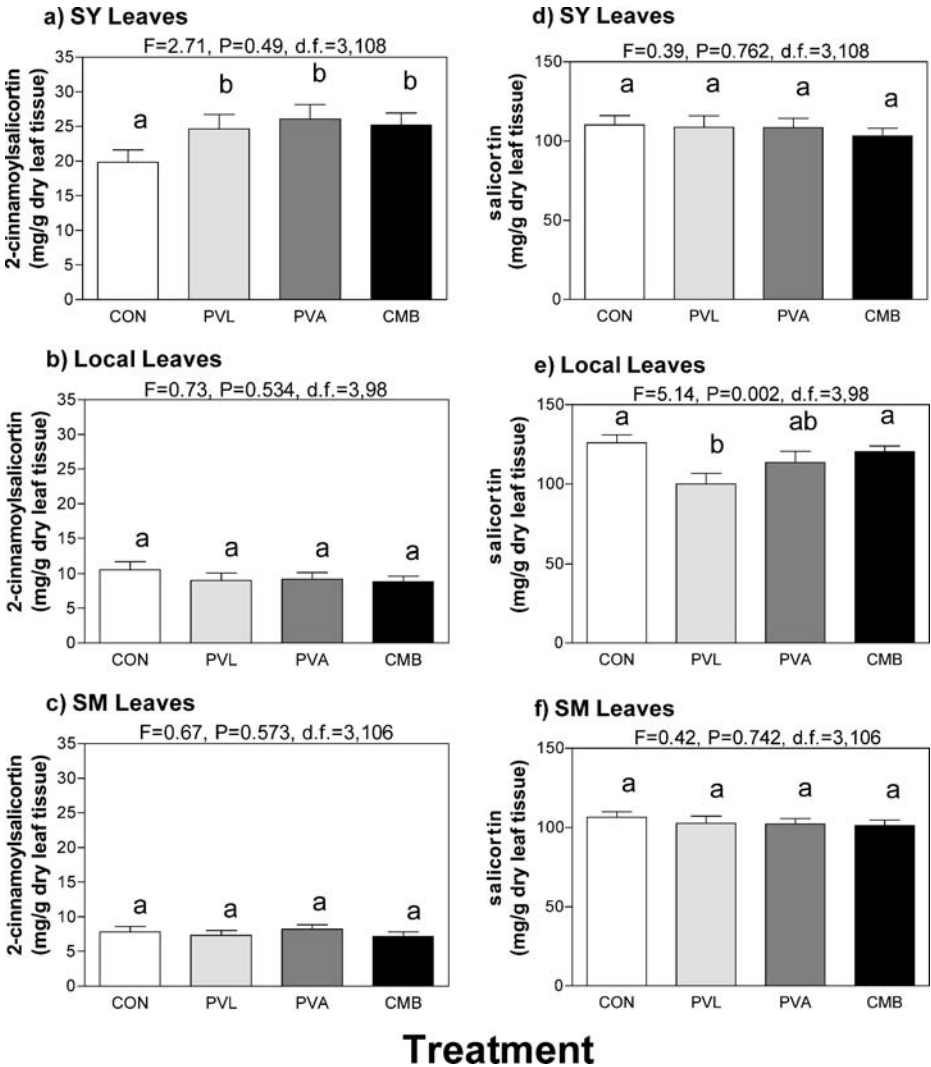


Fig. 1 The effects of treatments on least square mean (\pm SE) 2'-cinnamoylsalicycortin (a, b, c) and salicycortin (d, e, f) concentrations in SY, local, and SM *Salix sericea* leaves. Treatments were compared by one-way ANCOVA with leaf tissue mass as a covariate. Different letters above bars indicate significant differences between treatments ($P=0.05$). ANCOVA results are presented above the graph. SY = systemic young leaves, local = local leaves, SM = systemic mature leaves, CON = clip cage controls ($N=26$), PVL = *Plagioderma versicolora* larvae ($N=28$), PVA = *P. versicolora* adults ($N=29$), CMB = *Calligrapha multipunctata bigsbyana* adults ($N=30$)

undamaged upper leaves. In contrast, there was no increase in salicycortin concentration. Thus, young *S. sericea* seedlings are capable of increasing the concentration of 2'-cinnamoylsalicycortin in young leaves in response to beetle herbivory. Whereas the three herbivore treatments caused similar patterns of induction in young undamaged leaves, *P. versicolora* larvae actually caused a significant drop in salicycortin levels within damaged leaves.

Table 1 Pearson's correlation coefficients (r) representing the relationship between leaf area consumed by herbivores and the concentrations of 2'-cinnamoylsalicortin and salicortin in SY, local, and SM *Salix sericea* leaves

Herbivore treatment	2'-Cinnamoylsalicortin			Salicortin		
	SY	Local	SM	SY	Local	SM
PVL	-0.293	-0.479**	-0.277	-0.443*	-0.592***	-0.253
PVA	-0.118	-0.134	-0.167	0.261	0.214	0.089
CMA	0.263	-0.155	-0.008	0.110	-0.063	-0.045

SY = Systemic young leaves, SM = systemic mature leaves, PVL = *Plagioderia versicolora* larvae, PVA = *P. versicolora* adults, CMA = *Calligrapha multipunctata bigsbyana* adults.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Patterns of induction are best examined in context of baseline levels of phenolic glycosides. The distribution of phenolic glycosides within the control seedlings varied according to the compound. The distribution of 2'-cinnamoylsalicortin showed a clear negative relationship with leaf age (e.g., the young sink leaves had the highest concentrations, whereas the oldest mature leaves had the lowest). The spatial distribution of salicortin, however, revealed a different pattern: local leaves had significantly higher salicortin concentrations than both systemic young and systemic mature leaves. This result is consistent with previous work on *S. sericea* seedlings (Orians, unpublished data). Similarly, Julkunen-Tiitto et al. (1995) found that whereas the concentrations of many phenolic glycosides are highest in the young shoot tips of *S. myrsinifolia*, the concentration of others (e.g., salicortin-2) are highest in the mature leaves. These results indicate that phenolic glycoside biosynthesis can continue after leaf transition from sink to source leaves (see also Kleiner et al., 1999).

Not only were 2'-cinnamoylsalicortin concentrations highest in youngest leaves, but the three beetle treatments induced significant increases in the concentration of 2'-cinnamoylsalicortin, but not salicortin, in these younger leaves. Ruuhola et al. (2001) also found phenolic glycoside induction to be greatest in leaves with constitutively higher concentrations and suggest that induction of phenolic glycosides was probably a result of increased biosynthesis. It is interesting that 2'-cinnamoylsalicortin, which rarely exceeds 2% dry leaf mass, was induced but that salicortin, which often exceeds 10% dry leaf mass, was not. This could be due to the fact that 2'-cinnamoylsalicortin is most likely synthesized by adding a 2'-cinnamoyl group to salicortin, and, thus, the observed increase may be a result of enhanced conversion of preexisting salicortin to 2'-cinnamoylsalicortin. Because salicortin levels decreased in the locally damaged leaves, it is tempting to speculate that this was the source of salicortin. There is, however, no evidence to date that phenolic glycosides are transported within the vascular system from leaf to leaf. Rather, we suggest that any decrease in salicortin concentrations in SY leaves went undetected because salicortin concentrations were so much higher than 2'-cinnamoylsalicortin concentrations.

We had expected the damaged leaves to exhibit the greatest induction, but none was observed. As expected, induction was absent in the SM leaves. Whereas SY leaves exhibited induction, the lack of 2'-cinnamoylsalicortin induction in damaged leaves suggests that this compound is not inducible in source leaves (see also Ruuhola et al., 2001). Compared to control plants, all three herbivore treatments exhibited lower mean

salicortin concentrations in locally damaged leaves, although the PVL treatment was the only treatment that showed a significant difference. This decrease probably represents chemical degradation (Clausen et al., 1989; Julkunen-Tiitto et al., 1995) and would suggest that 2'-cinnamoylsalicortin is less prone to degradation. Clausen et al. (1989) reported that crushing *P. tremuloides* leaves induced a rapid (within seconds to minutes) enzymatic conversion of salicortin to salicin and 6-HCH. Although few of the samples in this study contained detectable quantities of salicin (personal observation), it is possible that salicin degraded even further in the days following wounding. Differences in local salicortin concentrations between the *Plagioder*a adults and larvae treatments may reflect different feeding strategies. Adult beetles feed by chewing on leaves, from the outer edges inward, whereas larvae feed by skeletonizing the leaves from the inner leaf outward, thereby causing damage to more leaf area per amount consumed and possibly causing more degradation. Alternatively, the lower concentrations found in larva-consumed tissues may reflect the fact that remaining vein tissue does not contain as much salicortin as mesophyll tissue.

Despite differences in the amount of leaf area removed, this did not have an effect on the amount of induction observed, as there were no significant positive relationships between phenolic glycoside concentration and the amount of leaf area consumed by herbivores. These data suggest that induction of 2'-cinnamoylsalicortin in *S. sericea* may be an “all- or none” response triggered by even small amounts of herbivory by these beetles. In previous experiments, mechanical damage designed to mimic herbivory did not produce an induced response (Fields, unpublished data), indicating that 2'-cinnamoylsalicortin induction in *S. sericea* occurs in response to a specific cue presented by the beetles. Would the same pattern of induction be observed following herbivory by a highly specialized beetle such as *Chrysomela* spp., which use phenolic glycosides as both feeding stimulants and as a source of defensive metabolites? We suggest that such herbivores would not elicit a strong induction of phenolic glycosides in willow because this could result in substantial costs to the plant. Although preliminary results in 2001 with *Chrysomela knabi* support this prediction (personal observation), we were unable to test this in 2002 because *C. knabi* were absent that year.

The ecological consequences of these induced changes are unknown. We suggest that induction could reduce subsequent feeding by herbivores such as *Plagioder*a, which are known to be sensitive to changes in phenolic glycoside concentration (Tahvanainen et al., 1985; Orians et al., 1997). Our data suggest a possible negative effect on beetle feeding: leaves with constitutively higher phenolic glycoside concentrations received significantly less larval *P. versicolor*a herbivory. Interestingly, there was no clear pattern or significant differences for adult *P. versicolor*a, suggesting that feeding preference may be a function of developmental stage. Although *C. multipunctata bigsbyana* also prefers foliage with moderate to low concentrations of phenolic glycosides (Orians et al., 1997), we found no association between leaf chemistry and leaf area consumed for this species.

One of the main questions raised by this study asks why 2'-cinnamoylsalicortin is inducible but salicortin is not. We suggest three possibilities that merit further investigation. First, salicortin production may be hindered during leaf expansion. Second, salicortin is known to be an oviposition/feeding stimulant for a number of beetles (Tahvanainen et al., 1985; Orians et al., 1997). If 2'-cinnamoylsalicortin is not increasing its concentrations, holding salicortin concentrations steady might represent a strategy to minimize future herbivory and oviposition. Third, plants may induce 2'-cinnamoylsalicortin because it is more bioactive. Although we favor the first explanation, we cannot reject the other two.

Experiments examining the effects of 2'-cinnamoylsalicortin and salicortin on beetle behavior and performance would be useful in assessing the plausibility of the second and third explanations.

In summary, there is accumulating evidence for specificity of induction in terms of which chemicals are induced, the developmental stage of the leaves that are induced, and the agent of damage (Stout et al., 1996; Agrawal, 2000; Ruuhola et al., 2001). For *S. sericea* seedlings, we observed induction of one of two phenolic glycosides but only in young sink leaves. Although all herbivores tested caused similar patterns of induction, the question whether more specialist beetles that can easily detoxify phenolic glycosides cause similar effects requires further study. Interestingly, the magnitude of induction was not influenced by the amount of leaf area consumed or the species/stage of herbivore feeding, indicating an all-or-none response triggered by even small amounts of damage by these beetles.

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spongy-fruited *Clistocarpa*, fleshy-fruited *Sarcocarpa*, and capsular-fruited *Chaenocarpa*. Whereas *Clistocarpa* consists of a single species, *Yucca brevifolia* Engelmann, the other two sections consist of some 20 species each. Plants produce up to several hundreds of flowers on single or multiple inflorescence stalks, and individual flowers are open and fragrant only after sunset. All reproductive behaviors of yucca moths, i.e., mating within host flowers, oviposition into ovaries, and active pollination, take place at night in all but one species (Powell and Mackie, 1966), and floral volatiles are thus suggested to be important sensory cues for these insects during host and mate search.

In contrast to the fig–fig wasp pollination mutualism, where the floral scents of many *Ficus* species have been chemically identified (Grison et al., 1999; Grison-Pigé et al., 2002a; Song et al., 2001) and where there is behavioral evidence for fig pollinator attraction to host volatiles (Grison-Pigé et al., 2002b; Hossaert-McKey et al., 1994; Song et al., 2001), data on the chemical ecology of the yucca–yucca moth mutualism are scarce. Recently, however, a research program was initiated to elucidate the role of floral scent in this classic insect–plant interaction and to analyze how the floral fragrance chemistry has evolved within the yucca lineage. So far, a detailed chemical analysis of the floral odor has only been conducted on the capsular-fruited *Yucca filamentosa* L. (Svensson et al., 2005). This species is native to southeastern USA, but has been spread by European settlers across eastern USA in modern times (Pammel, 1925). It relies on two distantly related *Tegeticula* species for pollination in different parts of its range: *Tegeticula cassandra* Pellmyr on the Florida peninsula and *Tegeticula yuccasella* Riley elsewhere (Pellmyr, 1999; Althoff et al., 2006). Gas chromatography and mass spectrometry (GC-MS) analyses of the floral headspace of *Y. filamentosa* revealed a complex blend of homoterpenes and long-chain aliphatic hydrocarbons, but also two dioxygenated compounds previously not reported as floral compounds in angiosperms (Svensson et al., 2005).

In this study, we report on the chemical characterization of the floral fragrance of a second species within *Chaenocarpa*. *Yucca elata* Engelmann occurs in southwestern USA and adjacent Mexico and appears to rely solely on *Tegeticula elatella* Pellmyr for pollination (Pellmyr, 1999). A recent amplified fragment length polymorphism (AFLP) based phylogeny of a subset of closely related *Tegeticula* pollinators has confirmed close relationship between *T. elatella* and *T. cassandra*, whereas *T. yuccasella*, the most abundant pollinator of *Y. filamentosa*, is nested within another species cluster of moths (Althoff et al., 2006). As no robust phylogeny is available for *Chaenocarpa*, the precise phylogenetic relationship between *Y. elata* and *Y. filamentosa* is not known. However, a phylogeographic analysis of the widely distributed, nonmutualistic *Tegeticula intermedia* Riley, which utilizes both yuccas as hosts, has revealed indirect evidence that these plants have probably been allopatric for several million years (Segraves and Pellmyr, 2004). This is also supported by paleoecological data showing an emergent barrier between eastern and western yuccas (Graham, 1999). The combination of a long history of isolation and dependence on distantly related pollinators in most parts of their ranges suggests that these yuccas may have diverged in their floral odor blends, and this hypothesis was tested here.

Methods and Materials

The Plants

Yucca elata inhabits semidesert grasslands in Arizona, New Mexico, northwestern Texas, and adjacent Mexico (Fig. 1). This 3- to 4-m-tall species produces single or multiple

rosettes on trunks, each with one or several paniculate inflorescence stalks. It flowers from early May to early June. In contrast, *Y. filamentosa* grows in sandy and rocky habitats in eastern USA. From a basal rosette, a single paniculate inflorescence stalk is produced. Plants flower from late April to mid July. Both yuccas produce up to several hundreds of white, bell-shaped flowers per inflorescence.

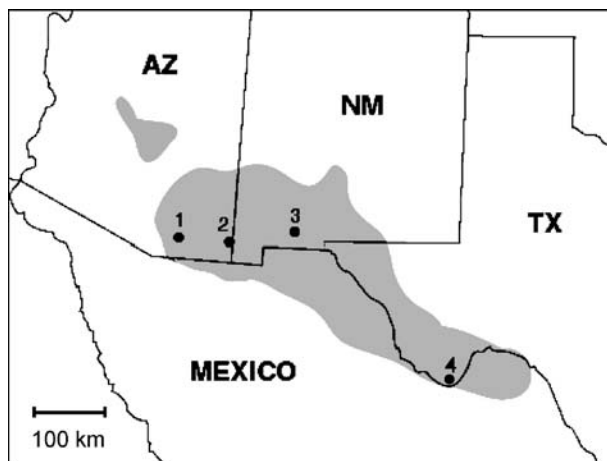
Floral Scent Collection

The floral fragrance of *Y. elata* was collected from four populations in 2004 and 2006: Benson (Cochise, Arizona; 31°58'04N, 110°17'40W), Portal (Cochise; 31°52'17N, 109°02'59W), Rock Hound State Park (Luna, New Mexico; 32°11'13N, 107°36'36W), and Big Bend National Park (Brewster, Texas; 29°15'00N, 103°15'00W; Fig. 1). The number of young flowers on each plant was counted before odor sampling to estimate release rates of volatile compounds on a per flower basis. The inflorescence was enclosed with a polyvinylacetate bag (406×444 mm), and a glass cartridge (7 mm i.d.) filled with 100 mg of Super Q absorbent (Alltech Associates, State College, PA, USA) was connected to the bag. Air passed through the filter at a rate of 200 ml/min using a PAS-500 personal air sampler (Supelco, Bellefonte, PA, USA). Odor collection was performed from 2000 to 2400 hours, corresponding to the maximum release of floral scent of *Y. elata* and peak activity of associated yucca moths, and after collection filters were eluted with 3 ml of hexane and extracts were stored at -18°C until analysis. Empty bags were used as ambient controls to check for possible contaminants emitted from a bag itself. Before GC-MS analysis, extracts were concentrated to 75 µl under N₂, and 5 µl of 0.03% toluene were added as an internal standard to each sample to enable crude estimation of release rates of compounds.

GC-MS Analysis of Floral Scent

Floral volatiles of *Y. elata* were analyzed using a Shimadzu GC-17A gas chromatograph, equipped with a DB-5 column (30 m×0.32 mm i.d., and 1-mm film thickness), and linked to a Shimadzu QP5000 mass spectrometer (EI: ionization energy=0.70 kV). Helium was used as carrier gas at a velocity of 43 cm/sec, and injector temperature was 270°C. Oven temperature was programmed for 50°C for 2 min after injection and then increased at 10°C/

Fig. 1 Distribution of *Y. elata*. Dots indicate sites of odor collection: (1) Benson, (2) Portal, (3) Rock Hound State Park, and (4) Big Bend National Park



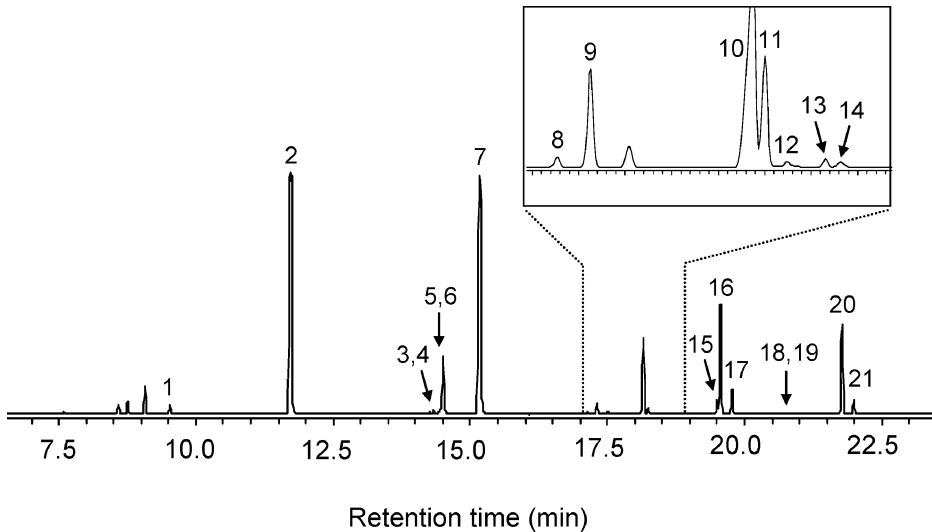


Fig. 2 Gas chromatogram of the floral headspace of *Y. elata*. Peak numbers correspond to compounds in Table 1

min to 275°C. Compounds in extracts were identified by comparing mass spectra and retention times with those of available reference compounds and by mass spectral matches to library spectra.

Analysis of Geographic Variation in Floral Scent

For each population, the relative abundance of individual compounds in the fragrance blend was calculated. Also, the coefficient of variation ($CV = \text{standard deviation} \times 100 / \text{mean}$) for each compound was calculated, using arcsine square-root-transformed ratios to better approximate a normal distribution. Two principal components analyses (PCA) were performed on arcsine square-root-transformed proportions of scent compounds. The first analysis included scent data from the four populations of *Y. elata* to screen for geographic variation in the odor blend. The second analysis also incorporated scent data from 10 populations of *Y. filamentosa* (Svensson et al., 2005) to test whether the floral odor blends of these two species differ. Before analysis, each variable was scaled to unit variance. The total release of floral compounds from *Y. elata* was quantified by the formula:

$$\text{Emission rate} = \frac{\left(\sum \frac{\text{peak area of compound}_x}{\text{peak area of IS}} \right) \times \text{amount of IS}}{\frac{\# \text{ flowers on plant}}{\text{h of sampling}}}$$

× extract volume after addition of IS

Total release rates of compounds were compared between populations using one-way analysis of variance. All statistics were performed using JMP 3.2.1 (SAS Institute, 1998). Leaves and flowers of *Y. elata* from each population were kept as vouchers and deposited at the A. C. Moore Herbarium at University of South Carolina.

Results

Analyses of floral headspace extracts of *Y. elata* revealed that this species produces a blend of homoterpenes and aliphatic hydrocarbons virtually identical to that of *Y. filamentosa* (Fig. 2). The same 21 compounds recently identified from *Y. filamentosa* were also found in all *Y. elata* individuals, with the exception of the six plants from Big Bend NP, which lacked β -myrcene. The same two dioxygenated compounds with a prominent peak at m/z 66 in the mass spectrum found in *Y. filamentosa* were also present in all *Y. elata* extracts (Fig. 2). Additional compounds, like β -pinene and *trans*- β -ocimene (not found in *Y. filamentosa*), were detected only in trace amounts in a few samples. Few compounds in small amounts were found in ambient controls.

Table 1 Mean percentage and CV of 21 floral volatiles from four populations of *Y. elata*

Compound	Site								Range of all populations (%)
	Benson (N=8)		Portal (N=11)		Rock Hound (N=9)		Big Bend (N=6)		
	%	CV	%	CV	%	CV	%	CV	
Monoterpenes									
1. β -Myrcene ^a	0.7	55.7	0.6	45.7	0.4	71.8	0	–	0–1.8
Homoterpenes									
2. (<i>E</i>)-4,8-Dimethyl-1,3,7-nonatriene ^a	35.4	20.1	31.8	18.8	35.9	29.9	28.0	13.8	15.4–71.0
4. Unknown homoterpene 1	0.5	28.8	0.8	25.7	0.4	45.2	0.2	4.1	tr–1.2
6. Unknown homoterpene 2	6.1	17.6	8.4	17.9	5.9	29.8	2.4	4.9	1.0–11.3
7. C ₁₁ -alcohol	25.1	12.5	25.4	5.6	26.7	12.8	23.8	14.8	19.4–35.9
11. Nerolidol ^a	2.5	43.2	2.4	42.3	1.6	51.6	3.2	42.7	0.1–5.3
Unknowns									
3. Unknown m/z 67 1 (C ₁₁ H ₁₈ O)	0.4	18.1	0.5	35.5	0.4	51.7	0.2	12.0	tr–0.9
5. Unknown m/z 67 2 (C ₁₁ H ₁₈ O)	1.5	17.2	2.1	15.3	1.3	31.0	0.3	20.1	0.2–2.7
10. Unknown m/z 66 1 (C ₁₁ H ₁₄ O ₂)	6.5	28.1	7.8	18.9	7.7	35.7	3.4	21.8	1.1–17.9
14. Unknown m/z 66 2 (C ₁₁ H ₁₄ O ₂)	0.1	118.6	0.1	20.8	0.1	40.1	0.1	105.3	tr–0.4
Aliphatic hydrocarbons									
8. Pentadecane	0.3	63.4	0.4	23.0	0.1	28.4	0.3	55.0	tr–0.8
9. Pentadecane	1.6	43.2	1.9	32.5	1.4	24.0	2.0	16.0	0.4–4.9
12. Hexadecene	0.2	34.9	0.1	33.7	0.1	48.4	0.3	9.8	tr–0.3
13. Hexadecane	0.1	35.7	0.1	22.0	0.1	28.7	0.3	26.7	tr–0.2
15. Heptadecadiene	1.8	35.1	1.8	24.2	1.3	28.1	2.7	5.5	0.2–3.8
16. 1-heptadecene ^a	9.8	14.3	8.5	9.5	9.1	18.4	15.7	11.7	5.2–23.2
17. Heptadecane ^a	2.4	26.4	1.5	17.9	1.8	26.8	4.8	7.7	0.5–5.7
18. Octadecene ^a	0.3	45.5	0.2	57.0	0.2	56.6	0.8	9.6	tr–0.7
19. Octadecane ^a	0.1	34.5	0.1	29.7	0.1	48.0	0.3	18.1	tr–0.3
20. Nonadecene ^a	4.2	19.7	5.3	11.0	4.9	25.5	10.5	8.2	0.5–12.5
21. Nonadecane ^a	0.3	25.1	0.3	16.4	0.4	33.2	0.8	18.7	0.1–1.1

Compound numbers are the same as in Fig. 2. Trace (tr.) indicates amounts less than 0.1% of total.

^aCompounds in which mass spectra and retention times were compared with that of reference compounds.

The relative ratios of 21 floral volatiles identified for *Y. filamentosa*, and also present in *Y. elata*, are shown in Table 1. Because of the absence of β -myrcene in Big Bend samples, this compound was excluded from the PCA. In the first PCA, four principal components with eigenvalues >1 explained 85.2% of the total variation found in floral fragrance data. On PC1, (*E*)-4,8-dimethyl-1,3,7-nonatriene was the only compound with strong negative loading, whereas all aliphatic hydrocarbons had similar positive loadings (Table 2). On PC2, the C_{11} -alcohol and the first eluting dioxygenated compound loaded positively, whereas (*E*)-4,8-dimethyl-1,3,7-nonatriene and a majority of the aliphatic hydrocarbons loaded negatively.

A score plot of the first two principal components in the initial PCA revealed little geographic variation of the odor blend in *Y. elata*. Considerable overlap in the fragrance blend was observed across populations, with the exception of samples from Big Bend, which formed a distinct cluster in odor space. However, in a second PCA including fragrance data also from *Y. filamentosa* (Svensson et al., 2005), the floral odor blends of the two yuccas largely overlapped (Fig. 3), and *Y. elata* from Big Bend was nested within *Y. filamentosa*. The total emission of floral volatiles in *Y. elata* did not differ between populations (Benson: $1.08 \pm 0.20 \mu\text{l flower}^{-1} \text{hr}^{-1}$; Portal: $1.16 \pm 0.21 \mu\text{l flower}^{-1} \text{hr}^{-1}$; Rock Hound: $1.03 \pm 0.17 \mu\text{l flower}^{-1} \text{hr}^{-1}$; Big Bend: $1.56 \pm 0.23 \mu\text{l flower}^{-1} \text{hr}^{-1}$; $F=1.12$, $df=3$, $P>0.10$).

Table 2 Loading of the first two principal components of the 20 compounds used in the PCA

Compound	PC1	PC2
Total variation explained by each factor (%)	39.9	25.6
Homoterpenes		
2. (<i>E</i>)-4,8-Dimethyl-1,3,7-nonatriene	-0.24	-0.23
4. Unknown homoterpene 1	0.00	0.42
6. Unknown homoterpene 2	-0.06	0.42
7. C_{11} -alcohol	-0.02	0.14
11. Nerolidol	0.02	0.09
Unknowns		
3. Unknown m/z 67 1 ($C_{11}H_{18}O$)	0.02	0.41
5. Unknown m/z 67 2 ($C_{11}H_{18}O$)	-0.12	0.40
10. Unknown m/z 66 1 ($C_{11}H_{14}O_2$)	0.02	0.27
14. Unknown m/z 66 2	0.13	0.02
Aliphatic hydrocarbons		
8. Pentadecene	0.18	0.13
9. Pentadecane	0.21	0.22
12. Hexadecene	0.32	0.10
13. Hexadecane	0.29	0.01
15. Heptadecadiene	0.27	0.17
16. 1-heptadecene	0.31	-0.10
17. Heptadecane	0.34	-0.07
18. Octadecene	0.34	-0.02
19. Octadecane	0.31	-0.09
20. Nonadecene	0.31	-0.07
21. Nonadecane	0.24	-0.15

Compound numbers are the same as in Fig. 2.

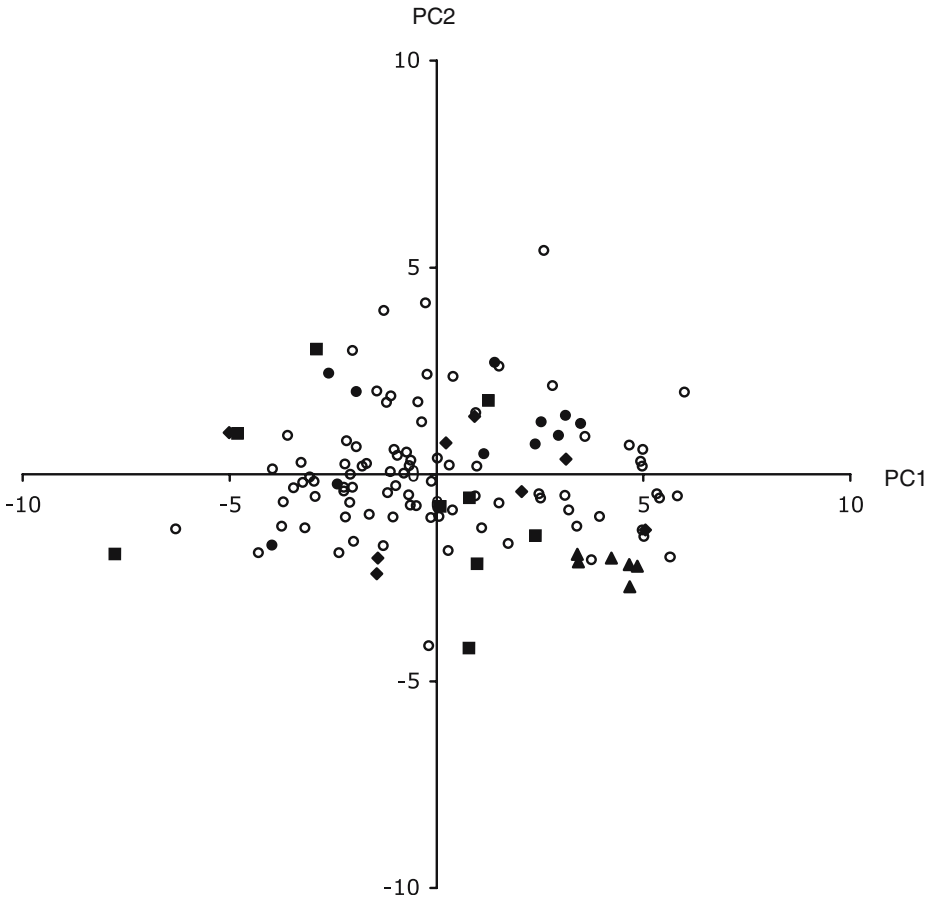


Fig. 3 Score plot of the first two principal components based on 20 compounds found in the floral headspace from four populations of *Y. elata* (■: Benson, $N=8$; ◆: Portal, $N=11$; ●: Rock Hound, $N=9$; ▲: Big Bend NP, $N=6$) and 10 populations of *Y. filamentosa* (from Svensson et al., 2005; ○, $N=87$)

Discussion

Contrary to our prediction of divergent scent composition with different pollinators, GC-MS analyses of the floral headspace of *Y. elata* revealed that this yucca produces a blend of floral volatiles virtually identical to the related, allopatric species, *Y. filamentosa*. The same 21 compounds identified in the floral scent of *Y. filamentosa* (Svensson et al., 2005) were also found in *Y. elata*. Furthermore, the relative ratios of these compounds within the odor blends of the two yuccas are very similar, as shown in the score plot in Fig. 3. Low variation in the fragrance blend was observed both within and among populations of *Y. elata*, similar to the pattern observed in *Y. filamentosa*, where no difference in the floral scent composition was observed between populations with different pollinators (Svensson et al., 2005). Whether low variation in the fragrance blend and the use of unique compounds reflect mechanisms for selective attraction of exclusive pollinators to host

flowers has yet to be tested. In fact, little is known about the adaptive significance of low variation in the floral odor blends in plants. Few studies have analyzed how this trait varies between populations (e.g., Azuma et al., 2001; Knudsen, 2002; Dötterl et al., 2005), and direct comparisons of the variation of floral compounds that are electrophysiologically active in pollinators with that of nonactive ones have only been conducted in sexually deceptive *Ophrys* orchids (Ayasse et al., 2000; Mant et al., 2005).

Although earlier studies suggested monophyly for the section *Chaenocarpa* (McKelvey, 1947), the phylogenetic relationships among capsular-fruited taxa have not been fully resolved. Available AFLP data support rapid diversification of this group (Pellmyr et al., unpublished data), and at this point, the hypothesis of a close relationship, or even sister species status, of *Y. elata* and *Y. filamentosa* cannot be tested. On the other hand, the phylogeographic work on *T. intermedia* by Segraves and Pellmyr (2004) indicates that the two yuccas may have been allopatric for a long time. Western populations of this yucca moth feed on several capsular-fruited yuccas, including *Y. elata* (Pellmyr, 1999; Segraves and Pellmyr, 2004), whereas eastern populations only use *Y. filamentosa* as host (Pellmyr, 1999). Both mtDNA and AFLP data suggest that this moth arose in the west and later spread eastward. There was a deep genetic split between populations east and west of the Mississippi River basin, corresponding to a divergence time of about three million years (Segraves and Pellmyr, 2004). Although their ancestral ranges are unknown, it is reasonable to assume that *Y. elata* and *Y. filamentosa* have been allopatric for a similar period of time. Still, their fragrance blends are similar, and such lack of divergence in a potential key trait mediating attraction of exclusive pollinators may indicate that strong stabilizing or purifying selection has been imposed on the fragrance blend. Floral traits under strong selection (e.g., tube length in hummingbird-pollinated flowers) are expected to show relatively little variation (Fenster, 1991). If scent is irrelevant to the obligate interactions between yuccas and yucca moths, we would expect measurable divergence in chemistry between isolated populations of *Y. filamentosa* and *Y. elata* simply because of genetic drift. The identification and synthesis of floral scent compounds and their attractiveness to obligate pollinators in the field will be critical steps toward a better understanding of how selection acts on both emitters and receivers in this pollination mutualism.

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Introduction

The pistachio twig borer, *Kermania pistaciella* (Lepidoptera: Oinophilidae), is one of the most important insect pests in plantations of pistachio, *Pistacia vera*, in Turkey and Iran. Females lay eggs close to shoot tips and fruit clusters. Larvae bore into and feed on terminal buds and in twigs and shoots formed the previous year. Larval feeding causes abscission of fruit buds and die-back of twigs (Küçükarslan 1966) and may directly damage fruit clusters (Mart et al., 1995). Pupation takes place in cocoons attached to the branch surface near larval exit holes. After 20–25 days, adults eclose, and females lay up to 60 eggs. The 1-month flight period of first-generation adults occurs in April–May (Küçükarslan 1966).

Many pistachio plantations in Turkey and Iran are treated annually with insecticides to reduce damage caused by *K. pistaciella* larvae. For example, in southeast Turkey in 2005, 548,600 trees were sprayed with insecticides to suppress *K. pistaciella* populations (Anonymous 2005). Identification of the *K. pistaciella* sex pheromone might be a first step toward pheromone-based control tactics (e.g., attract and kill) of *K. pistaciella* populations. We report the identification, synthesis, and field testing of (2*S*,12*Z*)-2-acetoxy-12-heptadecene as the major component of the sex pheromone of *K. pistaciella*.

Methods and Materials

Experimental Insects In early March 2001, in several locations of the Nizip province of Gaziantep (Turkey), sections of pistachio tree branches bearing pupal cocoons were cut off and sent under import permit from Agriculture and Agri-Food Canada (Food Production and Inspection Branch) to Simon Fraser University. Insects were kept at 24°C in mesh cages under a photoperiod of 13-hr light/11-hr dark. Eclosed males were transferred to and kept individually in filter paper-lined Petri dishes. The abdominal tip with pheromone gland of 1- to 2-d-old females was removed and extracted with hexane for 15 min.

Analyses of Extracts Aliquots of 1 female equivalent (FE) of such extracts were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD; Arn et al., 1975; Gries et al., 2002), employing a Hewlett-Packard (HP) 5890 gas chromatograph fitted with a column (30 m×0.25 or 0.32 mm ID) coated with DB-5, DB-23, or DB-210 (J&W Scientific, Folsom, CA, USA). Helium was used as carrier gas (35 cm/sec), with temperature programs described in the captions of Figs. 2 and 4. GC-mass spectrometry (MS) of compounds eliciting responses from antennae and of synthetic standards employed a Saturn 2000 Ion trap GC-MS (Varian Instruments) fitted with the DB-5 column referred to above.

Instrumentation Nuclear magnetic resonance (NMR) spectra of synthetic compounds were taken on a Varian AS500 spectrometer at 499.77 MHz for ¹H and 125.68 MHz for ¹³C spectra with chemical shifts reported in parts per million relative to tetramethylsilane (¹H, δ 0.00) and CDCl₃ (¹³C, δ 77.00). Elemental analyses were performed using a Carlo-Erba model 1106 elemental analyzer. Optical rotations were measured with a Perkin-Elmer 341 polarimeter.

The major candidate pheromone component in gland extracts was isolated by high-performance liquid chromatography (HPLC), employing a Waters LC 625 HPLC equipped with a Waters 486 variable wavelength UV–visible detector set at 210 nm, HP Chemstation

software (Rev.A.07.01), and a reverse phase Nova Pak[®] C₁₈ (3.9×300 mm) column (Waters) eluted with acetonitrile (1 ml/min).

The absolute configuration of the HPLC-isolated natural pheromone component **B** and the enantiomeric excess of synthetic standards were determined by analyses of samples on a custom-made chiral GC column coated with a 1:1 mixture of heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)- β -cyclodextrin and OV-1701 (König et al., 1992; Pietruszka et al., 1992).

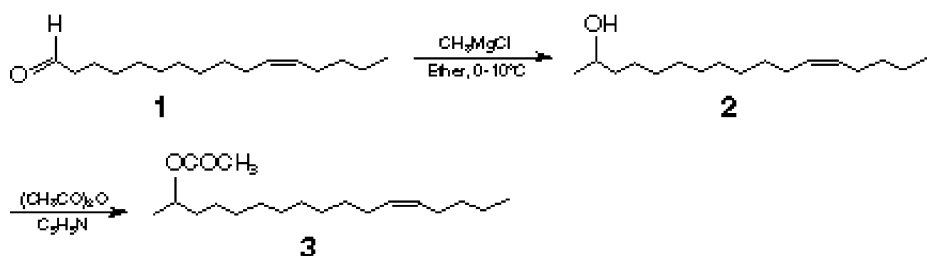
Syntheses

(*Z*)-12-Heptadecen-2-ol (compound **2** in scheme 1, Fig. 1). Racemic (*Z*)-12-heptadecen-2-ol was synthesized starting with (*Z*)-11-hexadecenal (**1**, scheme 1, Fig. 1; Bedoukian Research, Danbury, CT, USA). Compound **1** (225 g, 0.915 mol) in 400 ml of ether was added dropwise under argon at 0–10°C in 3 hr to a stirred solution of methylmagnesium chloride (3 M in ether; 333 ml, 1 mol). The mixture was allowed to warm to room temperature, quenched with 200 ml of saturated aqueous NH₄Cl, and extracted with ether (3×200 ml). Extracts were combined, washed with brine, dried (anhydrous MgSO₄), filtered, and evaporated *in vacuo*, affording 233 g of (*Z*)-12-heptadecen-2-ol (**2**, 99% pure by GC, 0.906 mol, 99% yield) as a slightly yellowish oil. ¹H NMR (CDCl₃) δ : 0.89 (t, 3H, *J*=7.1 Hz), 1.18 (d, 3H, *J*=6.3 Hz), 1.21–1.49 (m, 24H), 2.05 (m, 1H), 3.78 (m, 1H), 5.34 (m, 2H). ¹³C NMR (CDCl₃) δ : 129.84, 129.82, 68.15, 39.34, 31.94, 29.74, 29.63, 29.59, 29.55, 29.51, 29.27, 27.16, 26.88, 25.76, 23.45, 22.32, 13.98. Anal. calcd. for C₁₇H₃₄O (%): C, 80.24; H, 13.47; found C, 79.96; H, 13.24.

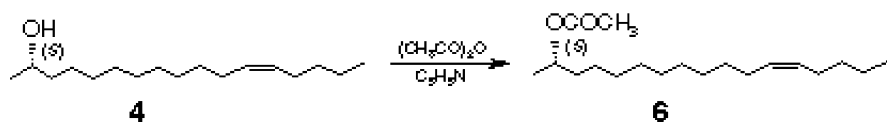
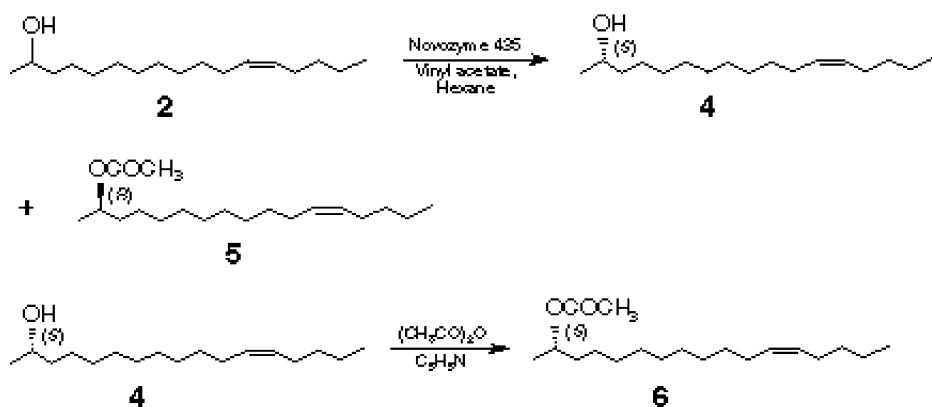
(*Z*)-2-Acetoxy-12-heptadecene (compound **3** in scheme 1, Fig. 1). Racemic (*Z*)-2-acetoxy-12-heptadecene was synthesized by adding 94 ml (0.99 mol) of acetic anhydride dropwise at room temperature to a stirred solution of 233 g (0.906 mol) of alcohol **2** in 100 ml (1.23 mol) of pyridine. After stirring for 6 hr, the reaction was quenched with water, and the product was extracted with ether/hexane (1:1) (3×250 ml). Combined extracts were washed successively with saturated aqueous NaHCO₃, HCl (10%), water, and brine, then dried (anhydrous MgSO₄). Filtration and removal of solvents afforded 270 g of (*Z*)-2-acetoxy-12-heptadecene (**3**, 99.5% pure by GC, quantitative yield). ¹H NMR (CDCl₃) δ : 0.87 (t, 3 H, *J*=7.0 Hz), 1.18 (d, 3 H, *J*=6.3 Hz), 1.21–1.35 (m, 22 H), 1.50 (m, 2 H), 2.00 (s, 3 H), 4.86 (m, 1 H), 5.33 (m, 2 H). ¹³C NMR (CDCl₃) δ : 170.68, 129.79, 129.77, 70.99, 35.88, 31.92, 29.72, 29.50, 29.49, 29.47, 29.42, 29.24, 27.14, 26.87, 25.37, 22.30, 21.31, 19.90, 13.94. Anal. calcd. for C₁₉H₃₆O₂ (%): C, 80.24; H, 13.47; found C, 79.96; H, 13.24.

(2*S*,12*Z*)-12-Heptadecen-2-ol, (2*R*,12*Z*)- and (2*S*,12*Z*)-2-acetoxy-12-heptadecene (compounds **4**, **5**, and **6** in scheme 2, Fig. 1). Enantioselective syntheses of these compounds were initiated by adding 70 mg of immobilized lipase Novozym 435 (10,000 units per gram, Sigma Chemical, St. Louis, MO, USA; Xiao and Kitazume, 1997) to a stirred solution of 0.600 g of racemic alcohol **2** (2.36 mmol; scheme 2, Fig. 1) and 0.53 ml of vinyl acetate (5.75 mmol, Aldrich Chemical, Milwaukee, WI, USA) in 3 ml of hexane. After stirring for 4 hr at 40°C, the Novozym-containing resin was filtered off, solvents were evaporated *in vacuo*, and alcohol **4** (0.280 g) and acetate **5** (0.340 g) were separated by flash chromatography [10 g of SiO₂, ether/hexane (1:20) as eluent]. Subsequent acetylation of alcohol **4** with acetic anhydride in pyridine and usual work-up (see above) afforded 0.320 g (1.08 mmol) of (2*S*,12*Z*)-2-acetoxy-12-heptadecene [**6**, 99.5% pure, 45.8% yield (theory: 50%)], [α]_D²⁰ = +0.83° (c 4.5; CHCl₃). The NMR spectrum of acetate **6** was consistent with that of racemic acetate **3**. The enantiomeric excesses (ee) of **4**, **5**, and **6** were

SCHEME 1



SCHEME 2



SCHEME 3

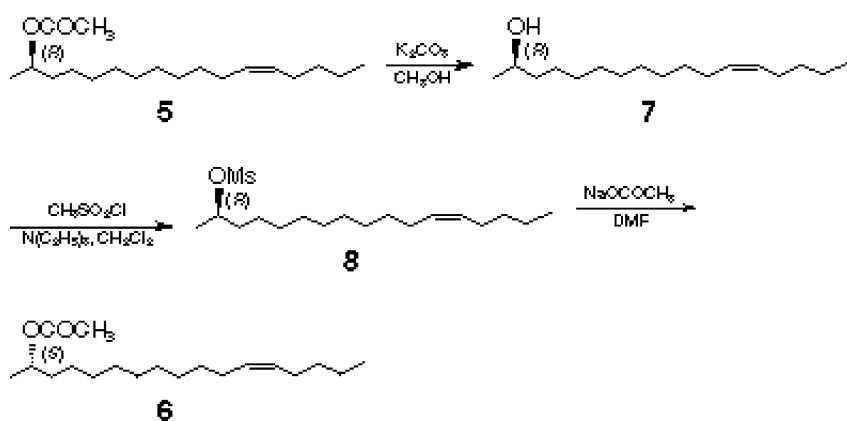


Fig. 1 Synthesis of racemic (*Z*)-12-heptadecen-2-ol (**2**) and (*Z*)-2-acetoxy-12-heptadecene (**3**) (scheme 1); enantioselective syntheses of (*2S*,*12Z*)-heptadecen-2-ol (**4**), (*2R*,*12Z*)-2-acetoxy-12-heptadecene (**5**) and (*2S*,*12Z*)-2-acetoxy-12-heptadecene (**6**) (scheme 2); and synthesis of (*2S*,*12Z*)-2-acetoxy-12-heptadecene (**6**) from (*2R*,*12Z*)-2-acetoxy-12-heptadecene (**5**) (scheme 3)

99, 95, and 99%, respectively, as determined by gas chromatography that separated enantiomers with baseline resolution (Fig. 2).

Additional quantities of **6** were obtained by a three-step synthesis (scheme 3, Fig. 1), based on inversion of the absolute configuration of **5**. A mixture of **5** (0.280 g) and K_2CO_3 (1.0 g) in methanol (10 ml) was stirred for 18 hr at room temperature. After removal of 9 ml of methanol *in vacuo*, ether (20 ml) and water (5 ml) were added to the reaction mixture. The organic layer was washed with water and brine, dried (anhydrous $MgSO_4$) and solvents were removed *in vacuo* to afford (2*R*,12*Z*)-12-heptadecen-2-ol (**7**, 95% pure by GC). Without further purification, alcohol **7** was mesylated at 0°C with methanesulfonyl chloride (1.5 ml) in the presence of triethylamine (2 ml) in dichloromethane (10 ml). After 1 hr, the reaction was quenched with aq. $NaHCO_3$. Extraction with ether (50 ml), washing of the extract with 10% HCl, water, and brine, and drying and evaporation of solvent afforded the (*R*)-mesylate **8**. Without further purification, mesylate **8** was stirred for 96 hr at 60–70°C in dry dimethylformamide (10 ml) with 3 g of sodium acetate. Usual work-up of the reaction mixture and purification of the product by flash chromatography yielded 0.180 g of pure **6** with 90% ee. Overall yield based on **5** was 64%.

Assignment of the absolute configuration of **6** was confirmed by an alternative synthesis, which comprised converting (*Z*)-1-bromo-9-tetradecene to the Grignard reagent and then to the monocuprate, adding (*S*)-propylene oxide and acetylating the resulting alcohol **4**. This

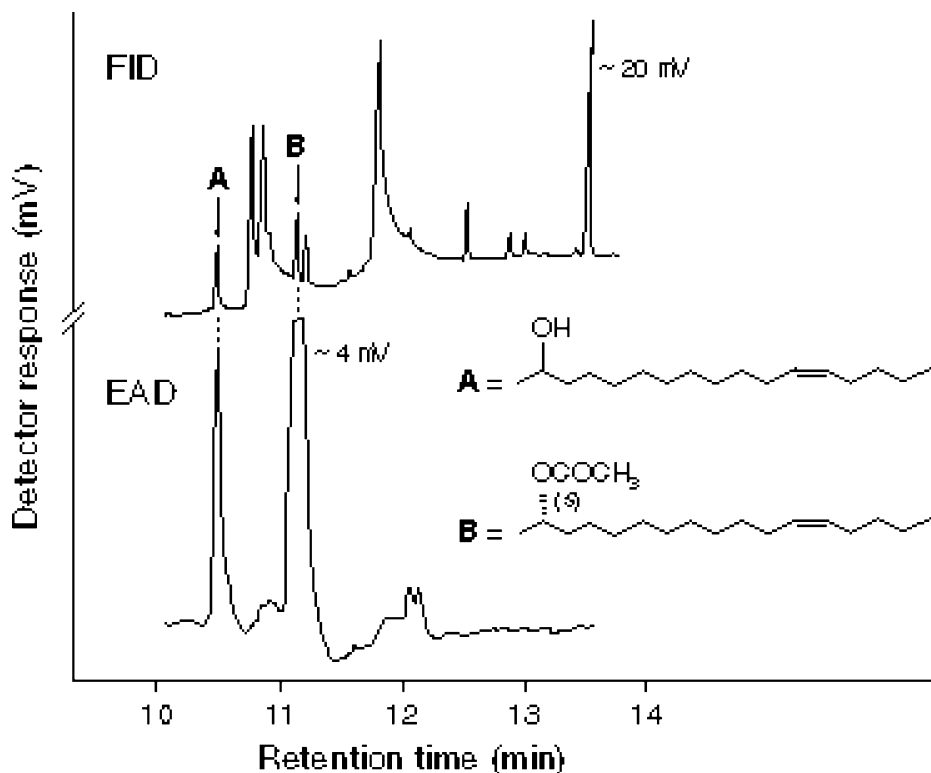


Fig. 2 Representative recording of flame ionization detector (FID) and electroantennographic detector (EAD: male *Kermania pistaciella* antenna) responses to one female equivalent of female *K. pistaciella* pheromone extract. Chromatography: DB-5 column; splitless injection, temperature of injection port and FID: 240°C; temperature program: 1 min at 50°C, then 20°C/min to 280°C

approach also proved conducive to large-scale synthesis of **6** (Britton and Khaskin, unpublished data).

Acetoxyheptadecanes (Table 1) were produced from the corresponding alcohols synthesized by Grignard reactions of suitable aldehydes with *n*-alkylmagnesium bromides. 2-Acetoxyheptadecenes with various (*E*)- and (*Z*)-double bond positions (Table 1) were synthesized from the corresponding hexadecenals according to scheme 1.

Field Experiments Field experiments were conducted in a pistachio orchard of the Gaziantep Pistachio Research Institute in Gaziantep, Turkey. Experiments employed a randomized complete block design with 8–10 replicates each. Delta-like traps were made from 2-L milk carton (Gray et al., 1984), coated with Tanglefoot (The Tanglefoot, Grand Rapids, MI, USA) and suspended from trees ~1.5 m above ground with ~16-m spacing. Traps were baited with a gray sleeve stopper (West Pharmaceutical Services, Lionville, PA, USA) impregnated with candidate pheromone components in HPLC-grade hexane.

Experiment 1 tested whether racemic (*Z*)-2-acetoxy-12-heptadecene was effective in attracting male *K. pistaciella*. Experiment 2 tested whether the corresponding alcohol, (*Z*)-12-heptadecen-2-ol, enhanced attractiveness of the acetate or was attractive by itself. Experiment 3 explored which enantiomer of (*Z*)-2-acetoxy-12-heptadecene was attractive and whether there were synergistic or inhibitory interactions between enantiomers. Considering the inhibitory nature of the *R*-enantiomer in experiment 3, experiment 4 retested the *S*-enantiomer by itself and in combination with either one or both enantiomer(s) of (*Z*)-12-heptadecen-2-ol. Doses used in field experiments are shown in Fig. 5.

Trap catch data were subjected to nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Scheffé test; Zar 1984; SAS/STAT 1988).

Table 1 Retention indices (RI) of synthetic standards and of components **A** and **B** in pheromone gland extracts of female *Kermania pistaciella*

Compounds	RI on GC column		
	DB-23	DB-5	DB-210
A in Fig. 2	2,376	1,890	2,093
B in Fig. 2	2,355	2,011	2,290
B1 (= hydrogenated B)	2,309	2,021	2,286
(<i>Z</i>)-12-Heptadecen-2-ol	2,376	1,890	2,093
(<i>Z</i>)-2-Acetoxy-12-heptadecene	2,355	2,011	2,290
(<i>Z</i>)-2-Acetoxy-10-heptadecene	2,342	2,002	2,281
(<i>Z</i>)-2-Acetoxy-11-heptadecene	2,348	2,007	2,284
(<i>E</i>)-2-Acetoxy-13-heptadecene	2,342	2,014	2,276
(<i>Z</i>)-2-Acetoxy-13-heptadecene	2,363	2,019	2,297
Heptadec-1-yl acetate	2,450	2,105	2,386
2-Acetoxyheptadecane (= B1)	2309	2021	2286
3-Acetoxyheptadecane ^a	2284	2001	2262
4-Acetoxyheptadecane ^a	2254	1981	2237
5-Acetoxyheptadecane ^a	2240	1970	2226
6-Acetoxyheptadecane ^a	2231	1965	2217
7-Acetoxyheptadecane ^a	2227	1962	2215

^a Mass spectra of 2-, 3-, 4-, 5-, 6-, and 7-acetoxyheptadecane did not have fragment ions diagnostic of the acetoxy position.

Results and Discussion

GC-EAD analyses of pheromone gland extracts from female *K. pistaciella* revealed two components (**A** and **B**) that elicited strong responses from male *K. pistaciella* antennae (Fig. 2). The mass spectrum of compound **B** (Fig. 3) with diagnostic fragment ion m/z 61 indicated an acetate functionality. Moreover, fragment ion m/z 236 [MW of $C_{19}H_{36}O_2$ (296)–60], instead of m/z 238 [MW (298)–60] expected for a saturated compound (Table 1), suggested that compound **B** had one double bond.

Hydrogenation (Millar and Haynes, 1998 and references cited therein) of pheromone gland extract, followed by repeated GC-EAD and GC-MS analyses, revealed a new EAD-active compound (**B1**) with different retention characteristics (Table 1). **B1** did not co-chromatograph with synthetic heptadec-1-yl acetate, suggesting that it might be a saturated secondary or tertiary acetate. It was determined to be 2-acetoxyheptadecane by GC-MS analyses and comparison of its GC retention indices (Table 1) with those of seven synthetic acetoxyheptadecanes (acetoxy group at C1, C2, C3, C4, C5, C6, and C7, respectively).

To determine the position of the double bond in 2-acetoxyheptadecene (**B**), HPLC-isolated **B** was treated with dimethyl disulfide (DMDS; Dunkelblum et al., 1985). The mass spectrum of the DMDS-derivative (MW=390) had adduct fragment ions [m/z 117 (26%) ($CH_3-S=CH-(CH_2)_3-CH_3$)⁺; m/z 213 (100%) ($273-HOAc$)⁺; m/z 273 (15%) ($CH_3-CHOAc-(CH_2)_9-CH=S-CH_3$)⁺; and m/z 390 (M^+ , 16%)] that revealed a double bond at C12. Synthetic (*Z*)-2-acetoxy-12-heptadecene, but not the (*E*)-isomer, and none of (*Z*)-2-acetoxy-10-heptadecene, (*Z*)-2-acetoxy-11-heptadecene, and (*Z*)- or (*E*)-2-acetoxy-13-heptadecene co-chromatographed with female-produced **B** (Table 1).

Comparative GC analyses of synthetic racemic (*Z*)-2-acetoxy-12-heptadecene, the synthetic *S*-enantiomer, and insect-produced **B** revealed that female *K. pistaciella* produces the *S*-enantiomer of **B** (Fig. 4). Component **A** (Fig. 2) had retention indices (Table 1)

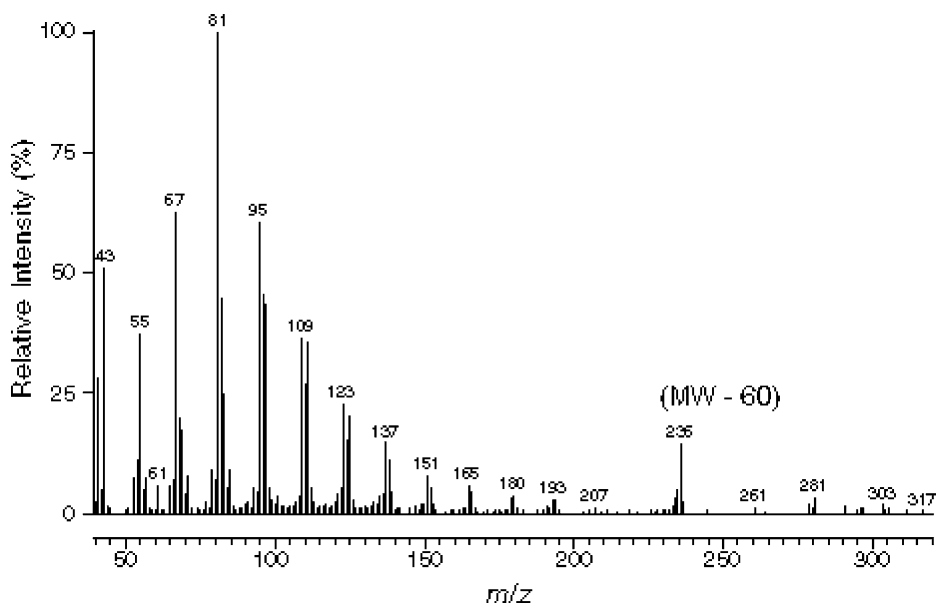


Fig. 3 Ion trap mass spectrum of compound **B** in Fig. 2 (compound **6** in Fig. 1)

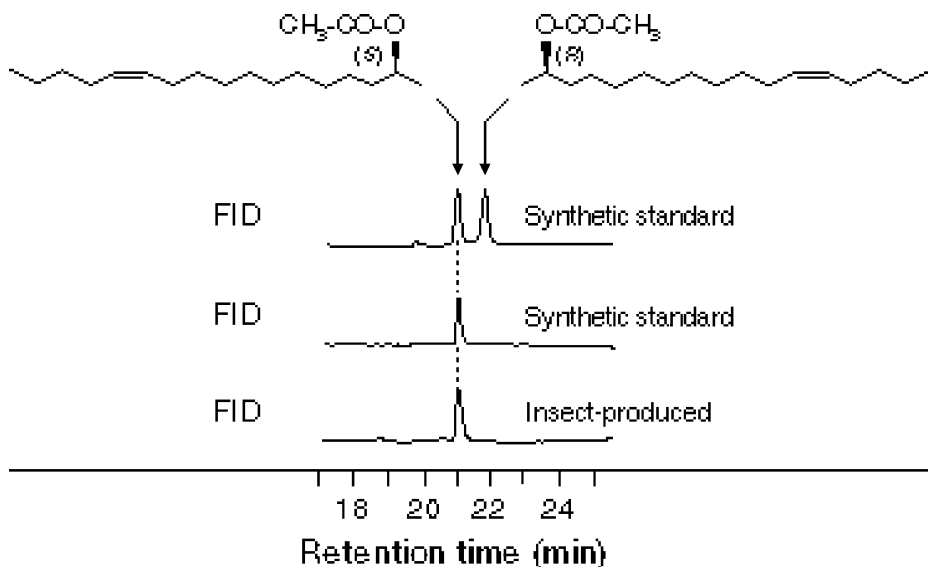


Fig. 4 Flame ionization detector (FID) chromatograms of racemic (*Z*)-2-acetoxy-12-heptadecene (**3** in scheme 1, Fig. 1), (*2S,12Z*)-2-acetoxy-12-heptadecene (**6** in schemes 2, 3; Fig. 1); and HPLC-isolated, insect-produced **B** (Fig. 2); chromatography: custom-made GC column coated with a 1:1 mixture of heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)- β -cyclodextrin and OV-1701 (König et al., 1992; Pietruszka et al., 1992); splitless injection; temperature of injection port and FID, respectively: 240 and 250°C; temperature program: 1 min at 100°C, then 10°C/min to 180°C (20 min)

indicative of an alcohol functionality. It was shown to be (*Z*)-12-heptadecen-2-ol by comparison of its GC and GC-MS data with those of authentic **2**.

In field experiment 1, traps baited with racemic (*Z*)-2-acetoxy-12-heptadecene (**3**) captured large numbers of male *K. pistaciella* (Fig. 5). The corresponding alcohol, (*Z*)-12-heptadecen-2-ol (**2**), was not attractive, and a 1:1 (w/w) mixture of **2** and **3** (as found in pheromone gland extracts) was significantly less attractive than **3** alone (Fig. 5, experiment 2). However, the addition of different ratios of alcohol **2** to the acetate **3** may have enhanced, instead of reduced, the attractiveness of the lure. This remains to be determined by further field tests. (*2S,12Z*)-2-Acetoxy-12-heptadecene attracted many males, whereas the *R*-enantiomer was not attractive and, when added to the *S*-enantiomer, significantly reduced its attractiveness (Fig. 5, experiment 3). Attractiveness of the *S*-acetate was reduced by either or both enantiomers of the corresponding alcohol (Fig. 5, experiment 4).

(*2S,12Z*)-2-Acetoxy-12-heptadecene is the first secondary acetate reported as a sex pheromone component in the Lepidoptera. In contrast, secondary alcohols have been identified in several species of the Lepidoptera, including *Stigmella malella* [(*S*)-(*E*)-6,8-nonadien-2-ol and (*S*)-(*Z*)-6,8-nonadien-2-ol (Tóth et al., 1995)], *Eriocrania cicatricella* [(*2R*)-heptan-2-ol (Zhu et al., 1995)], *Eriocrania sangii* [(*2S,6Z*)-nonen-2-ol (Kozlov et al., 1996)], *Eriocrania semipurpurella* [(*2S,6Z*)-nonen-2-ol and (*2R,6Z*)-nonen-2-ol (Kozlov et al., 1996)], and *Orgyia detrita* [(*11S,6Z,9Z*)- and (*11R,6Z,9Z*)-6,9-heneicosadien-11-ol (Gries et al., 2003)].

Straight-chain secondary acetate sex pheromones also commonly occur in cecidomyiid midges, including the Hessian fly *Mayetiola destructor* [(*2S,10E*)-2-acetoxy-10-tridecene; Foster et al., 1991; Harris and Foster, 1991; Millar et al., 1991], pea midge *Contarinia pisi* [(*S,S*)-2,11-diacetoxytridecane, (*S,S*)-2,12-diacetoxytridecane and 2-acetoxytridecane;

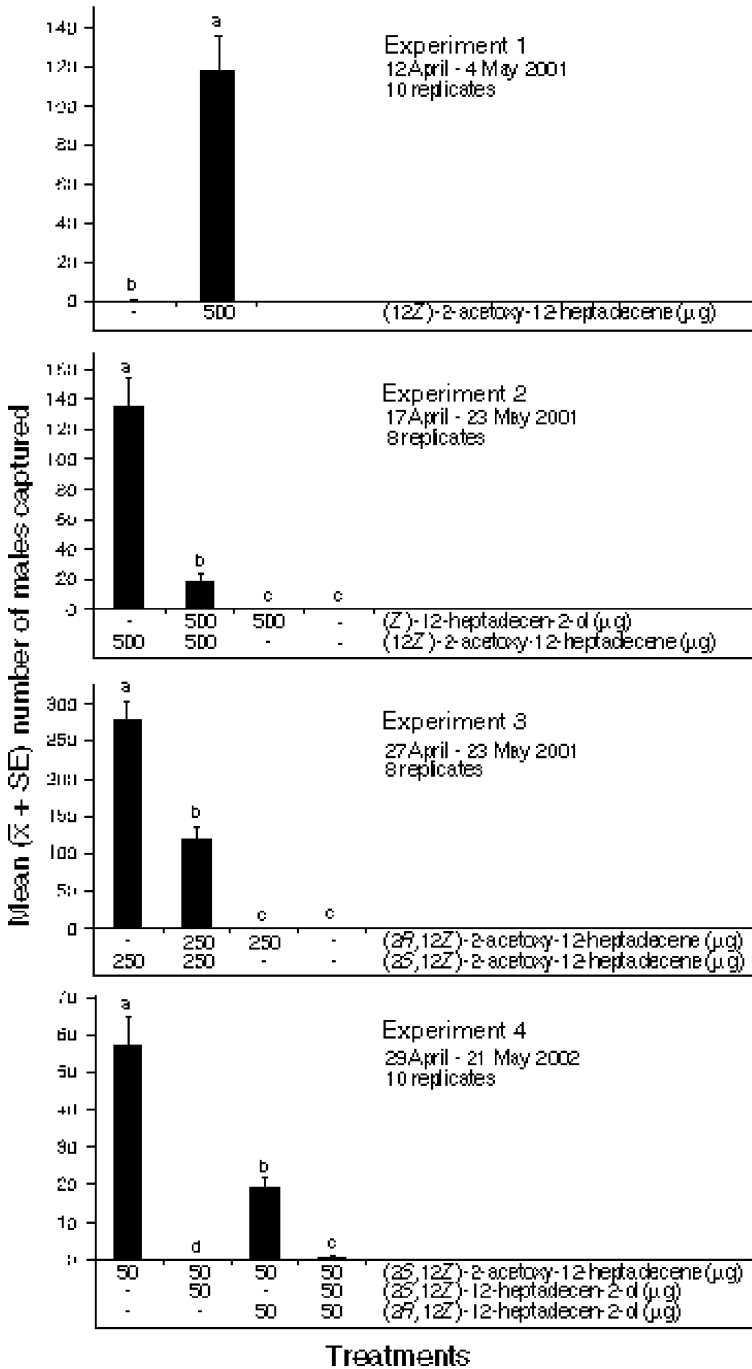


Fig. 5 Mean numbers (+SE) of male *Kermania pistaciella* captured per experimental period in experiments 1–4 in sticky traps baited with various candidate pheromone components, placed in pistachio orchards of the Gaziantep Pistachio Research Institute in Gaziantep, Turkey. In each experiment, bars with different letter superscripts are significantly different; nonparametric analysis of variance by ranks (Friedman’s test) followed by comparison of means (Scheffé test), $P < 0.05$ (Zar 1984; SAS/STAT 1988)

Hillbur et al., 1999, 2000, 2001], Douglas fir cone gall midge *Contarinia oregonensis* [(2*S*,4*Z*,7*Z*)-2-acetoxy-4,7-tridecadiene; Gries et al., 2002], *Aphidoletes aphidimyza* [(*R*,*S*)-2,7-diacetoxytridecane; Choi et al., 2004], swede midge *Contarinia nasturdii* [(*S*,*S*)-2,9-diacetoxyundecane, (*S*,*S*)-2,10-diacetoxyundecane, (*S*)-2-acetoxyundecane; Hilbur et al., 2005], and western red cedar cone midge *Mayetiola thujae* [(*S*,*S*)-2,12-diacetoxyheptadecane, (*S*,*S*)-2,13-diacetoxyheptadecane, (*S*,*S*)-2,14-diacetoxyheptadecane; Gries et al., 2005]. Cecidomyiid midges and *K. pistaciella* belong to discrete taxonomic orders (Diptera and Lepidoptera, respectively) of the Insecta. However, these two orders are related, which might explain the similarity of some of their sex pheromone structures.

With the *K. pistaciella* pheromone identified and shown to attract large numbers of male moths, it may be possible to develop pheromone-based tactics for control of *K. pistaciella*. In a 75-ha experiment in commercial pistachio orchards in Iran in 2005, plots treated with a proprietary attract-and-kill formulation containing chiral pheromone had significantly fewer larvae-infested fruit bunches (>120,000 assessed) than insecticide-treated plots or control plots (unpublished data; two PCT patent applications filed in 2006).

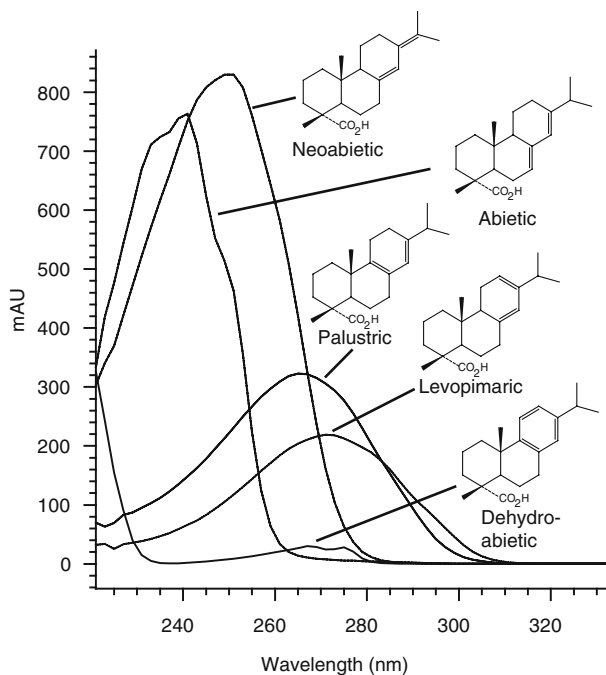
Acknowledgements We thank Wittko Francke for lending us a custom-made chiral column and for review of the manuscript, Eberhard Kiehlmann and one anonymous reviewer for constructive comments, Micky Yang for elemental analyses, Sharon Oliver and Renée Picard for word processing, and Bob Birch for graphical illustrations. The research was supported by a Discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) and by an NSERC-Industrial Research Chair to G.G. with Phero Tech Int., SC Johnson Canada, and Global Forest Science as industrial sponsors.

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Fig. 1 Structures and spectra of abietanes. A 1-mM mixture of five common abietanes (20 μ l injections) was analyzed by HPLC (see “Methods and Materials”) and spectra obtained at the apex of each peak. Retention times for the abietanes are given in Fig. 2



conifers (Wagner et al., 1983; Larsson et al., 1986). The activity of individual plant defense compounds commonly varies with the presence of other compounds, with interactive effects such as synergism frequently yielding results not predictable from analysis of individual components (Berenbaum, 1985). Moreover, concentrations of phytochemicals are typically highly variable in nature, due to both environmental and genetic differences. Thus, it is commonly necessary to analyze large numbers of samples to determine ecological significance. Based on these considerations, it is desirable to have an easy, quantitatively reliable method for separating resin acids from plant tissues. A significant analytical challenge arises from the similarity of their structures (Fig. 1).

The most common method used for resin acid analysis is gas chromatography (GC) of the methyl esters (Zinkel and Engler, 1977) with detection by flame ionization or mass spectrometry. However, this method has disadvantages, including instability of the derivatized samples (Latorre et al., 2003) and hazards of methylating reagents (potentially explosive and carcinogenic). Furthermore, considerable sample preparation is typically required with crude biological samples, in addition to derivatization. This may include extraction, partitioning of acid components into liquid or solid phase, solvent evaporation, redissolving sample in GC-compatible solvent, and removing moisture from the sample prior to injection.

There have been recent attempts to provide simple and robust analytical methods for resin acid analysis by high-performance liquid chromatography (HPLC). Reversed-phase HPLC of underivatized resin acids in river water does not resolve the structural isomers studied (abietic, isopimaric, and pimaric), and detection by negative ion electrospray mass spectrometry does not provide isomeric speciation (McMartin et al., 2002). Determination of dehydroabietic and abietic acids in Chinese medications (Lee et al., 1997) and adhesive (Lee et al., 1994) with UV detection is reported but without analysis for other abietanes.

Likewise, coumarin ester derivatives of nonaromatic resin acids are not resolved (Volkman et al., 1993).

Here, we report a simple method for the analysis of abietanes by reversed-phase HPLC. Advantages include: (1) no sample derivatization is required and therefore related expenditures and hazards are avoided; (2) extraction and chromatographic conditions are mild, and therefore, the sample components, and consequently their biological activities, should be unchanged; and (3) all components of the HPLC mobile (methanol, acetic acid, and water) phase are volatile and therefore recovery of compounds from fractionated sample is simplified. These benefits are particularly advantageous in biological studies that require rapid analysis of plant materials and screenings for biological activities.

Methods and Materials

Chemicals Abietic (90–95%), neoabietic (99+%), palustric (90–95%), levopimaric (95+%), and dehydroabietic (99+%) acids were purchased from Helix Biotech (British Columbia, Canada).

Sample Preparation Stock solutions of standard resin acids were prepared in 95% ethanol at approximately 2 mg/ml. These solutions were diluted using positive displacement syringes to prepare solutions of defined concentrations according to absorbance at λ_{\max} and literature (Joye and Lawrence, 1967) molar absorption coefficients: abietic at 241 nm ($24,150 \text{ M}^{-1} \text{ cm}^{-1}$), neoabietic at 252 nm ($24,540 \text{ M}^{-1} \text{ cm}^{-1}$), palustric at 266 nm ($9,060 \text{ M}^{-1} \text{ cm}^{-1}$), levopimaric at 272 nm ($5,800 \text{ M}^{-1} \text{ cm}^{-1}$), and dehydroabietic at 268 nm ($698 \text{ M}^{-1} \text{ cm}^{-1}$) and 276 nm ($774 \text{ M}^{-1} \text{ cm}^{-1}$).

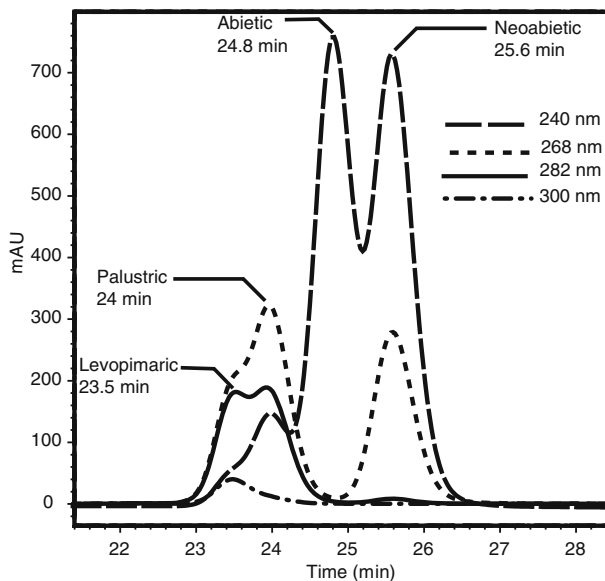
White spruce (*Picea glauca*) samples were collected from 10 mature trees in a planted stand in Dane County, WI, USA, in August. For the preparation of extracts, approximately 500 mg of phloem were cut into 2-mm pieces, extracted with 4 ml methanol, filtered through glass wool, and the extracts stored at -20°C . Extracts were filtered through 0.45- μm Teflon syringe filters prior to HPLC.

Instrumentation and Software A Hewlett-Packard (Palo Alto, CA, USA) series 1050 HPLC, fitted with an Alltech (Deerfield, IL, USA) Alltima C18 column (5 μm , $250 \times 4.6 \text{ mm}$) and a Hewlett-Packard diode array detector was used. An isocratic mobile phase with a ternary solvent system (85%, 5%, and 10%; methanol, 5% acetic acid, water, respectively) was run at a flow rate of 1 ml/min. Data were analyzed with Agilent ChemStation (Santa Clara, CA, USA) for LC 3D software.

Results and Discussion

Method Development Abietanes have distinctive spectra that we used here, together with chromatographic separation, to distinguish and quantify the resin acids by HPLC. The spectra of the individual components (Fig. 1), chromatographically separated from a 1-mM mixture of standards, show λ_{\max} values of 240 nm (abietic), 250 nm (neoabietic), 266 nm (palustric), 272 nm (levopimaric), and 266 nm (dehydroabietic). This is in excellent agreement with spectra obtained with the standards run separately, indicating little or no

Fig. 2 Multiwavelength analysis. HPLC chromatographs obtained from a 1-mM mixture of abietanes (Fig. 1) are shown at wavelengths to optimize detection and differentiation between components. Dehydroabietic acid ($rt=15.5$ min) is well resolved from the other four abietanes and detected at 268 nm

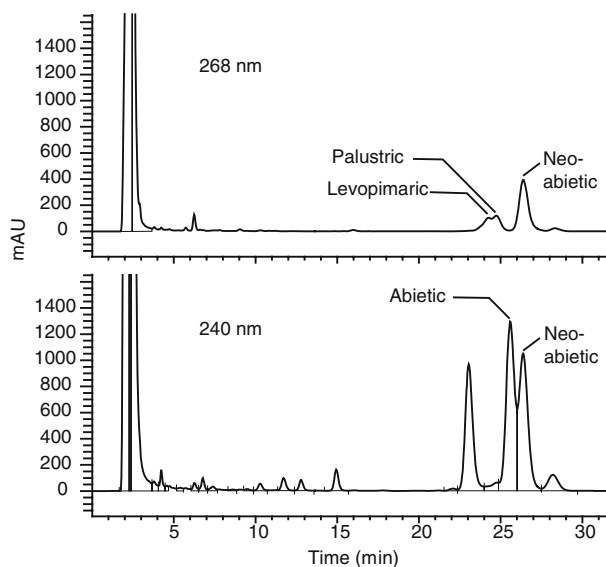


interference in the mixture. Neoabietic and abietic acids have the greatest response factors in the diagnostic wavelength region, followed by palustric, levopimaric, and dehydroabietic acids. These response factors are also in reasonable agreement with literature values of the relative molar absorption coefficients at the corresponding λ_{\max} values (see “Methods and Materials”).

Although detection of analytes at λ_{\max} provides the greatest sensitivity (absorbance units), it may not allow the determination of component concentrations if peaks are not chromatographically resolved. However, the overlay spectra of the abietanes (Fig. 1) indicated that it might be possible to distinguish the abietanes in a mixture and minimize interferences by careful choice of detection wavelengths. Also, detection at an isosbestic point provides summation of component concentrations at a wavelength of equal response. The purpose is to allow quantification in terms of absolute concentration, instead of arbitrary absorbance units, even in cases where chromatographic separation is not complete. Specifically, at 282 nm, palustric and levopimaric acid give equal response; at 240 nm, abietic and neoabietic acid give equal response; at 268 nm, neoabietic acid is detected without interference from abietic acid; and at 300 nm, levopimaric acid is preferentially detected over palustric acid.

Chromatograms of a 1-mM mixture at these four wavelengths are presented in Fig. 2 and provide a test to determine whether a multiwavelength approach can provide quantitative results. Detection at 268 nm allows baseline resolution for neoabietic acid ($rt=25.6$ m) without interference from other components, even its nearest neighbor abietic acid ($rt=24.8$ m), because it does not absorb at this wavelength. Both abietic and neoabietic acids are detected with essentially the same response at 240 nm with little interference from levopimaric and palustric acids. Similarly, palustric and levopimaric acid give the same response at 282 nm with no interference from neoabietic acid (chromatographically resolved) or abietic acid (no absorbance at 282 nm). Levopimaric and pimaric acids can be further distinguished at 300 nm, and concentrations determined without interference from the other abietanes. Dehydroabietic acid ($rt=15.5$ min) is well resolved from the other four abietanes and detected at 268 nm. For

Fig. 3 Abietanes in *P. glauca*. HPLC analysis of methanol extract (20 μ l injection) from *P. glauca* is shown with detection at 240 and 268 nm



convenience, response factors for the abietanes can be normalized to that of abietic acid at 240 nm by using standards determined individually: 240 nm: abietic (100%), neoabietic (102%), levopimaric (7.3%), palustric (19%); 268 nm: abietic (0%), neoabietic (40%), levopimaric (26%), palustric (40%); 282 nm: abietic (0%), neoabietic (1.1%), levopimaric (24%), palustric (22%); 300 nm: abietic (0%), neoabietic (0%), levopimaric (5.5%), palustric (1%).

Accordingly, the concentrations of abietanes in the 1-mM mixture were in close agreement with predicted levels (from simple dilution of stocks to prepare the mixture). For illustration, Fig. 2 chromatograms results are: abietic (1.08 mM at 240 nm), neoabietic (1.0 mM at 268 nm; 1.1 mM at 240 nm), levopimaric (1.08 mM at 282 nm; 1.02 mM at 300 nm), palustric (1.21 mM at 282 nm, 1.14 mM at 268 nm), and levopimaric–palustric sum (2.06 mM at 282 nm peak area). Peak heights are used for response factors to minimize the contribution of neighboring analytes, except in the case of levopimaric–palustric acid summation where peak areas can be used without interferences. This may allow in principle a more accurate determination of palustric by subtracting levopimaric acid concentration (1.02 mM at 300 nm) from levopimaric–palustric sum (2.06 mM at 282 nm).

The basis for the analytical technique described here relies on both the optimal combination of wavelengths for detection and the chromatographic resolution of the peaks. The degree of resolution we observe (Fig. 2) is similar to that reported previously with a Hypersil C8 column and a mobile phase of methanol–water–propanol (Rigol et al., 2003). However, the order of elution of the abietanes in their case is different (levopimaric–neoabietic < abietic < palustric). It is noteworthy that the coelution of levopimaric and neoabietic was problematic with detection by negative ionization/mass spectrometry because no fragmentation was observed (Rigol et al., 2003). The analytes of identical m/z 301 were not distinguished from each other, in contrast to the multiwavelength UV detection method presented here. We have included acetic acid in the mobile phase to stabilize pH conditions for the acid components, thus improving reproducibility and peak shape.

Detection of Abietanes in *P. glauca* The HPLC of methanol extract from *P. glauca* phloem is shown in Fig. 3. Spectral analyses of the analytes at peak apices correspond to standards using automated spectral library search. As illustrated before with standards, the following is observed: abietic (1.83 mM at 240 nm), neoabietic (1.39 mM at 268 nm and 1.44 mM at 240 nm), levopimaric (0.54 mM at 282 nm, 0.49 mM at 300 nm, and 0.56 mM at 268 nm), palustric (0.46 mM at 282 nm and 0.42 mM at 268 nm), and levopimaric–palustric sum (1.04 mM at 282 nm peak area). This corresponds to 4.0 mg abietic, 3.1 mg neoabietic, 1.2 mg levopimaric, and 0.95 mg palustric acids per gram of dry extracted phloem. The averages \pm standard deviations for 10 samples are 3.1 ± 1.6 mg abietic, 3.1 ± 2.2 mg neoabietic, 2.4 ± 2.6 mg levopimaric, and 2.2 ± 2.2 mg palustric acids per gram dry extracted phloem. The concentration of dehydroabietic acid was too low, or the interferences in the oleoresin too high, for reliable detection. A major peak at 23 min (observed at 240 nm) has a spectrum similar to that of abietic acid. This unknown was observed in all *P. glauca* samples tested.

In summary, the analytical method presented here for common abietanes has advantages particularly useful in ecological studies where large numbers of samples are required for statistical validation, and where it is important to retain the biological activities of the plant constituents. This is demonstrated with complete methanol extracts of conifer phloem with no prior fractionation. Therefore, all components are available from the chromatographic separation for subsequent chemical or biological characterizations. Samples are not derivatized (in contrast to typical GC methods), and therefore biological activities of fractionated components are retained. Components include not only the diterpenes but also monoterpenes, sesquiterpenes, and phenolics in oleoresin. The method is simple and rapid, employing robust reversed-phase HPLC. Detection at four wavelengths and spectral analysis provides confirmation and/or refinement for quantitative determination of abietic, neoabietic, levopimaric, and palustric acids.

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recognition of host plants by larvae of *M. sexta*. To answer this question, we examined various solanaceous species by standard methods and isolated fractions that were eluted in a standard chromatographic program. Fractions that showed activity in feeding bioassays were analyzed for the presence of indioside D and/or other active components.

In this work we report the isolation and identification of a new compound, a different furostan glycoside, from *S. surattenses*, which appears to account for stimulation of feeding by *M. sexta* larvae on this plant.

Methods and Materials

Plants

Solanum surattenses was grown from seeds (personal seed collection of MH) in a greenhouse under 16:8 L/D, RH=75%, and temperature=28°C until plants reached a height of approx. 0.75 cm (3–4 months).

Extraction and Isolation

The foliage that was about 0.75 cm high was extracted with hot water (approximately 5 ml g), and the solid debris was filtered off. The resulting extract was concentrated under reduced pressure to a small volume. The extract was applied to a C₁₈ reverse-phase preparative column and eluted under medium pressure with increasing concentrations of MeOH (0%, 25%, 50%, 75%, and 100%) in water, and five fractions were collected (1–5). Fractions 2–4 were combined and rechromatographed on a second C₁₈ column by eluting with increasing concentrations of MeOH (0%, 25%, 35%, 45%, 55%, 75%, and 100%) in water. Seven fractions (A–G) were collected, and fraction F was further separated by HPLC using a Discovery RP Amide C₁₆ semiprep column (Supelco, 25 cm×10 mm, 5 μm) using a water/acetonitrile gradient system (0% acetonitrile at 0 min; 30% at 5 min; 63% at 30 min and 100% at 50 min; flow rate 2.2 ml/min) and monitored at 254 nm by a diode array detector (Program I). The active fraction, between 20.7 and 25.5 min, as identified by bioassay experiments, was collected and rechromatographed on an amide C₁₆ analytical column (Discovery, Supelco, 15 cm×4.6 mm, 5 μm) using a slightly different water-acetonitrile gradient system (0% acetonitrile at 0 min; 23% at 10 min, 30% at 40 min, and 100% at 50 min; flow rate 1.0 ml/min) (Program II), and an active fraction (compound **1**) was isolated (yield ~0.5 mg/gm leaf material).

Instrumentation

CID mass spectra were recorded using a Waters-Micromass (Manchester, UK) Quattro I triple quadrupole mass spectrometer equipped with an electrospray ion source. The source temperature was held at 85°C. The argon gas pressure in the collision cell was adjusted to attenuate precursor ion transmission by 50%, and laboratory-frame collision energy was optimized for each experiment. Samples were infused into the ESI source as acetonitrile–water solutions at a rate of 5 μl min⁻¹ via a syringe pump. Solutions were prepared by dissolving samples in 80:20 (v/v) of acetonitrile/deionized water mixture (LC-MS grade acetonitrile; Riedel-de Haën, Seelze, Germany) and 0.1 ml of 1.0% (v/v) formic acid in water (~25% in H₂O, Fluka, Buchs SG, Switzerland). Accurate mass measurements were obtained by electrospray ionization mass spectrometry (ESIMS) using an Autospec high-

resolution mass spectrometer (Micromass) with PEG as the reference material. All nuclear magnetic resonance (NMR) experiments were performed on a Bruker AVANCE 600, with a 5 mm TXI probe at 30°. All 2D NMR spectra were acquired in CD₃OD using standard Bruker pulse sequence. Signal at 3.3 ppm for MeOH was used as an internal reference to calibrate the spectra. Spectra were analyzed using Bruker XWINNMR version 3.5 software. Chemical shifts are presented as ppm.

Bioassays

Choice assays were conducted using 1.4 cm diam discs cut from fresh cowpea (nonhost) leaves as the substrate. Four discs were presented to each larva. Two of the discs were treated with 20 μ l (10 μ l on each side) of the test solution diluted in methanol, and the two control discs were treated with same amount of methanol. Treated and control discs were pinned alternately in a 15 cm Petri dish lined with moist filter paper. One 3rd- or 4th-instar *M. sexta*, reared on potato foliage, was randomly selected and placed at the center of each dish. After a period of 30–120 min, we recorded whether a treated or control disc was eaten. Distinct biting was also recorded as stimulatory activity. In general, if the sample was active, insects fed on one of the treated discs within the first 15 min.

Statistical Analyses

2 \times 2 Tables were prepared for the number of treatments and controls that were eaten or not, and Fisher's exact tests were conducted to determine whether the extracts were active or not.

Results and Discussion

Bioassay-guided fractionation of methanolic extracts led to the isolation of a single compound (**1**), which showed significant feeding stimulant activity (Table 1). Forty-two g leaf equivalents (gle) of extract yielded 19.9 mg of compound **1**. Throughout the isolation process, most of the fractions were active at a concentration of 0.1 gle, but higher

Table 1 Feeding Responses of *Manduca Sexta* (3rd Instars) to Fractions of *Solanum surattenses* Extracts and Compound **1**

Extract/Fractions	Conc. in gle*	No. fed on treatments	No. fed on controls	Total of treatments	P value (Fisher's exact test)
Fraction E+F	0.02	8	0	12	<0.01
	0.01	7	0	12	<0.01
Fraction F (20.7–25.4 min)	0.01	9	0	12	<0.01
Fractions of F ^a					
F1 (15.5–31.3 min)	0.01	0	0	12	1
F2 (31.3–32 min)	0.01	5	2	12	0.185
F3 (32–40 min)	0.05	21	2	24	<0.01
Compound 1	0.2	7	0	12	<0.01
	0.1	10	0	12	<0.01

gle: Gram leaf equivalent.

^aFraction F at 20.7–25.4 min was refractionated into F1–F3 on C16 amide column (Discovery, Supleco) using program II.

concentrations were necessary to demonstrate activity of the highly purified material. Some loss of activity occurred with repeated fractionations, and compound **1** eluted as a broad peak with a long trailing edge.

Compound **1** was isolated as white amorphous powder. A positive-ion ESIMS showed a signal at m/z 1071 for $(M+Na)^+$, and additional accurate ESIMS provided a molecular formula of $C_{51}H_{84}O_{22}$ [1049.189 Da was observed for $(M+H)^+$ ($C_{51}H_{85}O_{22}$), (1049.553 Da calculated)]. 1D 1H NMR spectral data provided conclusive evidence of a steroidal glycoside structure. Under positive-ion ESI conditions, MS–MS of the quasimolecular ion peak, m/z 1071, peaks for product ions were observed at m/z 1031 (loss of water), 885 (loss of a deoxyhexose), 869 $[(M+H) - 162]^+$ (loss of a hexose), 723 $[(M+H) - 162 - 146]^+$ (loss of a hexose and a deoxyhexoses), 577 $[(M+H) - 162 - 146 - 146]^+$ (loss of two deoxyhexose and a hexose) and 415 $[(M+H) - 162 - 146 - 146 - 162]^+$ (loss of two hexoses and two deoxyhexose), 397 $[(415+H) - 18]^+$. The results suggested the presence of four sugar molecules, two of which are deoxyhexoses and the other two hexoses. The fragmentation pattern also suggested branched substitutions of sugars since fragmentation could result in a loss of either a hexose moiety or a rhamnose moiety. The presence of four sugars was confirmed by the presence of four NMR peaks at δ 5.14, 4.83, 4.49, and 4.22 for the anomeric protons. Moreover, the COSY, HSQC- TOCSY, and 1HNMR spectra suggested the two deoxyhexoses to be rhamnose and the two hexoses to be glucose based on the coupling constants observed for protons of each hexose moiety (Table 2). Also, the anomeric protons of two rhamnose moieties showed a small coupling constant of 1.3 Hz, indicating that they were β -substituted at C-1. Similarly, the large coupling constants of around 8 Hz indicated the glucose moieties were α -substituted. Furthermore, 1H NMR showed the presence of six methyl groups, four as doublets at δ 1.24, 1.24, 0.99, and 0.94, and two as singlets at δ 1.04 and 0.82, respectively. The signals at δ 1.24 and 1.24 correlated to sugar moieties in COSY and HSQC-TOCSY and were assigned to two rhamnoses. The multiplicity of methyl group signals suggested that the aglycone is a steroidal molecule. The signal at δ 3.58 suggested the presence of a 3-*O*-substituted OH group, which was correlated to the protons at δ 2.44 (dd, $J=12, 2.33$ Hz) and 2.28 (dd, $J=12, 12$ Hz) of C-4, and confirmed the α -orientation of the hydroxyl group. The downfield shift of H-4ab and a broad signal at δ 5.39 suggested presence of $C_5:C_6$ unsaturation. This was further confirmed by ^{13}C NMR and HMBC correlations. Peaks at δ 3.71, and 3.38 showed a correlation to signals at δ 1.73 and 0.94. Also in TOCSY, they correlated to a quaternary carbon signal at δ 110.0 ppm. The signal at δ 4.56 and its correlation to the doublet at δ 0.99 in TOCSY, along with COSY data established the presence of a furan ring. Thus, we concluded that compound **1** was a furostan derivative. The reported NMR values for the steroidal part compared well with some of the literature values reported for furostan derivatives, and the configuration at carbon 25 matched with 25*R* (Table 2) (Aquino et al., 1986; Yahara et al., 1996). Linkages between sugar moieties were ascertained by chemical shifts of ^{13}C of sugars and by HMBC and ROESY correlations. The anomeric proton at δ 4.49 showed a correlation with the proton at δ 3.58 and 2.44 in ROESY, thus indicating that the glucose is attached to C-3, and all these protons are on the same side of the molecule. Similarly, the signal at δ 5.15 correlated to 4.49 and 3.58. The signal at δ 3.39 correlated to the signal at 100.91 in HMBC, and thus established that one of the rhamnose molecules is attached to glucose at the C-2' position. The anomeric proton at δ 4.29 showed a correlation to the proton at δ 3.51 of C-4 in ROESY. The anomeric carbon at δ 101.8 showed a correlation to the proton at δ 3.58 in HMBC, thus establishing the substitution on C-3'' of rhamnose molecule. This was further substantiated by the shift to a higher value (78.0 ppm) for the carbon attached to it. The fourth anomeric proton at δ 4.22 of the second glucose

Table 2 NMR Values of Compound **1** in CD₃OD

Position	$\delta^1\text{H}$	Splitting	J values (Hz)	^{13}C (ppm)
1	1.07, 1.87			37.1
2	1.62, 1.59			29.3
3	3.58	d		76.6
4	2.44, 2.29	dd	13, 2.33	38.1
5				140.0
6	5.37	d	3.1, 4.8	121.6
7	1.99, 1.50			32.5
8	1.65			31.2
9	0.96			52.0
10				38.1
11	1.58, 1.55			20.8
12	1.20, 1.78			39.6
13				41.8
14	1.13			56.3
15	1.99, 1.25			31.8
16	4.56	dd	7.1, 14.8	80.8
17	1.76			62.6
18	0.82	s		15.3
19	1.04	s		18.4
20	2.10	m		39.5
21	1.00	d	7	14.4
22				110.0
23	1–2, 1.67	m		31.2
24	1–2, 1.68			27.7
25	1.73			33.5
26	3.71, 3.38			74.6
27	0.94	d	6.7	16.0
1'	4.49	d	7.8	99.1
2'	3.40	dd		77.8
3'	3.59	dd	9.2, 9.2	77.8
4''	3.51	dd (t)	9.2, 9.2	78.3
5'	3.38	1h m		74.5
6'	3.78, 3.63	dd	1.8, 12.2	60.5
1''	4.83	d	1.3	101.8
2''	3.82	dd	1.7, 8.9	71.1
3''	3.58	dd	8.9, 8.9	78.0
4''	3.38	dd	9.1, 9.1	72.5
5''	3.92	dq	6.2, 9.1	69.3
6''	1.24	d	6.2	16.6
1'''	5.14	d	1.3	100.9
2'''	3.91	dd		70.7
3'''	3.65	dd		71.0
4'''	3.38	dd	9.1, 9.1	72.3
5'''	4.12	dq	6.2, 9.1	68.3
6'''	1.23	d	6.2	17.0
1''''	4.22	d	7.7	103.2
2''''	3.17	dd	7.9, 9.12	73.7
3''''	3.34	dd		76.7
4''''	3.29	m		77.7
5''''	3.25	m		70.3
6''''	3.85, 3.65	dd	2.0, 12	61.4

moiety showed a correlation to the signal at δ 3.71 and 3.37 of C-26 protons in ROESY and HMBC. Furthermore, the ^{13}C NMR shift of C-26 was similar to that of compounds in which C-26 is substituted with a glycoside (Yahara et al., 1996). Therefore, we could establish that compound **1** is 26-*O*- β -D-glucopyranosyl-(25*R*)-furosta-5-ene-3- β -yl-*O*- α -L-rhamnopyranosyl-(1''-2')-*O*- α -L-rhamnopyranosyl-(1'''-3'')-*O*- β -D-glucopyranoside (Fig. 1). To the best of our knowledge, this compound has not been previously reported.

Acceptance of *S. surattenses* as a host plant by *M. sexta* larvae can be explained by the stimulatory effect of compound **1**. Compound **1** has the same steroidal structure as indioside D, which was reported as a feeding stimulant for *M. sexta* from potato foliage (del Campo

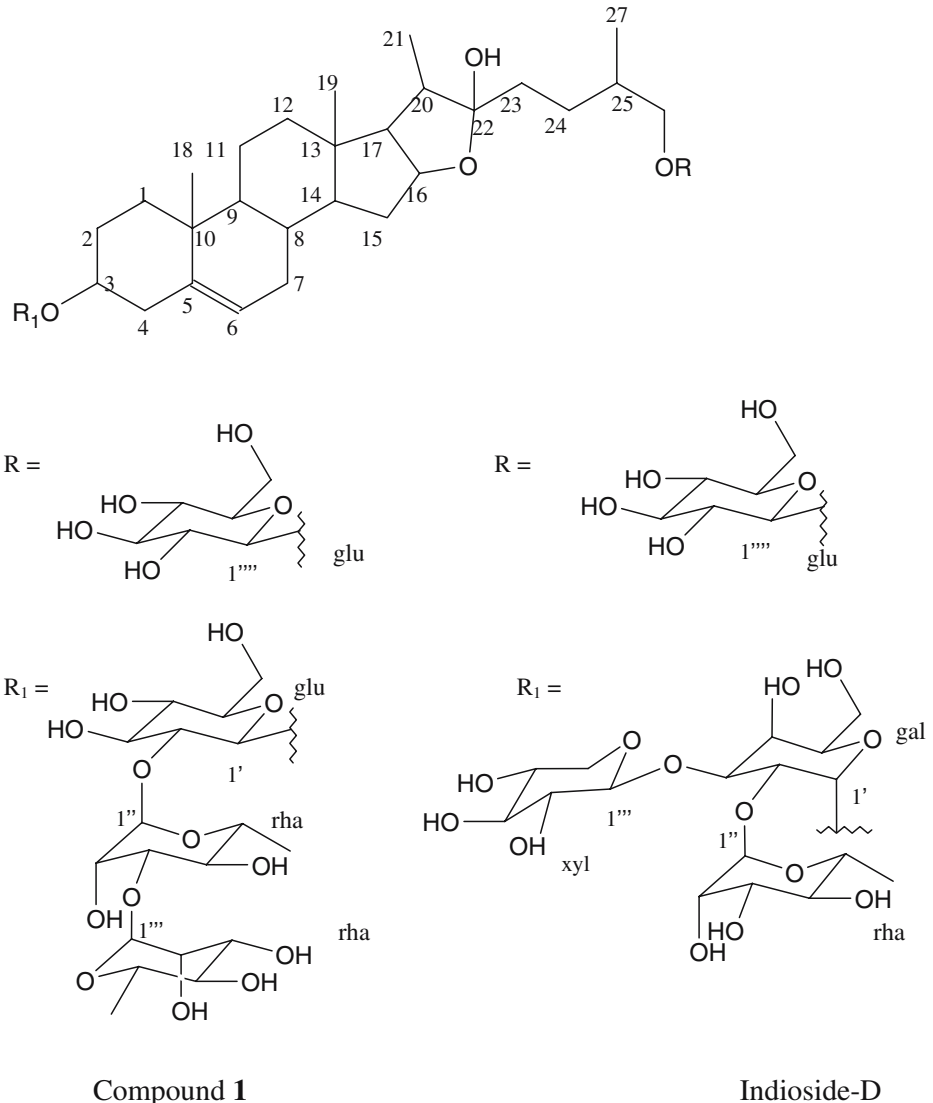


Fig. 1 Chemical structures of compound **1** and indioside-D

et al., 2001), but it differs in the types of sugars and in their attachment. Indioside D has branched sugar substitution, whereas the sugars in compound **1** are not branched.

Solanaceous plants are rich in steroidal and triterpenoid glycosides, and many of these, alone or in combination, may be capable of serving as recognition factors for this insect. Other fractions of *S. surattenses* extracts showed varying amounts of feeding stimulatory activity at different concentrations, suggesting that more than one compound is involved in recognition of this plant. Furthermore, recent studies with potato foliage have indicated that other compounds may also be involved in stimulating feeding by *M sexta* larvae (Müller and Renwick, 2001). Additional chromatographic fractions in potato extract were active in bioassays, but the active compounds were not isolated and identified. These results suggest that *M. sexta* may become “tuned” to any one of several related steroidal glycosides.

M. sexta is one of about 800 species of hawkmoths (Family: Sphingidae) worldwide that develop on a variety of host plants (Oehlke, 2005). The genus *Manduca* (tribe: Sphingini), consisting of approximately 70 species, is widely distributed throughout North and South America and in the Caribbean Islands. The main hosts are in the Solanaceae, Verbenaceae, Boraginaceae, Bignoniaceae, Oleaceae, and Annonaceae, although a few of them feed on some other families (Mechaber and Hildebrand, 2000; Oehlke, 2005). All of these plant families are rich in biologically important chemical constituents that include terpenoids, iridoids, lignans, flavonoids, quinones, alkaloids, etc., and their glycosides.

Larval food is selected by adult *M. sexta* females during oviposition, and the female's choice of plant for oviposition is based on stimulants that may include visual, volatile, and contact cues (De Boer and Hanson, 1984; Fraser et al., 2003). Furthermore, oviposition stimulants and feeding stimulants may not be the same compounds or even the same type of compounds. Several researchers have studied the role of host volatiles that are involved in attracting the moth to the plant (Fraser et al., 2003), but little is known about the contact oviposition stimulants. It is likely that the different host plant families are related in having similar oviposition stimulants that attract the females to lay eggs on them. del Campo et al. (2001) have shown that the presence of indioside D alone was sufficient to stimulate feeding by *M. sexta* larvae. However, after their experience with this compound in their diet, larvae became dependent on it for continued feeding. This dependency has been related to development of receptor sensitivity to the compound. This would suggest that larvae have the plasticity to develop receptor sensitivity to a compound and thus may be capable of developing sensitivity to different chemicals in different host families, based on their first experience (De Boer and Hanson, 1984). In addition, acceptance of a plant for oviposition or feeding is dependent on the presence of other components in the host, either stimulants or deterrents that may contribute to a balance of positive and negative stimuli that results in acceptance or rejection of a plant.

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odor blend could be a useful attractant in detecting the presence of the biocontrol agent, *D. elongata*, in stands of saltcedar newly colonized by the beetle.

Keywords Biological control · Chrysomelidae · Coleoptera · *Diorhabda elongata* · Electrophysiology · Field evaluation · Green leaf volatiles · Host-odor attractants · Saltcedar · *Tamarix ramosissima*

Introduction

The leaf beetle *Diorhabda elongata* Brullé (Coleoptera: Chrysomelidae) has been successfully introduced into the United States from China as a biocontrol agent for saltcedar (*Tamarix* spp., Tamaricaceae) (DeLoach et al., 2003, 2004). Saltcedars were originally imported from Eurasia as ornamentals and for erosion control of streambanks and river channels. However, these fast-growing shrubs or small trees are invasive and have become a major component of western riparian ecosystems (Friedman et al., 2005), causing serious environmental and economic damages (Zavaleta, 2000; Shafroth et al., 2005).

In an earlier study (Cossé et al., 2005), we identified the male-produced aggregation pheromone of *D. elongata* as a 1:1 blend of two seven-carbon compounds, (2*E*,4*Z*)-2,4-heptadienal (2*E*, 4*Z*-7:Ald) and (2*E*,4*Z*)-2,4-heptadien-1-ol (2*E*, 4*Z*-7:OH) and demonstrated attraction to the synthetic compounds in the field. The pheromone was identified in collections of volatiles from beetle-infested saltcedar by using electrophysiological analysis of these collections as a key tool. We noted that the antennae of male and female beetles responded to a number of compounds from the host foliage in addition to the pheromone components. Here, we report the identification and quantification of several saltcedar-emitted compounds and their electrophysiological and field activity for *D. elongata* adults.

Methods and Materials

Insects

Adult *D. elongata* used in this study were collected from a field population at Lovelock, NV, USA, or obtained from a colony maintained by one of us (DWB) at the United States Department of Agriculture Agricultural Research Service (USDA-ARS) Exotic and Invasive Weed Research Unit, Albany, CA, USA. The beetles originated from sites near Fukang, in the Xinjiang province of northwestern China (DeLoach et al., 2003; Lewis et al., 2003). Upon arrival at the USDA-ARS National Center for Agriculture Utilization Research (NCAUR) in Peoria, IL, USA, adult beetles were kept at 25°C under a 17-hr light/7-hr dark photoperiod. *Tamarix ramosissima* Ledebour was grown year-round in a greenhouse facility at NCAUR to provide food for the beetles. The beetles were used for electrophysiological tests and also to create feeding damage on saltcedar foliage.

Collections of Volatiles

Two types of collection techniques for volatiles were used: solid-phase microextraction (SPME) and Super-Q/activated charcoal filters. The SPME collections allowed for rapid

detection of changes in saltcedar volatile profiles, whereas the Super-Q/charcoal collections served as the source of saltcedar volatiles for compound quantification and gas chromatographic–electroantennogram detection (GC-EAD) analysis.

Volatile saltcedar compounds, emitted during the first 30 min of experiments and directly after beetles were placed on the saltcedar foliage, were collected simultaneously on polydimethylsiloxane/divinylbenzene (65 μm) fibers of SPME needles (Supelco, Bellefonte, PA, USA) and on collection filters (Cossé et al., 2002) containing a mixture (40 mg, 1:1) of Super-Q porous polymer (80–100 mesh, Alltech, Deerfield, IL, USA) and activated charcoal (Darco G-60, EM Science, Cherry Hill, NJ, USA).

Collections of volatiles were made from saltcedar foliage (branch approximately 75 cm long), either infested with beetles (about 200, both sexes but predominately males) or without beetles. In either case, the foliage was placed in a vertical glass tube (5 cm inside diameter [i.d.] \times 65 cm) with the freshly cut end protruding from the lower opening into a beaker of water. A piece of fine mesh nylon cloth with a small central hole for the saltcedar stem was secured around the lower end of the tube to keep the beetles contained. In some cases, collections of volatiles were made specifically from the flowering parts on the foliage. The collections were made at 25°C under a 17-hr light/7-hr dark photoperiod.

Using several reducing glass adapters, the top of the tube (female 55/50 ground glass joint) was fitted with an adapter with a male 24/40 joint on one end and a threaded fitting with an air-tight “O” ring seal on the other (Ace Glass, Vineland, NJ, USA, #5028-30). The threaded airtight fitting held one leg of a T-shaped glass tube (0.5 cm outside diameter). A second leg received the SPME needle through a rubber septum, and the last leg was connected to the Super-Q/charcoal filter assembly, which was, in turn, connected to a vacuum line (for diagram, see Bartelt and Zilkowski, 1999).

With the vacuum line, air was drawn through the collection tube and past the SPME fiber at 25 ml/min during the SPME sampling. After 30 min, the SPME needle was removed and the flow rate adjusted to 1 l/min for the remainder of the 24-hr collection period. Foliage was replaced on a daily basis and any dead beetles in the collector were replaced with fresh ones. Collected volatiles were rinsed from the filters into vials using methylene chloride (400 μl) after every 24 hr. 1-Octanol (10 μl of a methylene chloride solution containing 250 ng/ μl) was added to each vial as a quantitative internal standard.

It was confirmed that the 6-carbon and larger compounds did not break through the Super-Q/charcoal filters by placing a second filter in series and analyzing both separately. However, collection filters of Super-Q or 60–80 mesh-activated charcoal (GraphTrap-GB, Alltech) alone did not trap 6-carbon compounds quantitatively, and the very fine powdered Darco G-60 charcoal created excessive airflow resistance in the filters. The mixture of Super-Q and Darco G-60 charcoal resolved both problems.

Quantification

The amounts of (*E*)-2-hexenal (2*E*-6:Ald), (*Z*)-3-hexenal (3*Z*-6:Ald), (*Z*)-3-hexen-1-ol (3*Z*-6:OH), and (*Z*)-3-hexenyl acetate (3*Z*-6:OAc) in the collections of volatiles were quantified by coupled GC–mass spectrometry (GC-MS) using selected ion monitoring (SIM) mode. Two ions were monitored for each compound, *m/z* 83 and 98 for 2*E*-6:Ald, *m/z* 82 and 100 for 3*Z*-6:OH, *m/z* 69 and 98 for 3*Z*-6:Ald, and *m/z* 67 and 82 for 3*Z*-6:OAc. The selected ions for the internal standard, 1-octanol, were *m/z* 70 and 84. Quantification was based on the italicized ions of the above-mentioned ion pairs, whereas the second ions served as qualifiers (proper ion ratios would support compound identity and purity in the GC peaks).

Serially diluted solutions of all the synthetic compounds and internal standard (0.01–30 ng/ μ l) were first analyzed by GC with flame-ionization detection (GC-FID) to verify proper concentrations and further analyzed by GC-MS in SIM mode to obtain linear calibration curves (log dose vs. log abundance). The linear calibration curves (average of three replicate injections for each concentration) served as the basis of the quantification. In SIM mode, compounds were still easily detectable at concentrations as low as 10 pg/ μ l.

Electrophysiology

GC-EAD analyses were made by methods and equipment generally described by Cossé and Bartelt (2000). GC-EAD connections were made by inserting a glass-pipette silver-grounding electrode into the back of an excised beetle head. A second glass-pipette silver-recording probe was placed in contact with the distal end of one antenna. Both pipettes were filled with Beadle–Ephrussi (Ephrussi and Beadle, 1936) saline.

Instrumentation

Volatile collections were analyzed by GC-FID and GC-MS. Samples were injected in splitless mode using Hewlett Packard 6890 (Palo Alto, CA, USA) instruments fitted with 30-m DB-1, DB-5, or DB-Wax capillary columns (0.25 mm i.d., with 1.0-, 0.25-, and 0.5- μ m film thickness, respectively, J&W Scientific, Folsom, CA, USA). Temperature programs were from 40 to 280°C at 10°C per minute. Inlet temperatures were maintained at 280°C, and GC-EAD effluent interface from postcolumn splitter was kept at 280°C. For SPME analysis, the GC inlet port was fitted with an SPME-optimized glass liner (Supelco). Mass spectrometry was performed using a Hewlett Packard 5973 instrument (electron impact, 70 eV). The Wiley mass spectral library, with 275,821 spectra, was available on the MS data system (Wiley, 1995).

Chemicals

The two pheromone components, 2*E*,4*Z*-7:Ald and 2*E*,4*Z*-7:OH, were synthesized according to Petroski (2003). The purities of the compounds were >95% by GC-FID; by GC-MS, impurities were other geometrical isomers. 3*Z*-6:Ald (50% in triacetin, Bedoukian, Danbury, CT, USA) was obtained in pure form by Kugelrohr distillation (room temperature, 40 mm Hg) and stored (–70°C) as a dilute solution in methylene chloride. *E,E*-Farnesene was obtained by gentle reflux (4 hr) of *E,E*-farnesol (1 g) with Dowex 50W-X4 cation exchange resin (2 g) in hexane (50 ml), followed by open column chromatography on silver nitrate-coated silica (25% AgNO₃, elution solvent 15% 1-hexene in hexane). All other compounds were purchased (Aldrich, Milwaukee, WI, USA) with purities >97%.

Field Lures

Two types of host odor lures were prepared for field testing. The first lure type consisted of four green leaf volatiles (GLV), 3*Z*-6:Ald (4 mg), 2*E*-6:Ald (4 mg), 3*Z*-6:OH (0.8 mg), and 3*Z*-6:OAc (0.6 mg), in mineral oil. The second lure type consisted of seven compounds, heptanal (0.1 mg), octanal (0.25 mg), nonanal (1 mg), *E,E*-2,4-heptadienal (0.2 mg), *E,E*-2,6-nonadienal, indole (2 mg), and *E,E*-farnesene (2 mg), in mineral oil. A bulk solution of the compounds needed for each lure type was prepared in mineral oil (enough of each for

50 lures, in a total volume of 25 ml). Then, aliquots (0.5 ml) of this mixture were placed in 1.5 ml polypropylene micro centrifuge tubes (Bio Plas Inc., San Rafael, CA, USA) together with a piece (1 cm long) of braided cotton roll (Richmond Dental, Charlotte, NC, USA). The emission rates of freshly prepared GLV lures were measured in an incubator, using the general reported methods and equipment reported earlier (Cossé et al., 2005). Specifically, volatiles were collected from lures at 25°C with a 150-ml/min airflow. Volatiles were collected periodically over 2 d, and the release rates of individual compounds were measured by GC-FID using 1-octanol as the quantitative internal standard.

Lures were stored at -20°C until needed. Prior to field deployment, the caps of the tubes were pierced with a single pinhole (1 mm) to allow compound release from the headspace.

Pheromone lures (Cossé et al., 2005) consisted of a 1:1 mixture of 2*E*,4*Z*-7:Ald and 2*E*,4*Z*-7:OH (500 µg of each component), released in a nearly 1:1 ratio at 7 µg component⁻¹ d⁻¹.

Field Study

Experiments were carried out between May and September 2004 in saltcedar stands along the Humboldt River (40°01'N, 118°31'W) 17 km southwest of Lovelock, NV, USA, where a population of *D. elongata* had been established (DeLoach et al., 2004).

Yellow sticky traps (15.5×30.5 cm, AgriSense, Pontypridd, UK) were attached to branches (oriented vertically) in the upper half of the trees, typically at a height of 2–4 m, and were placed in trees that were at least 10 m apart and were similar in size, foliage density, and in accessibility to beetles flying from downwind. There were always other saltcedar trees near the treatment trees and usually between them as well. The protective paper was removed only from one side of the trap, so that the sticky side was oriented downwind. The traps were set out in midafternoon, when flight activity usually began to intensify, and trap counts were made the following morning. Thus, each replicate lasted 1 d. A wire, wrapped around the bait tube, was used to secure the lure to a trap.

The field experiments were carried out in two types of saltcedar stands. Defoliated saltcedar consisted of saltcedar that had previously experienced complete defoliation and had little or no foliage present during the experiment. For the experiment beginning on May 6, 2004, the stand had experienced defoliation the previous season, and adult beetles had overwintered in the leaf litter beneath the stand. They were newly emerged from overwintering when the experiment was performed. Later in the season, newly defoliated saltcedar stands were the result primarily of heavy infestation with *D. elongata* larvae. The second stand type had little or no previous defoliation; adults were just beginning to move into these stands, whose trees had relatively lush green foliage during the tests.

Three sets of experiments were deployed. The first set was a preliminary assessment of the host odor lures. The two types of host odor lures (four-component and seven-component) were first combined on traps and compared to unbaited control traps in a paired design ($N=12$), followed by the pairwise comparison of only the four-component GLV lures and unbaited controls ($N=6$). In addition, there was one comparison of the seven-component lure vs. unbaited control.

The second experiment, deployed in both saltcedar stand types, was a more systematic pairwise comparison of the GLV lures and unbaited controls, throughout the 2004 season, starting on May 6, when the overwintered beetles were becoming active, and finishing on September 14, when all of the beetles had entered reproductive diapause (DeLoach et al., 2004). The first trapping day in both types of saltcedar stands had 18 replications per

treatment followed by six replications per treatment for all other days. Trapped beetles were sexed periodically (Cossé et al., 2005). Sweep net samples were taken on June 28 in both types of saltcedar stands and included both flying beetles and those present on foliage. Captured beetles were counted and sexed.

The third experiment measured the effects of combining pheromone and GLV lures and was set up on August 11, 2004, at least 200 m away from the nearest saltcedar trees. Traps used in this experiment were two-sided yellow sticky panel traps (15.2×30.5 cm, both sides exposed, Seabright Laboratories, Emeryville, CA, USA). Treatments were pheromone lure, GLV lure, pheromone and GLV lures combined, and unbaited control. A randomized complete block design was used (four traps per block), and there were four replicates of each treatment.

Statistical Analyses

Counts of *D. elongata* adults on traps were transformed using $\log(X+1)$ to stabilize variance. The data from the pairwise experiments were analyzed by paired *t*-tests, whereas data from the randomized complete block design were submitted to analysis of variance and means were compared using the least significant difference test.

Results

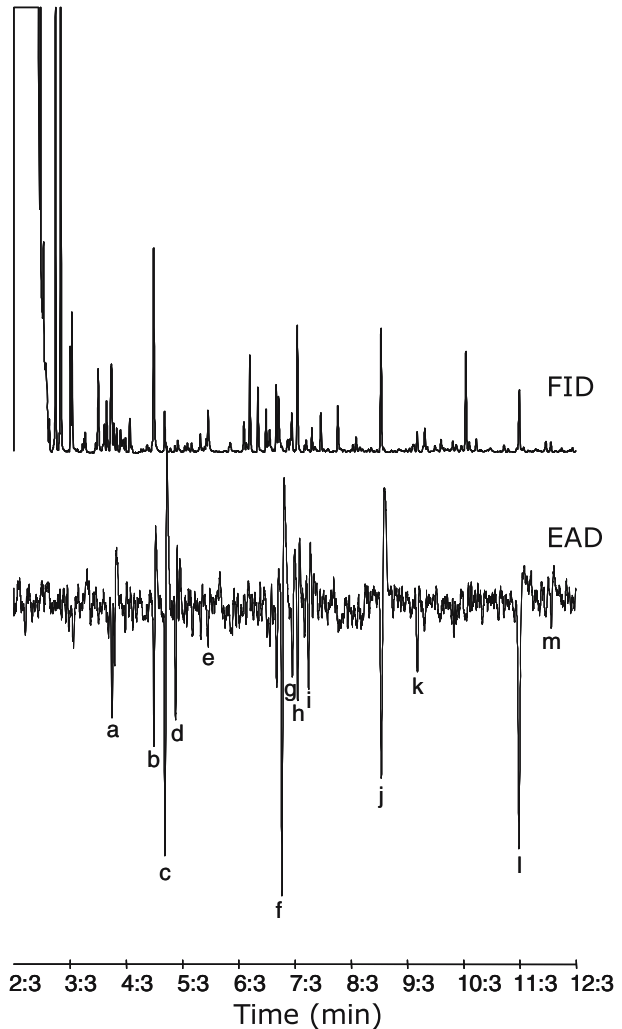
GC-EAD Analysis of Saltcedar Volatiles

GC-EAD comparisons of volatiles collected by Super-Q/charcoal from beetle-infested saltcedar foliage and foliage alone showed that male and female antennae responded to a variety of compounds (Fig. 1). Compounds that triggered male and female antennal responses were identified as 3Z-6:Ald, 2E-6:Ald, 3Z-6:OH, 2E-6:OH, 1-hexanol, heptanal, 3Z-6:OAc, octanal, (2E,4E)-2,4-heptadienal, benzyl alcohol, nonanal, (2E,6Z)-2,6-nonadienal, indole, and (E,E)-farnesene. By GC-MS, only trace amounts of the same compounds could be detected in collections of volatiles from uninfested saltcedar, and the antennal activity to these was in most cases inconclusive. More consistent GC-EAD activity from uninfested saltcedar could be obtained from collections of volatiles of crushed saltcedar foliage followed by sample concentration. Additional collections of volatiles from the flowering parts of saltcedar showed a relative abundance of benzyl alcohol, which was easily detected by the antennae of both sexes. Male and female antennae responded also to the two pheromone compounds, 2E,4Z-7:Ald and 2E,4Z-7:OH, which were present in beetle-infested foliage collections. Identities of GC-EAD-active compounds were verified by GC-MS and GC-EAD analysis of authentic standards.

Collections of Volatiles by SPME

Thirty minutes after placing the beetles on saltcedar foliage, several GLV compounds were already easily collected by SPME and detected by GC-MS (Fig. 2A), whereas these compounds were almost absent in the SPME collections from uninfested saltcedar foliage (Fig. 2B). Compounds that were consistently more abundant in the beetle-infested SPME collections were 3Z-6:Ald, 2E-6:Ald, 3Z-6:OH, 3Z-6:OAc (coeluting with compound d in Fig. 2A), nonanal, and decanal. SPME collections from uninfested saltcedar showed only

Fig. 1 Simultaneously recorded gas chromatogram with FID and EAD of female *D. elongata* antenna to volatiles collected from male and female *D. elongata* adults feeding on saltcedar. Letters denote antennal activity by: (Z)-3-hexenal (a), (E)-2-hexenal (b), (Z)-3-hexen-1-ol (c), (E)-2-hexen-1-ol (d), 1-hexanol (e), (Z)-3-hexenyl acetate (f), octanal (g), (2E,4E)-2,4-heptadienal (h), benzyl alcohol (i), nonanal (j), (2E,6Z)-2,6-nonadienal (k), indole (l), and (E,E)-farnesene (m)



trace amounts of 3Z-6:Ald, 2E-6:Ald, nonanal, and decanal. In addition, SPME collections from beetle-infested saltcedar also showed the presence of the two male-produced pheromone components, 2E,4Z-7:Ald and 2E,4Z-7:OH. The most abundant and earliest detectable GC-EAD-active compounds in collections of volatiles from beetle-infested saltcedar were selected for further analysis.

Quantification of GLVs from Saltcedar

Quantification of four GLVs, 3Z-6:Ald, 2E-6:Ald, 3Z-6:OH, and 3Z-6:OAc, collected from saltcedar, with and without the feeding beetles, revealed that beetle-infested saltcedar foliage emitted about 50 times more of the four compounds than did uninfested foliage (Table 1). Nearly equivalent amounts of each of the four compounds were released by uninfested foliage, whereas proportionally greater amounts of 2E-6:Ald and lesser amounts

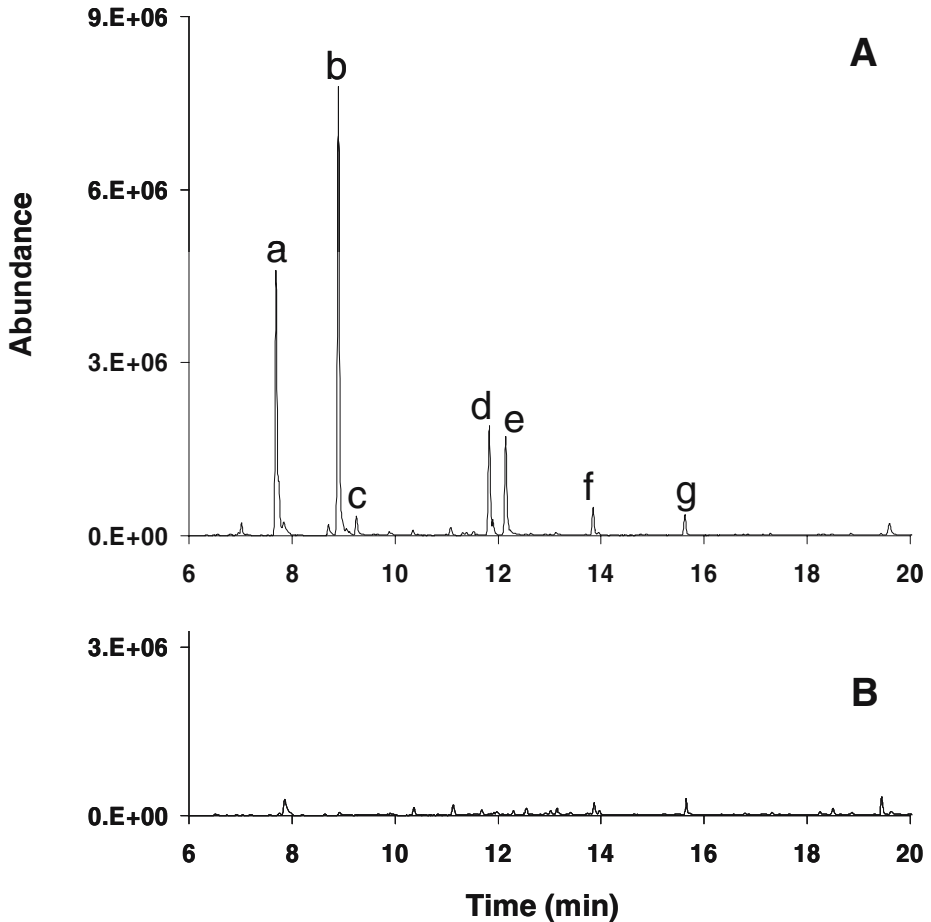


Fig. 2 GC-MS profiles of SPME-collected volatiles during the first 30 min of a 24-hr collection of volatiles from (A) saltcedar foliage infested with feeding adult *D. elongata* and (B) saltcedar foliage alone. Letters above the GC-MS peaks denote the presence of (*Z*)-3-hexenal (a), (*E*)-2-hexenal (b), (*Z*)-3-hexen-1-ol (c), (2*E*,4*Z*)-2,4-heptadienal (d), (2*E*,4*Z*)-2,4-heptadien-1-ol (e), nonanal (f), and decanal (g)

of 3*Z*-6:Ald and 3*Z*-6:OAc were released by infested foliage. Preliminary analysis of Super Q/charcoal collected saltcedar emissions (Cossé et al., 2005) showed that some of the less volatile compounds (2,6-nonadienal, indole, and *E,E*-farnesene) could only be detected towards the end of the 24-hr collection period.

Field Lures and Field Study

After 24 hr, the GLV field lures released approximately 30 µg/hr and in a ratio of nearly 32:3:1:1 (2*E*-6:Ald/3*Z*-6:OH/3*Z*-6:Ald/3*Z*-6:OAc; Table 2). Preliminary field trapping experiments captured 314±152 (Mean ± SD, *N*=12) beetles per trap per day in response to the two host odor baits combined, whereas the unbaited traps caught 32±60 (Mean ± SD, *N*=12) beetles per trap per day. The GLV lure alone caught 254±94 (Mean ± SD, *N*=6)

Table 1 Quantities of four GLVs released from saltcedar, *Tamarix ramosissima*, foliage infested with and without adult leaf beetles, *Diorhabda elongata*

Compound ^a	Foliage+200 Beetles		Foliage	
	Mean ± SD (μg/d) (N=8)	%	Mean ± SD (μg/d) (N=5)	%
3Z-6:Ald	1.26±0.57	4.7	0.13±0.02	24.3
2E-6:Ald	16.97±4.90	63.1	0.11±0.06	21.3
3Z-6:OH	6.17±3.71	23.0	0.13±0.07	24.6
3Z-6:OAc	2.48±1.02	9.2	0.15±0.08	29.8
Total	26.87±8.57		0.51±0.17	

^a For compound abbreviations, see text.

beetles per trap per day, whereas the unbaited traps caught 12±24 (Mean ± SD, N=6) beetles per trap per day. A single pair of traps comparing the trap catch of the seven-compound host odor lure (16 beetles per trap per day) with that of the unbaited control (five beetles per trap per day) elicited a much lower response. Therefore, the GLV lure was selected for further field testing.

The second set of field trapping experiments demonstrated the attractiveness of the GLV lures to male and female *D. elongata* throughout the season and in the two types of saltcedar stands (Table 3). Significantly more adults were attracted to traps baited with GLV compared to the control traps during seven different periods in defoliated saltcedar stands. Similar results were obtained in saltcedar stands with abundant foliage during four trapping periods. In both types of saltcedar stands, the response ratio between GLV and control treatments was higher early in the season and slowly declined throughout the season (Table 3). Relatively high numbers of beetles were trapped on May 6 and August 12 in the defoliated saltcedar stands.

Trap counts showed a strong bias towards males in both baited and unbaited traps (Table 3). Similar male bias was found in aerial sweep net samples, collecting 38 males and nine females in areas with foliage and seven males and three females in defoliated areas. The observed sex ratio was consistent between the two saltcedar foliage categories (2×2 contingency table, $X^2=0.58$, 1 *df*, $P=0.44$; overall, 79% males). Sweep net samples of beetles from saltcedar trees showed a considerably more balanced sex ratio, with 43 males and 26 females from defoliated saltcedars (from 30 plants) and 29 males and 24 females from saltcedars with foliage (from 15 plants). Again, the sex ratio was not significantly different between the two saltcedar stand types (2×2 contingency table, $X^2=0.72$, 1 *df*, $P=0.40$; overall, 59% males). However, the proportion of males in the aerial sample was significantly higher than in the sample from the trees (after summing over stand type in the

Table 2 Quantities of four GLVs emitted from field lures for *Diorhabda elongata*

Hour	Total μg/hr	Mean ± SD (μg/hr) (N=3)							
		3Z-6:Ald		2E-6:Ald		3Z-6:OH		3Z-6:OAc	
		Mean	%	Mean	%	Mean	%	Mean	%
1	32.1	0.8±0.2	2.5	27.1±3.9	84.4	3.6±0.5	11.2	0.6±0.1	1.9
5	38.1	1.6±0.5	4.2	31.6±3.6	82.9	4.1±0.4	10.8	0.8±0.01	2.1
24	29.4	1.0±0.7	3.4	25.1±4.5	85.4	2.6±0.4	8.8	0.8±0.2	2.7
48	22.3	0.6±0.2	2.7	20.9±1.8	93.7	2.1±0.3	9.4	0.6±0.1	2.7

For compound abbreviations and applied dosages, see text.

Table 3 Response of *Diorhabda elongata* to sticky traps baited with four GLVs in defoliated and foliated saltcedar, *Tamarix ramosissima*, stands in Lovelock, NV, May–September 2004

Date	Response			GLV/Ctrl ^a	% Male Catch	
	Mean ± SD (Beetles per Trap per Day)				GLV	Ctrl
	GLV	Ctrl	<i>t</i>			
Defoliated Saltcedar						
6-May	293.9±135.4	22.1±50.8	12.14**	42.4	–	–
18-June	8.7±6.8	0.8±1.2	3.46*	11.2	–	–
28-June	13.7±13.9	1.0±1.3	4.87*	12.9	96.5	100
21-July	46.6±20.4	5.2±5.0	11.01**	12.1	95.8	73.7
23-July	20.8±12.8	2.7±2.8	7.59**	8.5	89.0	90.5
12-Aug.	156.6±45.3	30.4±15.9	8.76**	6.0	–	–
14-Sept.	60.8±75.5	1.0±0.9	7.99*	43.9	77.3	100
Saltcedar with Foliage						
18-June	34.7±43.2	1.0±2.4	3.54*	7.2	–	–
28-June	11.5±8.8	1.2±1.2	7.24**	10.1	96.6	100
6-July	19.4±20.0	4.1±5.2	3.81*	4.2	–	–
25-July	51.3±59.9	20.2±34.2	4.18*	4.0	92.1	65.4

For bait composition and emission rates, see Table 2.

^a From the statistical analysis, ratios are based on log-transformed means.

* $P < 0.01$.

** $P < 0.001$.

above data sets, a 2×2 contingency table for sex vs. aerial/tree sampling gave $X^2 = 6.82$, 1 *df*, $P < 0.01$).

In the third field experiment, adult *D. elongata* were attracted to traps baited with pheromone, GLV, or both, in numbers that were significantly higher than those found on the unbaited control traps (Table 4). There was no significant difference between the numbers of *D. elongata* attracted to traps baited with GLV lures compared to those attracted to traps baited with pheromone lures. The combination of GLV and pheromone lures attracted the highest number of beetles, with approximately six times more *D. elongata* than to GLV lures alone, and approximately four times more *D. elongata* than to pheromone lures alone (Table 4).

Table 4 Response of *Diorhabda elongata* to sticky traps baited with four GLVs (for bait composition and emission rates, see Table 2), a 1:1 mixture of 2E,4Z-7:ALD and 2E,4Z-7:OH (pheromone) (for bait composition and emission rates, see text), and a combination of GLV and pheromone in saltcedar, *Tamarix ramosissima*, stands in Lovelock, NV, August 12, 2004

Lure	Response
	Mean ± SD (beetles per trap per day) ^a
Pheromone+GLV	90.4a
Pheromone	23.7b
GLV	15.9b
Control	3.1c
<i>F</i> statistic	24.28 (<i>df</i> =3,12)
<i>P</i>	<0.001

^a Means followed by the same letter do not differ by least significant difference test, $P > 0.01$.

Discussion

In this study, we focused on possible biologically active saltcedar volatiles released during feeding by *D. elongata*. The experimental results demonstrated that at least 15 volatile compounds were antennally active to male and female *D. elongata*. Collections of volatiles from uninfested saltcedar showed relatively small amounts of GC-EAD-active material, but collections from beetle-infested foliage showed that the feeding activity of the beetles significantly increases the amounts of GC-EAD-active saltcedar volatiles released into the air. GLVs (Visser and Avé, 1978; Visser, 1986) especially were released in relative abundance and could be easily detected during the first 30 min after feeding began, whereas relatively lesser amounts could only be detected in the samples collected over a 24-hr period. Numerous studies have demonstrated the quantitative and qualitative differences between volatiles emitted by herbivore-infested plants compared to the volatiles emitted by uninfested plants (for reviews, see Dicke et al., 1998; Dicke and Vet, 1999; Dicke and van Loon, 2000), and several of these studies have shown the role of the identified GC-EAD-active compounds in tritrophic interactions (for reviews, see above). In this study, we focused only on GC-EAD-active material. Qualitative differences in these compounds were not noted between beetle-infested and noninfested plants, but the quantitative differences were dramatic.

The behavioral significance of the identified GLVs was demonstrated in the field. A blend of four of the most abundant, antennally active GLVs, released at compound ratios mimicking the beetle-infested plant release ratios, was highly attractive to male and female *D. elongata* in areas where most of the saltcedar stands were completely defoliated. Furthermore, this synthetic blend was still highly attractive to male (and, to a lesser extent, female) *D. elongata* in areas with an abundance of beetles and saltcedar foliage.

Further evidence for the importance of the GLV blend was demonstrated with the observed synergistic effect when pheromone and GLVs were combined (Table 4). It should be noted that this synergistic effect was demonstrated in previously uninfested saltcedar stands. As such, the combination of the pheromone and the green leaf odor blend could be a useful attractant in detecting the presence of *D. elongata* in newly colonized areas. This would be a useful tool in the saltcedar biocontrol program.

In beetle taxa with aggregation pheromones, pheromone emission occurs at the feeding/breeding site, and host-derived volatiles are potent synergists of the pheromones for some taxa (Borden, 1985; Bartelt, 1999). Only recently has the synergistic interaction of host compounds and pheromones been shown for chrysomelid beetles (Soroka et al., 2005; Tóth et al., 2005).

Field observation of *D. elongata* showed that the population density is not uniformly distributed among the saltcedar bushes. Especially in newly colonized stands of saltcedar, the presence of adult beetles is highly aggregated with, for example, a single plant containing hundreds of feeding beetles being surrounded by many other similar-looking plants without any beetles. In those instances where a small number of beetles landed and started feeding on a previously uninfested saltcedar bush, it was quickly (<30 min) followed by the arrival of numerous other beetles on the same saltcedar branch (personal observations by authors Cossé, Bartelt, and Bean). If the original group of pioneer beetles contained males, one could envision that the arrival of the additional beetles was caused by the release of the male-specific aggregation pheromone, the release of attractive host volatiles as a result of the feeding action, or both stimuli.

Our earlier study dealing with the identification of the male-specific aggregation pheromone of *D. elongata* showed that males feeding on saltcedar foliage released a 1:1 mixture of 2E,4Z-7:Ald and 2E,4Z-7:OH, and that this mixture attracted significant numbers of males and females to traps placed in saltcedar stands in various stages of

defoliation (Cossé et al., 2005). In this study, the pheromone could be detected within the first 30 min after placing the beetles on the saltcedar foliage in the collector.

Certain chrysomelid beetles, such as *Diabrotica* spp. (for review, see Metcalf and Metcalf, 1992) and *Leptinotarsa decemlineata* (Dickens, 1999, 2000, 2006), are attracted to blends of some of the same GC-EAD-active compounds found in this study. For example, benzyl alcohol and indole, two volatile components associated with the blossoms of *Cucurbita* spp., were both antennally active and attractive to adult *Diabrotica undecimpunctata howardi* and *Diabrotica virgifera virgifera* in field studies (Andersen and Metcalf, 1986; Lampman et al., 1987). Antennae of *L. decemlineata* responded to several volatile potato foliage compounds, including 3Z-6:OH, 2E-6:OH, 3Z-6:OAc, nonanal, decanal, and indole (Dickens, 1999). However, Dickens (2000) showed that some blends containing the GLVs 3Z-6:OH and 2E-6:OH can also be unattractive or repellent for *L. decemlineata*. Also, cereal leaf beetles, *Oulema melanopus*, did not respond to a GC-EAD-active oat volatile, 3Z-6:OAc, in field tests (Rao et al., 2003). Behavioral activity to GLVs has also been demonstrated with other Coleoptera; for example, scarab beetles, *Melolontha melolontha* (Reinecke et al., 2005) and *Phyllopertha horticola* (Ruther and Mayer, 2005), are strongly attracted to single or blends of specific GLV compounds. In addition, GLVs significantly enhanced the responses of the smaller European elm bark beetle, *Scolytus multistriatus*, and boll weevil, *Anthonomus grandis*, to their respective pheromones (Dickens et al., 1990).

Future field tests with *D. elongata* will be necessary, using single components and different blends and blend ratios of saltcedar volatiles, to fully investigate the behavioral significance of all of the EAD-active compounds.

The field bioassays showed a male-biased sex ratio that was surprisingly strong on some of the trapping dates. It is possible that males were more attracted to the host plant odors, but with similar male sex ratios in unbaited control traps and aerial sweeps, a more likely explanation is that males are simply the more active fliers, especially because the beetle sex ratio on the foliage was far less male-biased. In previous field tests of the aggregation pheromone blend, only a slight male bias was noted in the captured beetles (Cossé et al., 2005). Again, it is possible that males are more strongly attracted to host plant odors than females and that this differential attraction is not as pronounced with aggregation pheromones. A more likely explanation is that the behavioral, physiological, and ecological context of these experiments is critical in the outcome, both in terms of attraction to the baits and the sex ratio of captured beetles. For instance, placement of traps outside of the areas where beetles are mating and ovipositing might bias the sex ratio toward males, if they are the primary colonizers. The physiological status of the beetles, whether it be reproductive, diapause, hungry, or fed, will also undoubtedly have a strong influence on response to behaviorally active compounds.

Future field studies might further investigate the hypothesis that male *D. elongata* are pioneers in the colonization of saltcedar and that successful establishment of new populations is being guided by both the release of the male-produced aggregation pheromone and some of the volatile compounds released by the host plants.

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Tannins can form stable complexes with proteins and can also act as metal chelators (e.g., Laks, 1989; Slabbert, 1992; Hättenschwiler and Vitousek, 2000). As a consequence, they play multiple roles in biogeochemical processes such as organic matter decomposition, nutrient dynamics, soil formation, and (heavy) metal transport (Kraus et al., 2003a). Through tannin–proteins complexes, N mineralization is affected, and organic matter decomposition and nutrient dynamics decrease or increase (Handley, 1961; Schimel et al., 1998; Fierer et al., 2001; Kraus et al., 2003a, 2004; Nierop et al., 2006). In the mineral soil compartment, tannins release metals from soil particles and can promote podzolization (Bloomfield, 1953, 1957; Coulson et al., 1960; Kaal et al., 2005), a soil-forming process that involves the release, translocation, and precipitation of Al, Fe, and organic matter.

Despite the many roles tannins play in soil processes and litter decomposition, they are difficult to detect in the same litter and soils (Tiarks et al., 1989; Schofield et al., 2001; Lorenz and Preston, 2002; Lorenz et al., 2004). Often, tannin concentrations are measured with colorimetric methods including the Folin–Ciocalteu and the Prussian Blue assays for total phenolics, the HCl–butanol and the vanillin assays for CT, and the potassium iodide assay for hydrolyzable tannins (Waterman and Mole, 1994; Yu and Dahlgren, 2000; Schofield et al., 2001; Hartzfeld et al., 2002). These methods require extraction prior to quantification, and indicate decreasing amounts of tannins upon litter decay (e.g., Schofield et al., 1998; Bradley et al., 2000; Lorenz et al., 2000; Maie et al., 2003; Nierop et al., 2006), suggesting that tannins have been decomposed or leached away. ^{13}C nuclear magnetic resonance (NMR) spectroscopy, however, suggests that a portion of tannins are inextricable and, therefore, escape the analytical windows of the assays developed for quantification (Benner et al., 1990; Lorenz et al., 2000; Yu and Dahlgren, 2000; Maie et al., 2003). Although NMR is a suitable method for detecting tannins in solid plant and soil material, tannin resonances interfere with those of lignin and polysaccharides, which may hamper obtaining detailed information on tannin amounts, structures, and possible transformations (Wilson and Hatcher, 1988; Preston et al., 1997; Parfitt and Newman, 2000; Lorenz and Preston, 2002; Maie et al., 2003). Along with an acid-catalyzed depolymerization method (Hernes and Hedges, 2000) and MALDITOF-MS (Behrens et al., 2003), thermally assisted hydrolysis and methylation (THM) offers an alternative to study both HTs and CTs in plants, litter, and soils (Garnier et al., 2003; Nierop et al., 2005). Although most products obtained by THM from tannins have also been found from lignin, the 1,3,5-trimethoxybenzenes derived from the A ring of CTs have never been identified from lignin, and are thus good markers of CTs in environmental samples (Nierop et al., 2005).

In this paper, we used THM to assess the fate of HTs and CTs after addition to Corsican pine litter and subsequent incubation. Knowledge of the amounts of tannins added, comparison between Folin–Ciocalteu and HCl–butanol assays that quantify extractable tannins and those present in litter, will provide clues to the fate of tannins in coniferous litter.

Methods and Materials

Litter collection, pretreatment, and general composition. Litter, i.e., material from the F1 horizon, was collected from a pine forest named Luizenberg, which is situated in the Amsterdamse Waterleiding Duinen, a coastal dune area with a partly decalcified sandy soil at the south of Zandvoort, west of Amsterdam (the Netherlands). The trees, Corsican pine (*Pinus nigra* var. *maritima*), were planted in 1935. Collected litter was air-dried at 30°C for 24 hr and subsequently sieved over 2 cm to remove coarse fragments and to obtain a rather homogenous substrate, which was stored at 2°C before experiments and analysis.

Condensed tannin collection. Needles from Corsican pine were collected by cutting branches from the trees in May 2003. Needles were dried under vacuum on the same day and subsequently ground to pass a 2-mm sieve and stored at 2°C before further treatment. Tannins were obtained by following the method described Preston (1999). Ground needles were extracted with hexane with a Soxhlet device to remove lipids. Residual needles were subsequently extracted by a mixture of acetone/water (7:3, v/v) for 24 hr. After centrifugation, the dark green solution was stored at 2°C, and the extraction/centrifugation sequence was repeated twice with the solid residue. All three solutions were combined, and the acetone was removed under vacuum. The resulting aqueous solution was subsequently extracted by dichloromethane 3–5 times to remove remaining lipids until the organic phase was colorless, followed by extraction with ethyl acetate to obtain an orange to brown solution. After freeze-drying, the crude tannins obtained were loaded on a Sephadex LH-20 column and eluted by methanol/water (1:1, v/v) to remove low molecular weight phenolics and tannin monomers. After switching the eluate to acetone/water (7:3, v/v), tannins were obtained from the Sephadex LH-20 column. The collected and combined water/acetone solution was rotary evaporated to give an aqueous solution that was freeze-dried to yield purified CTs. Tannins were characterized by solution ^{13}C NMR, having a procyanidin/prodelphinidin ratio of content of 37:63, an average chain length of 6.6%, and 85% *cis* configuration (Nierop et al., 2005).

Hydrolyzable tannins. Tannic acid (TA), a commercially available tannin, is often used as hydrolyzable tannin. Although TA is a rather reactive compared with other tannins and as such not a very representative HT (Kraus et al., 2003b), it is used by many researchers and serves as a useful tannin for comparison. TA of Merck was used, which has an average molecular weight of 1700 (=10 gallic acid units linked to the sugar core), and based on ^{13}C NMR, was pure (Kaal et al., 2005).

Incubation experiment. Five g (on absolute dry basis) of air-dried litter were weighed into Petri dishes, after which 5 ml water were added to give a moisture content of 100% (w/w). All filled Petri dishes were stored in an isothermal room (20°C) for 4 d to acclimate. Next, a series of control, TA-containing and CT-containing samples were prepared as follows.

To control samples, another 5 ml of water were added to obtain litter material with a moisture content of 200% (w/w). To the TA series, 5 ml of a solution of 20 g/l TA were added, which yielded litter material with the same moisture content as the control samples, and in addition 100 mg of TA per 5 g litter (on a dry weight basis). For the CT series, the same procedure as for the TA samples was followed, but using 5 ml of a solution of 20 g/l CT. Per time step ($t=1, 2, 4, 7, 14, 21, 28, 35, 42, 56, 70,$ and 84 d) during the incubation series, four replicates were used for each experiment (control, TA, and CT). Consequently, apart from the initial situation ($t=0$ d, only control samples), for each time step, 12 dishes were prepared as described, which accounted for 148 dishes in total. All preparations were incubated at a constant room temperature of 20°C.

Extraction and tannin assays. Total phenolics and CTs were determined with the Folin–Ciocalteu assay and the HCl–butanol assay, respectively (Waterman and Mole, 1994; Yu and Dahlgren, 2000), using 0.7 g of litter (DW basis), that was extracted by 40 ml of acetone/water (7:3, v/v) for 24 hr in the dark at 20°C. Total phenolics in the extracts obtained were determined with TA as the standard for all samples. Although it is preferable to use CT as a standard in case of the CT-amended series (Kraus et al., 2003b), control samples appeared to have certain levels of total phenolics as determined by Folin–

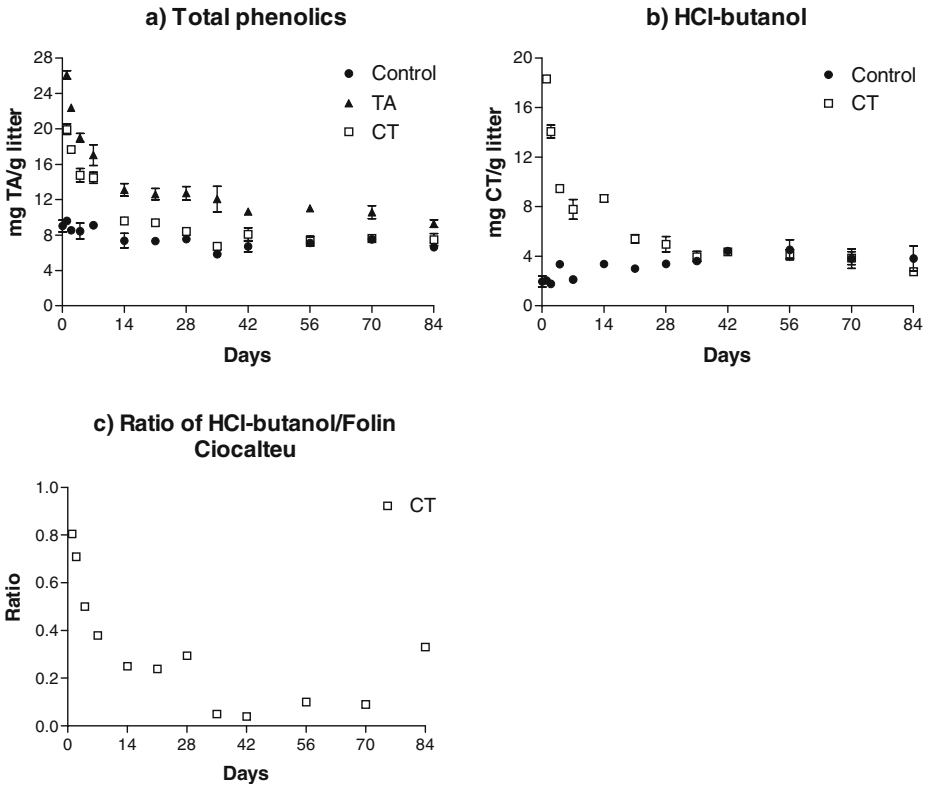


Fig. 1 (a) Total phenolic concentrations (Folin–Ciocalteu assay) in acetone/water (70:30, v/v) extracts from litter during the incubation experiment, (b) CT concentrations (HCl–butanol assay) in the same acetone/water extracts, and (c) the ratio of CTs measured by HCl–butanol assay divided by total phenolics measured by Folin–Ciocalteu assay in acetone/water extracts for the CT series (corrected for levels in control series). Errors bars indicate SEM ($n=4$)

Ciocalteu, whereas no response was found using HCl–butanol. Therefore, to allow comparison with control samples, TA was also used as the standard for the Folin–Ciocalteu assay for the CT-amended series. For the HCl–butanol assay, CT derived from Corsican pine was used as the standard.

Thermally assisted hydrolysis and methylation-gas chromatography/mass spectrometry. Litter samples were freeze-dried and milled. Prior to THM, they were pressed onto Curie-point wires, after which a droplet of a 25% solution of tetramethylammonium hydroxide (TMAH) in water was added. Samples were subsequently dried by a 100-W halogen lamp. THM was performed by heating the sample for 5 sec at 600°C. The Horizon Instruments Curie-Point pyrolyzer was connected to a ThermoQuest Trace GC 2000 gas chromatograph, and the products were separated by a fused silica column (J&W, 30 m, 0.32 mm i.d.) coated with DB-1 (film thickness 0.50 μm). Helium was used as carrier gas. The oven was initially kept at 40°C for 1 min; then it was heated at a rate of 7°C/min to 320°C, and maintained at that temperature for 10 min. The column was coupled to a Finnigan Trace MS quadrupole mass spectrometer (Electron Ionization; ionization energy 70 eV, mass range m/z 45–600,

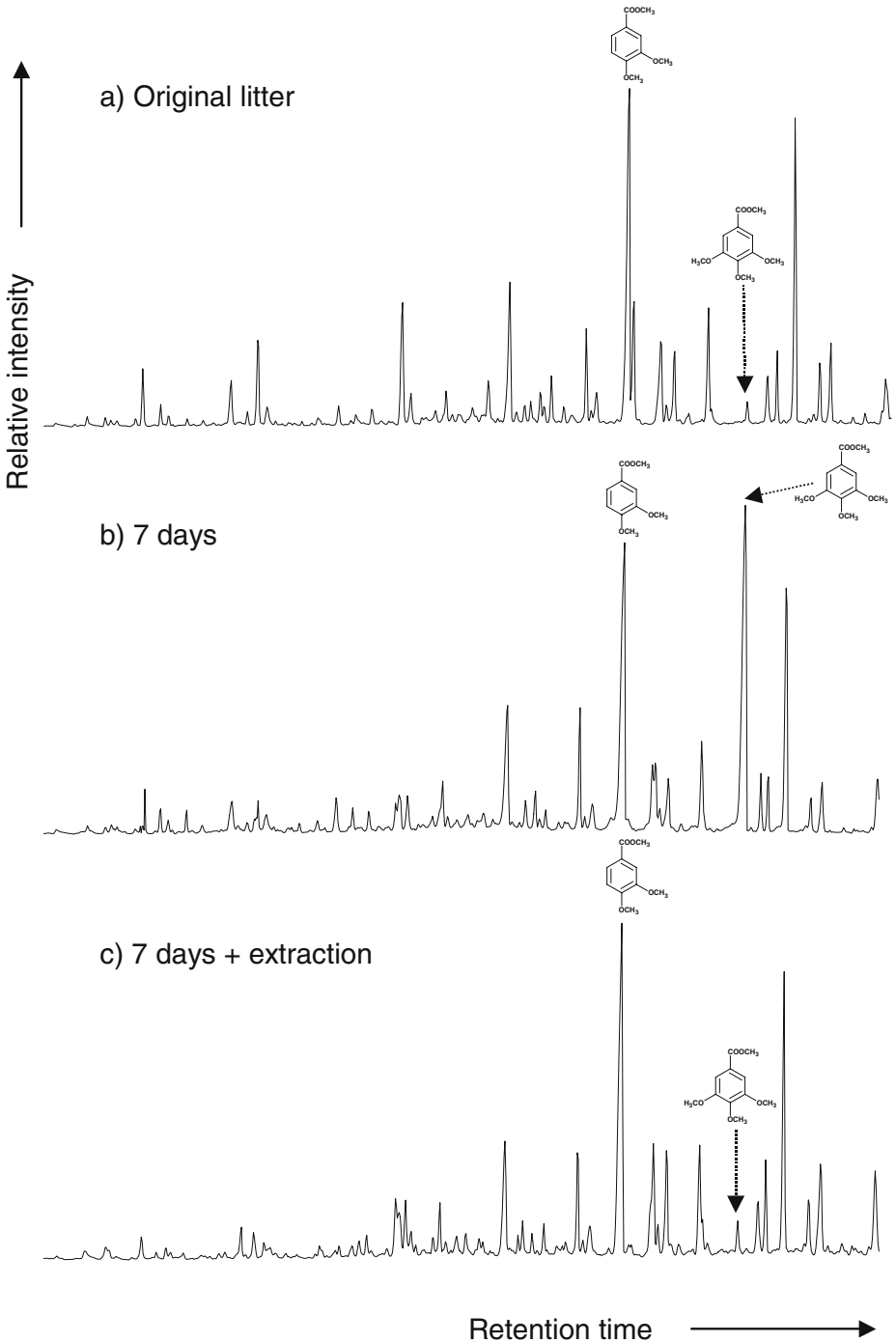


Fig. 2 Gas chromatograms of products from thermally assisted hydrolysis and methylation (THM) for (a) original litter, (b) TA-amended litter after 7 d incubation, (c) TA-amended litter after 7 d incubation and followed by acetone/water extraction. Methylated gallic acid and methylated vanillic acid are marked to emphasize the relative differences

cycle time 1 sec). Identification of the compounds was carried out by interpretation of their EI spectra with a NIST library, by their GC retention times, and by comparison with literature data.

Results

Total phenolics and condensed tannins. Total phenolics expressed as TA equivalents for the control litter decreased from 9.0 mg TA/g litter initially to 6.6 mg TA/g litter after 84 d incubation (Fig. 1a). For TA, the first day after addition, TA contents represented 26.1 mg TA/g litter and declined rapidly to 9.3 mg TA/g litter finally, while CT after 1 d started with 20.0 mg TA equivalents/g litter and ended at 7.5 mg TA equivalents/g litter. Using the HCl–butanol assay to measure CTs specifically, the control litter contained small amounts of CTs (2–4 mg CT/g litter), and the CT-amended litter exhibited a decline similar to that obtained by Folin–Ciocalteu (Fig. 1b). Only a few TA-amended samples were analyzed with the HCl–butanol assay, and yielded the same amounts of CTs as the control samples. For clarity, they were not included in the graph. The amount of CTs, as measured by the HCl–butanol assay, divided by the amount of total phenolics, as measured for the CT series (and corrected for the total phenolics present in control litter), decreased with time (Fig. 1c)

Thermally assisted hydrolysis and methylation To elucidate whether decreasing amounts of TA and CT, as determined by the Folin–Ciocalteu and HCl–butanol assays, were due to decomposition or whether they remained in the litter as inextricable compounds, THM was applied to litter samples to assess the appearance of TA and CT added through the subsequent incubation period followed by acetone/water extractions.

TA produces only methylated gallic acid (3,4,5-trimethoxybenzoic acid, methyl ester, or methyl trimethoxy benzoate) upon THM (Nierop et al., 2005). The initial litter revealed a small peak of 3,4,5-trimethoxybenzoic acid, methyl ester. Figure 2 shows the THM chromatogram of the TA-amended samples. After acetone/water extraction, the methylated gallic acid peak reduced to a height similar to control litter. For comparison, the peak representing (methylated) vanillic acid was marked as well. To show the resemblance between TA as measured by Folin–Ciocalteu and the relative amounts of TA detected by THM, Fig. 3 displays the decline as observed by both methods.

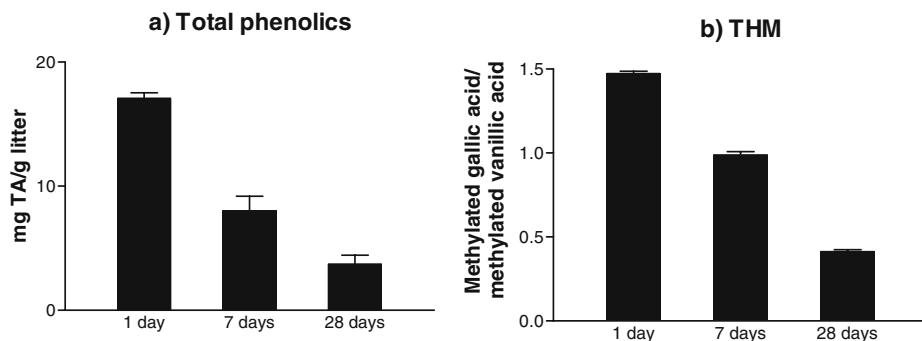
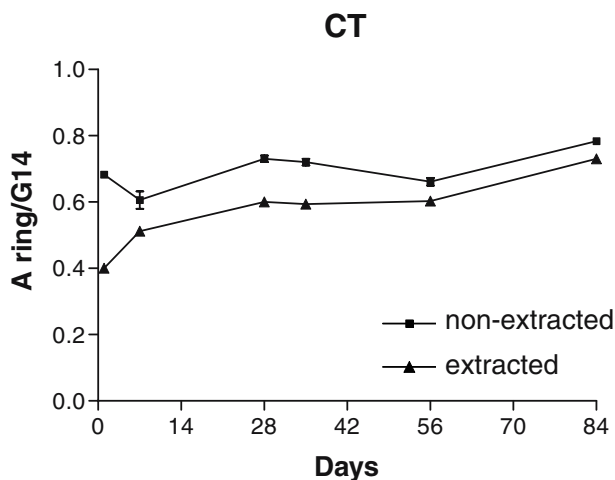


Fig. 3 (a) Total phenolics for TA-amended litter after 1, 7, and 28 d incubation, and (b) the methylated gallic acid/methylated vanillic acid ratio to indicate the relative abundance of TA in the same samples. Both total phenolics ($n=4$) and methylated gallic acid/methylated vanillic acid ratios ($n=3$) are corrected for untreated litter; errors bars indicate SEM

Fig. 4 Ratio of relative abundance of A ring products of CTs (1,3,5-trimethoxybenzene + 2-methyl-1,3,5-trimethoxybenzene) to G14 [*threo* 1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxy propane] derived from lignin as a measure of CT concentrations for both extracted and nonextracted CT-amended litter. Errors bars indicate SEM ($n=3$)



With THM, CTs decompose into many products, of which the 1,3,5-trimethoxybenzenes are the most abundant. They are derived from the A rings, and are the only ones typical of CTs (Nierop et al., 2005). Applying THM to the initial litter yielded very small peaks of 1,3,5-trimethoxybenzene and 2-methyl-1,3,5-trimethoxybenzene. The most abundant peaks in the chromatograms were from methylated vanillic acid, most of which is derived from lignin rather than B ring products of CTs. To enable (semi-)quantification, the peak areas of 1,3,5-trimethoxybenzene and 2-methyl-1,3,5-trimethoxybenzene were integrated and related to that of G14 (*threo* 1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxy propane), which is an aromatic product typical of lignin (Clifford et al., 1995) but not of tannins (Nierop et al., 2005). Figure 4 shows that without extraction the amounts of CTs remained at a constant level. As the peaks were very small and did not really increase upon CT addition, we also incubated samples with 5 times as much CTs (100 mg/g), and this was incubated for 28 d. Peaks of A ring products clearly increased, and after 28 d, peaks with and without extraction remained relatively high (Fig. 5).

Discussion

For TA, the behavior can be explained rather easily. TAs are degraded and used as a carbon source (Kraus et al., 2004; Nierop et al., 2006). The decline in TA concentrations on Folin–Ciocalteu shows this, and since TAs were not present in the litter after acetone/water extractions as determined by THM, the Folin–Ciocalteu assay reflects the actual concentration of TA in litter samples. Based on these data, TA does not seem to exist in strong complexes with proteins or other organic matter components. In addition, THM is a sensitive method to detect and quantify the TA (HT) in environmental samples. However, this approach works only well when CT concentrations are low, as THM produces methyl esters of 3,4,5-trimethoxybenzenes from prodelphinidin B rings (Nierop et al., 2005). Moreover, HTs occur only in dicots that also contain syringyl-lignin, which in turn yields a.o. methylated syringic acid upon THM (Clifford et al., 1995). Because methylation of syringic acid gives the same product as when gallic acid is methylated, there are limitations to the use of THM in identifying HTs unambiguously (Filley et al., 2006).

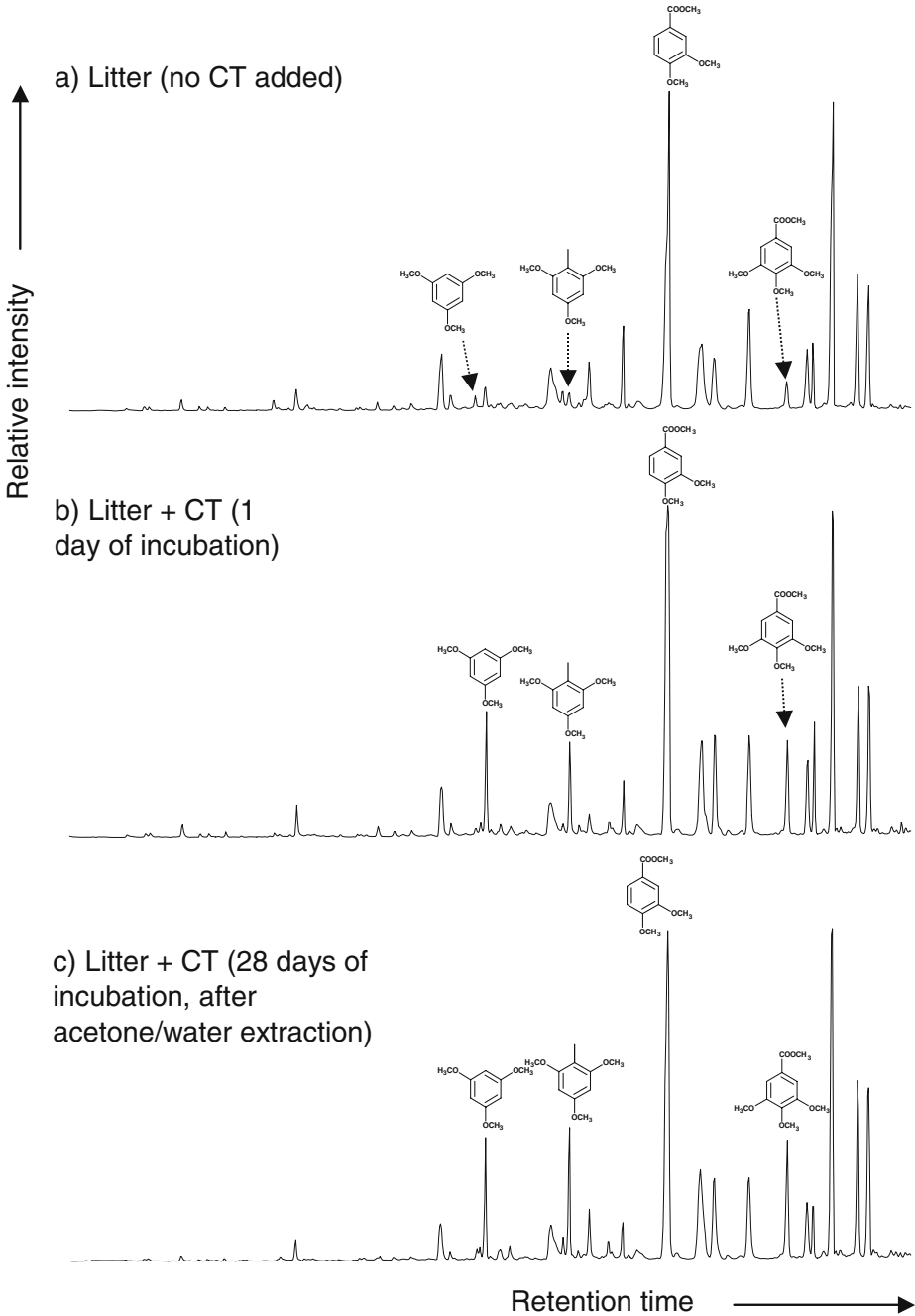


Fig. 5 Gas chromatograms of products from thermally assisted hydrolysis and methylation (THM) for (a) original litter, (b) CT-amended litter after 1 d incubation, and (c) CT-amended litter after 28 d incubation followed by acetone/water extraction

By contrast, CTs are more difficult to extract from litter samples. With increasing incubation time, extractable amounts of CTs decrease, and the ratio of extractable CTs (using HCl–butanol)-to-extractable phenolics (using Folin–Ciocalteu) declines as well (Fig. 1c). The latter suggests that the fraction of total extractable phenolics recognized as CTs becomes smaller in the course of the incubation. As HCl–butanol oxidatively cleaves the interflavanoid bonds (Porter et al., 1986; Waterman and Mole, 1994), longer CTs produce more colored products than shorter ones, because they contain more such linkages (Kraus et al., 2003b). The possible decrease in the average chain length of extractable CTs may be attributable to either increasing inextricability of longer chain CTs, or degradation in the form of depolymerization of CTs resulting into smaller and “simpler” phenolics. A similar behavior was found for sorption of CTs to quartz particles coated with iron oxides (Kaal et al., 2005). THM of CTs produces many compounds that are also identified upon THM of lignin (e.g., Clifford et al., 1995). B rings bearing two hydroxyl groups yield the same THM products as found for guaiacyl lignin; similarly, B rings containing three hydroxyl groups give products encountered for syringyl lignin. However, A ring products, i.e., 1,3,5-trimethoxybenzenes, are not identified after THM of lignin, and these products serve as excellent markers for CTs in any kind of litter.

The fate of CTs in soils has long been an unsolved problem (Lorenz and Preston, 2002). The low degree of extractability or yield may be attributable to CT decomposition, leaching through the soil profile, sorption to mineral phases, or the formation of strong tannin–protein/organic matter complexes (Hättenschwiler and Vitousek, 2000). In addition, chemical transformations such as oxidation and (de)polymerization may impose difficulties in identifying and quantifying tannins (Tiarks et al., 1989). There appears to be no single mechanism that applies to any soil system. From the incubation experiment using Corsican pine litter, the extent of CT decomposition is small (Nierop et al., 2006), and leaching and interactions with mineral soil particles are absent, so that the main reason for the inextricability (Fig. 4) must be the formation of (strong) tannin–organic N complexes (Benner et al., 1990; Hernes et al., 2001; Maie et al., 2003). This is supported by prolonged effects of CTs seen for net N mineralization in particular (Nierop et al., 2006), and the chemical modifications observed for CTs in decaying mangrove leaves (Hernes et al., 2001).

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Introduction

Generalist phytophagous insects feed on a wide variety of plant species. However, their host choice is of ecological importance, and the host species on which females oviposit affects the development and fitness of offspring (Jaenike, 1978; Singer, 1983; Courtney et al., 1989). In many herbivorous species, young instars are more vulnerable to natural enemies and are less efficient at escaping natural enemies than imagoes (Nomikou et al., 2003). It would be advantageous for adult herbivores to discriminate among risky and safe host plant species, and thus to select high-quality host plants whatever the risk is when the threat of natural enemies is low (Freitas and Oliveira, 1996). Moreover, natural enemies may exert selection pressure on the host plants used by phytophagous insects (Ohsaki and Sato, 1999; Stireman and Singer, 2003; Singer and Stireman, 2003). For example, a plant that contains defensive allelochemicals or precursors can be chosen by a phytophagous insect for the protection it provides against natural enemies (Stamp, 2001; Shiojiri et al., 2002). Plants can also change phytophagous aspects so that the attractiveness of their by-products (e.g., frass) to the parasitoids is modified (Mondor and Roland, 1997).

Grape berry moths, *Lobesia botrana* (Lepidoptera: Tortricidae) and *Eupoecilia ambiguella* (Lepidoptera: Tortricidae), are among the most serious insect pests in European viticulture. Both are polyphagous and may occur sympatrically in vineyards, but their relative frequency depends on climatic factors (Thiéry, 2005). The larval host plant, including the grape cultivar, modifies several patterns involved in the reproductive output of *L. botrana*, for example, the development time of the larvae (Savopoulou-Soultani et al., 1994; Thiéry and Moreau, 2005; Moreau et al., 2006a) and larval metabolism. As a result, infochemicals produced by larvae may be modified as in other species (Lill and Marquis, 2001; Thorpe and Day, 2002). The host plant ingested by *L. botrana* larvae during its development may also modify parasitism of eggs by the generalist egg parasitoid *Trichogramma cacoeciae* (Thiéry, Pizzol, and Wajnberg, unpublished data).

Female *L. botrana* exhibit preferences among the different grape cultivars (Maher et al., 2001), and larvae feed on the plant on which the eggs are laid, most of the larvae developing close to the oviposition site. Therefore, the chemical cues related to frass are always located close to the larvae themselves, and thus provide pertinent local information for a larval parasitoid. Frass is often a relevant cue for natural enemies during the host location process (Auger et al., 1990; Reddy et al., 2002; Steidle and Van Loon, 2003), and it can be expected to be relevant also to our model/system.

Several studies have shown that parasitoids and predators can be efficient biological control agents against the different grape berry moths. As much as 50–60% of the moth larvae can be naturally controlled (Luciano et al., 1988; Coscolla, 1997; Thiéry et al., 2001; Bagnoli and Lucchi, 2005; Thiéry et al., 2006). The main parasitoids reported in most European vineyards belong either to Hymenoptera (Trichogrammatidae, Ichneumonidae, Braconidae or Pteromalidae) or to Diptera (Tachinidae; Thiéry et al., 2001, 2006). The pteromalid wasp *Dibrachys cavus* is a generalist, gregarious ectoparasitoid of more than 200 species belonging to different families (Peck, 1963, Universal Chalcidoidea Database, 2003). It has been found on overwintering pupae of *L. botrana* in France (Causse et al., 1984), Italy (Marchesini and Della Monta, 1994), and Spain (Coscolla, 1997). This parasitoid is a multivoltine species that can attack each generation of *L. botrana* (Faure and Zolstarewsky, 1925). *D. cavus* parasitizes fifth instars of *L. botrana* just before pupation and not pupae (Faure and Zolstarewsky, 1925; Chuche, Xuéreb and Thiéry, unpublished observations).

The goal of our study is to determine which components of the host odor attract *D. cavus* from a distance. Thus, we tested the specificity of such an attraction and whether it varied

according to the larval food plant. First, we tested the response of *D. cavus* females to host larvae without frass, to silk, and to frass alone, and whether it discriminated among different host species on the basis of their odor. Second, we tested the influence of host larval food and frass for attractiveness.

Methods and Materials

Insects

Our *D. cavus* strain originates from grapes harvested in an experimental vineyard (INRA Bordeaux, La Ferrade). Insects were reared in the laboratory for successive generations on *L. botrana* larvae under a 16:8 (light–dark) photoperiod, at $23 \pm 1^\circ\text{C}$, and 45–50% relative humidity. Under these conditions, adults *D. cavus* emerged ca. 20 days after oviposition on the larval cuticle. Emerging adults were collected daily, except during the weekends, transferred into new boxes containing fifth instars *L. botrana*, and supplied with a 20% honey–water solution. We used a strain of *L. botrana* reared without diapause under the conditions described above on a semisynthetic diet (control medium) as described in Thiéry and Moreau (2005), and a strain of *E. ambiguella* reared in our laboratory (same conditions as for *L. botrana* except 60–70% relative humidity). All experiments were performed in a climate chamber under controlled conditions as described above. *D. cavus* were all 48-hr-old, mated, fed females with no previous oviposition experience. They were tested only once. The frass of four phytophagous insect species was tested: three Lepidoptera—*L. botrana*, *E. ambiguella*, and *Sphinx ligustri* (Sphingidae)—and an Orthoptera selected as a control: *Chorthippus brunneus* (Acrididae). The Sphingidae and the Orthoptera used have never been described as *D. cavus* hosts.

Plants, Insect Diets, Frass Collection, and General Procedure

Grape cultivars supplied as larval food were compared using a standardized procedure adapted from that described by Thiéry and Moreau (2005) and Moreau et al. (2006a). *L. botrana* larvae were reared in enclosed plastic cups (diam: 3 cm on base and 4 cm on top; height: 3 cm) with five larvae per cup, filled with 15 ml of artificial diet. This medium was identical to the two references quoted above. Plant material was freeze dried, and a fine powder was made using a blender as described in Moreau et al. (2006a). A limited range of host plants was tested, two grape cultivars: *Vitis vinifera* (Vitaceae) cv. Pinot noir and Riesling, the presumed ancestral host of *L. botrana*: *Daphne gnidium* (Thymelaeaceae; Maher and Thiéry, 2006), another possible host plant of *L. botrana*: *Rosemary officinalis* (Lamiaceae; Katerinopoulos et al., 2005), and tansy: *Tanacetum vulgare* (Asteraceae) used as a possible repellent source for *D. cavus*. We used bunches of *V. vinifera* harvested in mid-July, *T. vulgare* flowers (β -thujone chemotype, cf. Gabel and Thiéry, 1994), leaves of *R. officinalis*, and flowers, and berry of *D. gnidium*. Plants were all collected on the INRA Bordeaux site.

Box lids were pierced to allow air circulation. Using a fine brush, five newly hatched larvae (age < 24 hr) were transferred into each box: 15 boxes for *R. officinalis* and *D. gnidium* diet, 30 for Pinot noir and *T. vulgare*, 45 for Riesling, and control.

The two grape moths were fed on the control medium and their frass was collected. *Sphinx* frass was harvested from a natural population of larvae collected from *Catalpa*

bignonioides (Bignoniaceae) leaves. Larvae of *C. brunneus*, a specialist of Poacea, were harvested from a natural population in the Limoges area, and their frass were collected.

Bioassays

Experiments were conducted by using a T-tube olfactometer made of transparent glass (0.4 cm ID; stem 10 cm, arms 5 cm). Each arm was connected to a small plastic chamber holding the odor source (Fig. 1). To avoid contact with the odor source and to hide the source, small pieces of black glass fiber tissue were placed at the ends of each arm of the olfactometer. Filtered air (0.3 l min^{-1}) was sucked through the olfactometer by a pump connected to a flowmeter. Females were individually introduced into the olfactometer and observed for at least 2 min. Females that did not express a choice after 2 min were discarded. A female that settled at least 10 sec near one of the container was scored.

To avoid a bias due to possible chemical marking of the walking tracks, the glass tube and the glass fiber tissue were changed after each test. The position of the T-tube was turned 180° after each test to avoid effects of any directional bias. The T tubes were washed in hot water (ca. 70°C), rinsed in purified high-performance liquid chromatography (HPLC) quality water, and dried in an oven at 60°C before each experiment. All tests were performed between 09:00 and 18:00. All experiments were conducted under artificial light at $24 \pm 1^\circ\text{C}$. A white circular paperboard arena (diam: 35 cm; height: 21 cm) surrounded the olfactometer to prevent visual perturbations and to create a homogeneous light. *D. cavus* females were offered different dual choices.

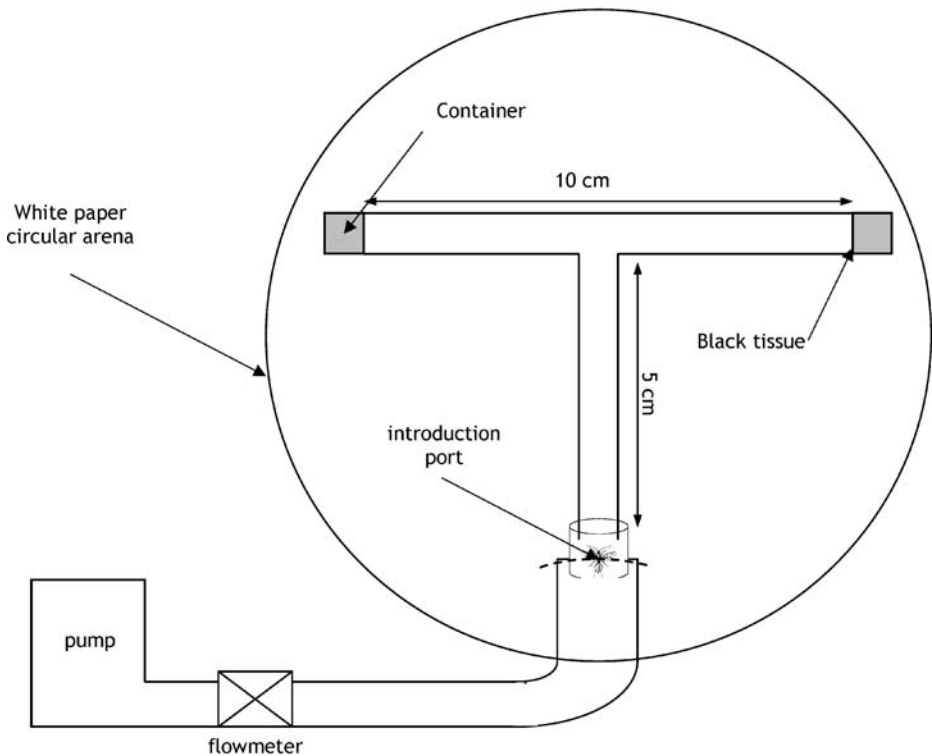


Fig. 1 T-tube olfactometer and set-up used in this study. Details are provided in the text

Responses to Larvae, Silk, and Frass

Attractiveness of fifth instar *L. botrana* or of their by-products (silk and frass) was tested. In this experiment, parasitoids were given the choice between one larva or 0.07 mg of silk, which corresponds to the average mass of a *L. botrana* cocoon, or 0.02 g of frass (i.e., the average production of two to three larvae in 24 hr or the larval production in 2–3 d) and a blank (no odor). Twelve cocoons of larvae reared on control medium were weighed to calculate the average amount of silk produced per larva. The average amount of frass produced per *L. botrana* larva was calculated from 19 fourth instars (L4) reared on control medium and by collecting their frass produced during 24 hr. The average amount collected in this condition was 9.96 ± 4.78 mg. Because of the small number of *E. ambiguella* and *S. ligustri* larvae, their attractiveness could not be tested.

Preferences for *L. botrana* Larvae Reared on Different Foods

The attractiveness of larvae fed on different plants (Pinot noir, Riesling, or *T. vulgare*) was compared with larvae fed on control medium.

Preferences for Frass Produced by Different Species

Frass produced by the three Lepidoptera species was compared at the identical mass mentioned above. To test the influence of the rearing host on the olfactory response of parasitoid females, some *D. cavus* were reared on *E. ambiguella*, as described for *L. botrana*. These parasitoids had the choice between frass produced by the two tortricids.

Preferences for *L. botrana* Frass from Different Larval Foods

Frass collected from larvae fed with different plants (Pinot noir, Riesling, *T. vulgare*, *D. gnidium*, or *R. officinalis*) was compared with those of larvae fed on control medium or on another plant supplemented medium.

Responses to Solvent Extracts

To isolate attractive cues, *L. botrana* frass was extracted by using solvents with different polarities. Three ml of solvent were added to 172.5 mg of dry frass (the production of ca. 300 L4). After 15 min at room temperature, frass was removed. Five different solvents were used: acetone, dichloromethane, hexane, methanol (99–99.80% purity, SDS, Peypin, France), or purified HPLC quality water. Each solvent was tested in the olfactometer, and none was found repellent or attractive. *D. cavus* females were given the choice between an extract and its respective solvent. Extracts and solvents were presented on a piece of Whatman paper (1 cm²). Different volumes were tested: 10, 30, and 60 µl, corresponding to 1, 3, and 6 larval equivalents, respectively.

Statistical Analysis

Statistical tests were performed using [Statistica Software \(Version 6.0, Stat Soft, Inc.\)](#). Attraction scores were analyzed using the Cochran *Q* test.

Results

Responses to Larval Silk and Frass

Female parasitoids were not attracted to larvae ($N=100$; $Q=0.1$; $P>0.05$) nor to silk ($N=99$; $Q=2.13$; $P>0.05$; Fig. 2). However, *D. cavus* females were attracted to the frass of the three Lepidoptera tested: *L. botrana* ($N=100$; $Q=20.60$; $P<0.001$), *E. ambiguella* ($N=100$; $Q=5.07$; $P=0.02$), and *S. ligustri* ($N=100$; $Q=4.06$; $P=0.04$). The frass produced by the grasshopper, *C. brunneus*, was not attractive ($N=100$; $Q=0.41$; $P>0.05$; Fig. 2).

Preferences for *L. botrana* Larvae Reared on Different Foods

There was no significant difference in the choice of *D. cavus* for larvae fed on control medium or on media supplemented with Pinot, Riesling, or tansy ($N=50$; respectively, $Q=0.53$; $Q=0$ and $Q=0$).

Preferences for Frass Produced by Different Species

The parasitoid was more attracted to the odor of from *S. ligustri* than from *L. botrana* ($N=100$; $Q=25.82$; $P<0.001$), or *E. ambiguella* ($N=100$; $Q=9.04$; $P=0.003$; Fig. 3). *D. cavus* was also more attracted to the frass from *L. botrana* than from *E. ambiguella* ($N=100$; $Q=4.74$; $P=0.03$). This preference was independent of the species on which *D. cavus* had been reared; *D. cavus* females reared from *E. ambiguella* also preferred *L. botrana* frass ($N=100$; $Q=7.04$; $P=0.008$).

Preferences for *L. botrana* Frass from Different Larval Foods

There was no significant difference in choice of *D. cavus* for frass from *L. botrana* fed on control medium or fed on supplemented medium ($N=50$; Pinot, $Q=0.35$; Riesling, $Q=3.13$; tansy, $Q=0.82$ and rosemary, $Q=1.39$). There was also no significant difference in choice of *D. cavus* for frass from *L. botrana* fed on different supplemented medium: Riesling vs. Pinot ($Q=1.26$), Pinot vs. tansy ($Q=0.20$), Riesling vs. tansy ($Q=3.27$), Riesling vs. daphne ($Q=1.09$), and Riesling vs. rosemary ($Q=0.09$). For all, $N=50$.

Fig. 2 Responses of *D. cavus* females to larvae, silk of *L. botrana*, or frass of different insect species. Lb: *Lobesia botrana*; Ea: *Eupoecilia ambiguella*; Sl: *Sphinx ligustri*; Cb: *Chorthippus brunneus*. White bars = no odor. * $P<0.05$ and ** $P<0.01$

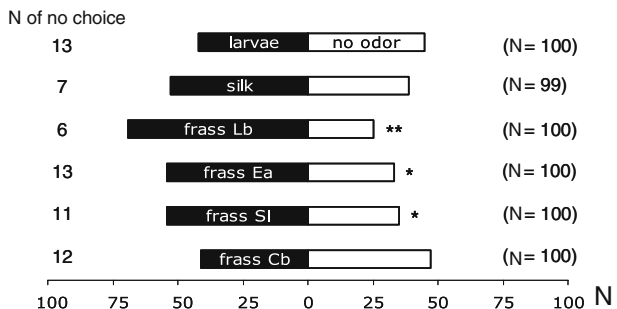
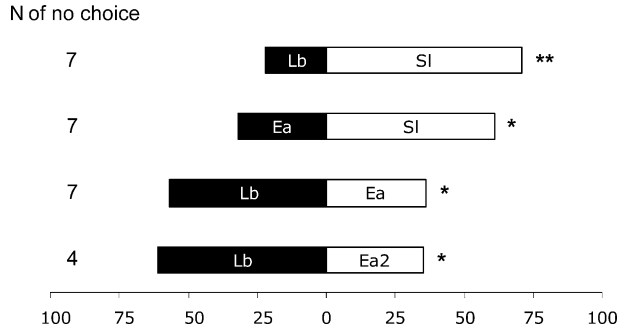


Fig. 3 Choice of *D. cavus* females between the odor of frass produced by caterpillars from different species. Lb: *Lobesia botrana*; SI: *Sphinx ligustri*; Ea: *Eupoecilia ambiguella*. Ea2: choice of females reared on *E. ambiguella*. All results: $N=100$. $*P<0.05$ and $**P<0.01$



Responses to the Solvent Extracts

D. cavus females responded in a dose-related way to extracts. None of the solvent extracts were attractive at the dose of 1 larval equivalent (Fig. 4). However, significant attraction was observed with water, dichloromethane, and methanol extracts applied at 3 larval equivalents ($N=50$; respectively $Q=12.8$; $P<0.001$; $Q=8$; $P=0.005$; $Q=4$; $P=0.045$). At the higher dose of 6 larval equivalents, only dichloromethane and water extracts elicited attractive responses as compared with solvent alone ($N=50$; respectively $Q=8.07$; $P=0.004$ and $Q=6.25$; $P=0.012$). Acetone and hexane extracts were not found to be attractive. Methanol elicited a similar percentage of females attracted, but because of a higher proportion of nonchoosing females, this result was not significant ($Q=3.2$; NS).

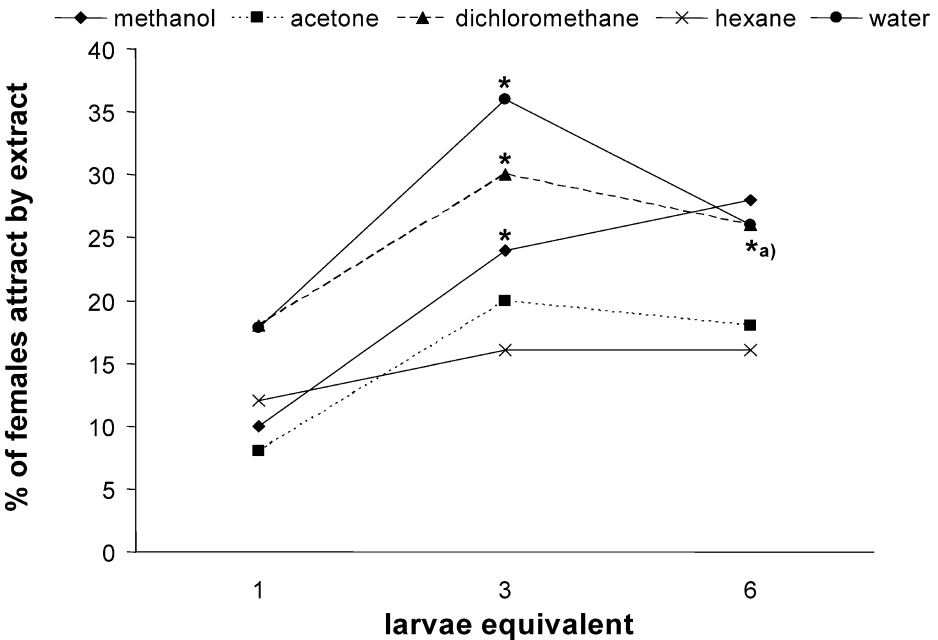


Fig. 4 Choice of *D. cavus* females between extracts of frass of *L. botrana* larvae and the correspondent solvent. All results: $N=50$. $*P<0.05$; *a), concerns the two treatments dichloromethane and water (see Results for details)

Thus, 3 larvae equivalents represented the optimally attractive dose, which corresponds to the amount of frass tested in the other experiments.

Discussion

The experiments demonstrate that *D. cavus* was attracted to frass produced by three species of Lepidoptera, but not by larvae alone. Moreover, *D. cavus* was not attracted to the silk produced by *L. botrana* larvae. Results revealed that larval food did not modify frass attractiveness, but that the moth species did.

Frass is a digestion residue containing nonabsorbed solids, water, scraps, diverse secretions, and microorganisms. Defecation is an essential physiologic process that also plays an important role in the ecology of host–parasitoid interactions (Weiss, 2003, 2006). Defecation behavior can partly be the result of natural selection, including predator/prey interactions, toxins or pathogens exposure, feeding, and social signalization (Weiss, 2003, 2006). For animals living near their feeding site, like *L. botrana*, presence and accumulation of frass can be disadvantageous because this can make prey more obvious to natural enemies. Hence, egestion products like frass can be perceived as an indicator of host presence (Bouchard and Cloutier, 1985; Reddy et al., 2002; Rogers and Potter, 2002).

D. cavus females were not attracted at distance to the different larvae tested or to their silk, but their frass represented an orientation cue. This could be extracted by different solvents, and *D. cavus* responded to these extracts in a dose-dependent way. Thus, frass bears chemical information able to attract this parasitoid. The volatiles extracted by water, dichloromethane, and methanol were attractive; thus, one may expect that a complex volatile signal made from intermediate to polar volatiles is needed to mimic the complete frass odor. Analytical comparisons of the different extracts were not performed in this work. However, such comparisons would be needed to confirm this point.

Although *D. cavus* is a generalist species, it was attracted in varying degree to the frass of the three Lepidoptera tested, and not at all to that of *C. brunneus*, which has not been described as a host. Generalist insects do exhibit preferences among their different hosts (Vinson, 1976; Janz, 2003; Maher and Thiéry, 2006). There is increasing evidence that natal or prenatal experience influences the preferences of the adult insect (Corbet, 1985; Vet et al., 1990; Rojas and Wyatt, 1999; Davis and Stamps, 2004). There are several examples of parasitoids responding to chemicals encountered during their larval or preimaginal instars (Corbet, 1985; Vet et al., 1990; Vet and Groenewold, 1990). The parasitoid *Nemeritis canescens* (Hymenoptera: Ichneumonoidea) reared on the nonhost insect *Meliphora grisella* (Lepidoptera: Pyralidae) develops an olfactory attraction for its rearing host (Alloway, 1972). In *D. cavus*, females reared on *L. botrana* were more attracted to *L. botrana* frass, but those reared on *E. ambiguella* were not specifically attracted to their developmental host frass. Therefore, we conclude that preimaginal conditioning to the rearing species did not occur in *D. cavus*.

Host alimentation can strongly influence parasitoid choices (Sauls et al., 1979; Benrey et al., 1997). Gandolfi et al. (2003) showed that rearing *Hyssopus pallidus* (Hymenoptera: Eulophidae) on *Cydia pomonella* (Lepidoptera: Tortricidae), fed on a medium supplemented with apple, enhances the attractiveness of the parasitoid to caterpillar frass. *D. cavus* has been found on numerous phytophagous species that feed on a wide range of host plants. However, none of the plants used in our experiment modified the attractiveness of the frass. Therefore, the plant on which the host feeds may be of minor importance to this parasitoid.

The experiments demonstrate that there is likely a general signal that makes Lepidoptera frass attractive to *D. cavus*. Actually, *D. cavus* is an extreme generalist, and generalists often use nonspecific chemical cues for host location rather than specific ones (Vet and Dicke, 1992; Thiéry and Gabel, 1993; Godfray, 1994; Steidle and van Loon, 2003). The olfactory signal used by this species may include general molecules indicating the occurrence of a wide range of possible hosts (e.g., Lepidoptera) and additional ones giving more specific information to discern among hosts.

This work is a first step toward understanding the relationships between *D. cavus* and two grape moths. We only focused on the responses of the wasp to odors, not considering the complete set of cues used by the wasp in its host recognition and acceptance. Indeed, the larval host plant influences the growth and survival of larvae of several generalist herbivores (Lazarević et al., 1998; Tikkanen et al., 1999; Serrano and Lapointe, 2002). In *L. botrana*, previous work showed that larval food modifies the larval development time (Savopoulou-Soultani et al., 1994; Moreau et al., 2006a,b; Thiéry and Moreau, 2005) and also their size with consequences on adult fitness. *L. botrana* larvae fed on different host plants may offer different food quality for their parasitoids, but *D. cavus* seems not to discriminate between the volatile information corresponding to such larvae.

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Zea mays · Parasitoids · Volatile Organic Compounds (VOCs) · Herbivore-Induced Plant Volatiles (HIPVs) · Host location · Associative learning · Tritrophic interactions · Indole · Shikimic acid · Glyphosate · Induced defenses

Introduction

Plants that are attacked by herbivorous arthropods are known to release a complex blend of volatile organic compounds (VOCs). These herbivore-induced plant volatiles (HIPVs) are exploited by predators and parasitoids as foraging signals that help them locate their herbivorous prey or hosts (Turlings and Wäckers, 2004; Arimura et al., 2005). At present, more than 1000 low-molecular-weight organic compounds have been reported to be emitted from plants, including alkanes, alkenes, alcohols, ketones, aldehydes, ethers, esters, and carboxylic acids (Dudareva et al., 2004; Niinemets et al., 2004). Although some of these compounds are constitutively emitted by undamaged, healthy plants, considerably higher amounts are emitted after herbivore damage and various HIPVs may even be synthesized *de novo* in response to damage (Turlings et al., 1990, 1998; Paré and Tumlinson, 1997). Some HIPVs are specific to certain plant taxa, for example, sulfur containing compounds in *Allium* plants (Dugravot et al., 2004) or glucosinolate breakdown products in Brassicaceae species (Scascighini et al., 2005), but others are common to many species (Fritzsche Hoballah et al., 2002; van den Boom et al., 2004). Common compounds include “green-leaf volatiles” (C₆ aldehydes, alcohols, and derivatives), cyclic and acyclic terpenoids, phenolic compounds, and nitrogenous compounds (Dicke, 1999; Paré and Tumlinson, 1997). These derive from at least three pathways. Green leaf volatiles are products of the enzymatic activity of hydroperoxide lyase (HPL), a component of the lipoxygenase (LOX) pathway, which results in multiple rearrangement of (*Z*)-3-hexenal (Bate and Rothstein, 1998; Blee, 1998). Terpenoids are synthesized via the isopentenyl pyrophosphate (IPP) intermediate following the classical mevalonate pathway or via an alternative IPP pathway with glyceraldehyde-3-phosphate and pyruvate identified as the direct precursors of IPP (Lichtenthaler et al., 1997). Finally, aromatic compounds, such as methyl salicylate (MeSA) and indole, are formed via the shikimic acid pathway (Bennett and Wallsgrove, 1994; Paré and Tumlinson, 1997).

In maize plants, the mechanisms of biosynthesis, induction, and release of HIPVs are well characterized (Turlings et al., 1998; Frey et al., 2000; Shen et al., 2000; Schnee et al., 2002; Gouinguéné et al., 2003; Schmelz et al., 2003a,b,c; Köllner et al., 2004; Lawrence and Novak, 2004; Ruther and Kleier, 2005), and the ecological significance of these compounds in tritrophic signaling has been demonstrated in laboratory and field experiments (Turlings et al., 1990; Bernasconi et al., 1998; Hoballah and Turlings, 2001; Fritzsche Hoballah et al., 2002; Rasmann et al., 2005). In particular, the role of green leaf volatiles and terpenoids in attracting natural enemies of the herbivores has been investigated in various experiments (D’Alessandro and Turlings, 2005; Hoballah and Turlings, 2005). Yet, it remains unclear which compounds are essential for attraction (D’Alessandro and Turlings, 2006). One group of compounds that has hardly been studied in the context of parasitoid attraction are the shikimic acid derived VOCs.

The main shikimic acid derived VOC released by maize seedlings after infestation with larvae of *Spodoptera* moths is indole (Turlings et al., 1998; D’Alessandro and Turlings, 2005). This compound is induced after treatment of maize seedlings with volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine], a fatty acid-amino acid conjugate found in the regurgitate of *Spodoptera* larvae (Alborn et al., 1997; Turlings et al., 2000). Indeed, jasmonic acid, which is involved in the induction of HIPVs in maize (Schmelz et al., 2003a), also appears

to be an integral part of volicitin-mediated induction of indole (Frey et al., 2000). Frey et al. (2004) identified an enzyme in maize, indole-3-glycerol phosphate lyase (Igl), which converts indole-3-glycerol phosphate to free indole. This differs from the enzyme BX1, which catalyzes the conversion of indole-3-glycerol phosphate to indole to form the direct defense compounds 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA) and 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), or tryptophane synthase, which produces the amino acid tryptophane (Frey et al., 1997). The selective activation of the evolutionarily similar genes *igl* and *bx1* suggests that the plants are capable of selecting direct or indirect defense mechanisms depending on the type of stress they are exposed to. Therefore, volatile indole was expected to be a key compound in the attraction of natural enemies of the herbivores.

Here we studied the importance of shikimic acid derived HIPVs, in particular indole, in attracting females of two parasitoid species, *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) and *Microplitis rufiventris* (Kokujev) (Hymenoptera: Braconidae). Both attack early instars of numerous lepidopteran moths, including many pests, and are known to use plant-provided VOCs in host location (Gouinguéné et al., 2003; Hoballah and Turlings, 2005). We manipulated the volatile blend emitted by maize seedlings (*Zea mays* var. Delprim) that had been fed upon by larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) by incubating the plants in glyphosate [*N*-(phosphonomethyl)glycine]. This compound inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Haslam, 1993; Schönbrunn et al., 2001) and strongly reduces the amounts of shikimic acid derived VOCs. Attraction of the odor from these inhibited plants was compared to that of the natural blends emitted by control plants. Subsequently, we tested an inhibited blend against an inhibited blend to which we added back a natural amount of synthetic indole, the major shikimic acid derived HIPVs.

C. marginiventris females, like many other female parasitoids, are able to learn and associate plant VOCs with the presence of suitable hosts during oviposition experiences (Turlings et al., 1993; D'Alessandro and Turlings, 2005). We therefore compared the responses of naïve and experienced female parasitoids, and in a series of learning experiments with *C. marginiventris* females, we estimated how well the wasps can learn to associate indole with host presence.

Methods and Materials

Insects and Insect Treatments The caterpillar *S. littoralis* (Boisduval) (Lepidoptera: Noctuidae) and the solitary endoparasitoids, *C. marginiventris* (Cresson) (Hymenoptera: Braconidae) and *M. rufiventris* (Kokujev) (Hymenoptera: Braconidae) were reared as previously described (Turlings et al., 2004). Adult parasitoids were kept in plastic cages at a male/female ratio of approximately 1:2 and were provided with moist cotton wool and honey as food source. Cages were kept in incubators (*C. marginiventris*, 25±1°C; *M. rufiventris*, 23±1°C; 16:8 hr L/D) and transferred to the laboratory 30 min before the experiments. We tested mated 2- to 4-d-old naïve and experienced females. The latter were given experiences by allowing them to oviposit 3–5 times into second instar *S. littoralis*. A metal screen was attached to the top opening (2 cm diam) of an odor source vessel of the same type as used in the olfactometer (see below). Approximately 20 larvae were placed on the screen, and individual wasps were allowed to oviposit in one or two larvae, while they were exposed to the odor of the source that was placed inside the vessel. This source was either an infested control plant, an infested inhibited plant, or synthetic indole only. Airflow and concentrations of volatiles

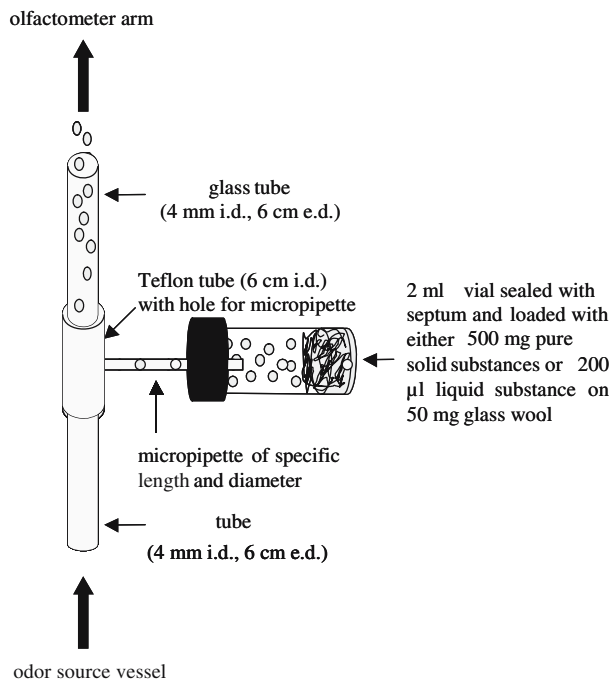
were the same as during the olfactometer bioassays (see below). Naïve females were neither exposed to the volatiles nor given any oviposition experience before testing. The different groups of wasps were kept separately in small plastic boxes with moist cotton wool and honey and released into the olfactometer 1–3 hr after the oviposition experiences.

Plants and Odor Sources Maize (*Z. mays*, var. Delprim) was sown in plastic pots (10 cm high, 4 cm diam) with commercial potting soil (Ricoter Aussaaterde, Aarberg, Switzerland) and placed in a climate chamber ($23 \pm 2^\circ\text{C}$, 60% r.h., 16:8 hr L/D, and $50,000 \text{ lm/m}^2$). Plants used for the experiments were 10–12 d old and had 3 fully developed leaves. The evening before the experiments, plants were cut with a razor blade at soil level, while the stem was held under water to prevent air entering the vascular system. Subsequently, they were placed in a vial (8 ml) filled with either deionized water (control plant) or in a 1 mM glyphosate [*N*-(phosphonomethyl)-glycine; Fluka, Buchs, Switzerland] solution (inhibited plant). Vials were wrapped in aluminum foil, and one vial containing a single plant was placed in an open odor source vessel of the olfactometer (described by Turlings et al., 2004). Two hr after incubation, plants were infested with 20 second instar *Spodoptera*, which were released in the whorl of the youngest leaf. After infestation, plants were kept under laboratory conditions ($25 \pm 2^\circ\text{C}$, $40 \pm 10\%$ r.h., 16:8 hr L/D, and 8000 lm/m^2) and were used for the experiments the following day, between 10 A.M. and 4 P.M.

To check whether the treatment of the plants with the inhibitor affected the larval feeding behavior, plants were collected after the bioassays, and the leaves were scanned into Adobe Photoshop 6.0. The total leaf area removed during the 24-hr feeding period was compared for the different treatments based on differences in pixels that indicated tissue removal.

Indole (purity $\geq 99\%$; Fluka) was released from a device consisting of a 2-ml glass vial that contained 500 mg synthetic indole (Fig. 1), which was connected, via a glass capillary,

Fig. 1 Schematic representation of the odor delivery system for synthetic VOCs



to a Teflon tube placed between two glass tubes connecting the top of an odor source vessel to an olfactometer arm. Preliminary experiments with various synthetic volatile compounds (e.g., MeSA, linalool, indole) showed that at room temperature this device allowed the constant release of pure compounds, and that their release rates could be controlled by adjusting the length and the diameter of the capillary tube (Duran, Hirschmann EM). The release rate was calibrated to the lower range of amounts of indole that was found for infested maize plants. Vials were prepared freshly the evening before the experiments and connected the following morning to the odor source vessels used for training or testing.

Olfactometer Bioassays All odor sources were tested for attractiveness to parasitoids in a four-arm olfactometer (described by D'Alessandro and Turlings, 2005) as indicated in Table 1. Cleaned and humidified air entered the odor source vessel at 1.2 l/min (adjusted by a manifold with four flowmeters; Analytical Research System, Gainesville, FL, USA) via Teflon tubing and carried the VOCs 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 (see "Collection and analyses of VOCs"). Incoming and outgoing air were balanced by a Tygon tube connected to a vacuum pump via another flow meter and a pressure gauge. Empty arms were connected to empty vessels and carried clean, humidified air only.

Wasps were released in groups of six into the central part of the olfactometer, and after 30 min the wasps that had entered an arm of the olfactometer 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." Experiments were replicated on eight different days, and for each replicate a total of six groups of six wasps were tested during a 3-hr sampling period, alternating between groups of naive and experienced wasps in experiments where three different groups were tested (total of 96 wasps/group), or only naive wasps in the other experiments (total 288 wasps). Ten neon tubes attached to a metal frame above the olfactometer provided approximately 7000 lm/m² at the height of the odor source vessels. All bioassays were carried out between 10 A.M. and 4 P.M.

Collection and Analyses of VOCs VOCs of each odor source were collected during the olfactometer bioassay on a Super-Q trap (25 mg, 80–100 mesh; Alltech Associates, Deerfield, IL, USA, described by Heath and Manukian, 1992). Each trap was attached horizontally to the elbow of the olfactometer and connected via Tygon tubing to a flowmeter (Analytical Research System) and a vacuum pump. Air carrying the volatiles was pulled through each trap for 3 hr at a rate of 0.6 l/min during each behavioral bioassay. Afterwards, the traps were extracted with 150 μ l dichloromethane (Suprasolv; Merck, Dietikon, Switzerland), and 200 ng of *n*-octane and *n*-nonyl acetate (Sigma, Buchs, Switzerland) in 10 μ l dichloromethane were added to the samples as internal standards. All extracts were stored at -76°C until analyses. Traps were washed with 3 ml dichloromethane before they were reused for a next collection.

VOCs of the experiments with control and inhibited plants were analyzed with a gas chromatograph (Agilent 6890 Series GC system G1530A) coupled to a mass spectrometer that operated in electron impact mode (Agilent 5973 Network Mass Selective Detector; transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33–280 amu). A 2- μ l aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 μ m film thickness; Alltech Associates). Helium at constant flow (0.9 ml/min) was used as carrier gas. After injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and

Table 1 Odor sources and experimental design

Figure	Odor sources				Wasp treatment	Replications of experiment	
	Arm 1	Arm 2	Arm 3	Arm 4			
3A	Control plant (infested)	Empty	Inhibited plant (glyphosate, infested)	Empty	<i>C. marginiventris</i>	Naive, control, inhibited	8
3B	Control plant (infested)	Empty	Inhibited plant (glyphosate, infested)	Empty	<i>M. rufiventris</i>	Naive, control, inhibited	8
4A	Inhibited plant and indole (glyphosate, infested)	Empty	Inhibited (glyphosate, infested)	Empty	<i>C. marginiventris</i>	Naive	8
4B	Inhibited plant and indole (glyphosate, infested)	Empty	Inhibited (glyphosate, infested)	Empty	<i>M. rufiventris</i>	Naive	8
5A	Indole	Empty	Empty	Empty	<i>C. marginiventris</i>	Naive, indole, control	8
5B	Control plant (infested)	Empty	Inhibited plant (glyphosate, infested)	Empty	<i>C. marginiventris</i>	Naive, indole, control	8

Further details on odor sources, number, and treatment of wasps are described in the text and in the figures. Treatments of the plants are given in parentheses.

subsequently to 200°C at 5°C/min followed by a post-run of 5 min at 250°C. The detected volatiles were identified by comparison of their mass spectra with those of the NIST 02 library, by comparison of their spectra and retention times with those of authentic standards, and by comparison of retention times with those in previous analyses (D'Alessandro and Turlings, 2005). Compounds that were not identified by comparing retention times and spectra with those of pure standards are indicated in Fig. 2 with superscript N, and their identity should be considered tentative. Twelve samples per treatment were analyzed in the SIM mode (ion 117, qualifier 95), and indole was quantified based on a calibration curve with known amounts of synthetic indole. All other compounds were only quantified in the full scan range based on comparison of their peak area with those of the internal standards (*n*-octane for compounds 1–14, *n*-nonyl acetate for compounds 14–27). A total of 18 samples were injected per treatment.

Statistical Analyses The functional relationship between parasitoids' behavioral responses and the different odor sources offered in the four-arm olfactometer was examined with a log-linear model (a generalized linear model, GLM). As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasi-likelihood functions to compensate for the overdispersion of wasps within the olfactometer (Turlings et al., 2004). 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. We

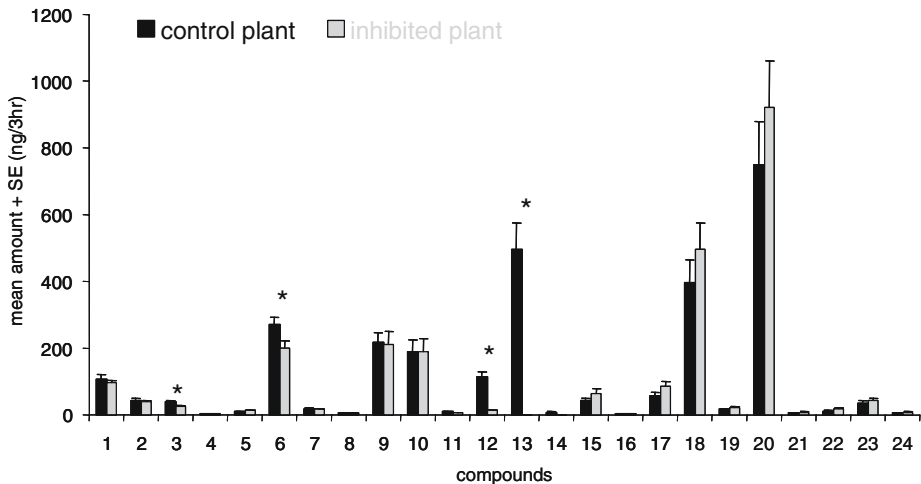


Fig. 2 Mean amount (\pm SE) (ng) of major VOCs recollected from cut *Spodoptera*-induced maize seedlings during 3 hr. Control plants were incubated in water, inhibited plants were incubated in a 1 mM glyphosate solution. Asterisks above bars indicate significant differences (*t*-test on $\log(x+1)$ transformed data, $P < 0.05$) in the amount of a specific compound. $N = 18$ per treatment. The compounds are as follows: **1**, (Z)-3-hexenal; **2**, (*E*)-2-hexenal; **3**, (Z)-3-hexen-1-ol; **4**, (Z)-2-penten-1-ol acetate; **5**, β -myrcene; **6**, (Z)-3-hexenyl acetate; **7**, (*E*)-2-hexenyl acetate; **8**, (Z)- β -ocimene^N; **9**, linalool; **10**, (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT); **11**, benzyl acetate; **12**, phenethyl acetate; **13**, indole; **14**, methyl anthranilate; **15**, geranyl acetate; **16**, unknown sesquiterpenoid; **17**, (*E*)- β -caryophyllene; **18**, (*E*)- α -bergamotene; **19**, unknown sesquiterpenoid; **20**, (*E*)- β -farnesene; **21**, unknown sesquiterpenoid; **22**, unknown sesquiterpenoid; **23**, β -sesquiphellandrene^N; **24**, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). The compounds are ordered in accordance with their retention on a nonpolar capillary column

tested “treatment” effects (=odor sources) for naive and experienced wasps separately, and we included “release” as an explanatory variable to avoid “pseudo-replications.” In addition, we tested if there was a significant effect of “experience” and an interaction between “treatment \times experience.”

The amounts of VOCs were analyzed by using *t*-tests. Amounts of VOCs that were not normally distributed were $\log(x+1)$ transformed prior to analysis. The amounts of indole quantified in the single ion mode were analyzed with a Kruskal–Wallis test. Differences between the treatments were analyzed with the Tukey’s test. Differences between the removed leaf areas after caterpillar feeding were analyzed with a *t*-test. All analyses were run on SigmaStat (Version 2.03).

Results

VOCs of Control and Inhibited Plants Herbivore-infested maize seedlings that were incubated with their cut stem in water (control plants) released a volatile blend consisting of 24 detectable VOCs, including green leaf volatiles, terpenoids, and shikimic acid derivatives (Fig. 2). Seedlings that were incubated in a 1 mM glyphosate solution released only trace amounts of indole and methyl anthranilate and had strongly reduced amounts of other shikimic acid derived VOCs (*t*-test: benzyl acetate, $t_{34}=1.741$, $P=0.092$; phenethyl acetate, $t_{34}=11.218$, $P<0.001$). The amounts of VOCs derived from other biochemical pathways were similar to those of the control plants, except for the somewhat reduced amounts of (*Z*)-3-hexen-1-ol ($t_{34}=2.306$, $P=0.027$) and 3-*Z*-hexen-1-ol acetate ($t_{34}=2.577$, $P=0.014$). In addition to the compounds quantified in Fig. 2, we also detected trace amounts of (*E*)-nerolidol, (*Z*)-jasmone, and some minor, unidentified compounds. These VOCs were not included in quantification analyses.

The single ion mode analyses of indole revealed that during the 3-hr bioassay periods we collected 835.44 ± 135.91 ng from control plants and only 0.55 ± 0.17 ng from inhibited plants. In bioassays where synthetic indole was added to the air stream with the odor of an inhibited plant, we detected 192.77 ± 11.46 ng. There was a significant difference between the amounts released by these three treatments (Kruskal–Wallis followed by Tukey test, $H_2=30.294$, $P<0.001$).

Inhibitor treatment did not affect the larvae’s feeding rate; the leaf areas removed by the larvae during a 24-hr feeding period were similar for control plants (4.73 ± 0.40 cm²) and for inhibited plants (4.81 ± 0.38 cm²) (*t*-test: $t_{20}=-0.15$, $P=0.882$).

Attractiveness of Inhibited vs. Control Plants Neither naïve nor experienced *C. marginiventris* females significantly distinguished between VOC blends emitted by *Spodoptera*-induced maize seedlings with strongly reduced amounts of shikimic acid derived VOCs (see above) and VOCs emitted by control plants (Fig. 3a, GLM, naive: $F_{1,15}=0.78$, $P=0.39$; experienced on inhibited blend: $F_{1,15}=3.91$, $P=0.067$; experienced on control blend: $F_{1,15}=0.43$, $P=0.52$). Yet, the type of experience had a significant effect on the choice of the wasps ($F_{1,60}=4.566$, $P=0.037$), implying that *C. marginiventris* females were able to detect the difference between the two odor sources. The responsiveness (the proportion of wasps entering an arm with one of the two treatments) was high and similar for all treatments, and only few wasps entered an empty arm (Fig. 3a).

Naive and experienced females of *M. rufiventris* significantly preferred the inhibited blend (Fig. 3b, GLM, naive: $F_{1,15}=11.04$, $P=0.005$; experienced on inhibited blend: $F_{1,15}=$

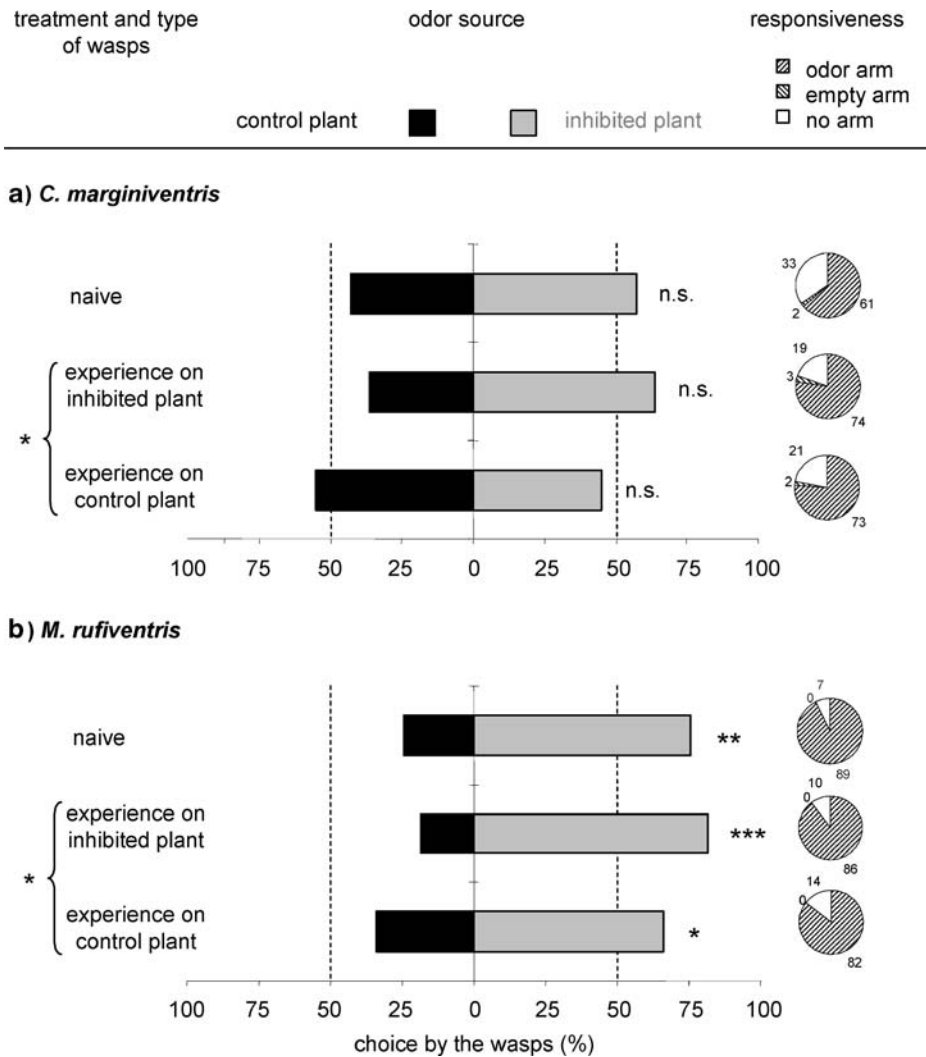


Fig. 3 Importance of shikimic acid derived VOC for the attraction of two parasitoid species. (a) Choice of *C. marginiventris* females and (b) *M. rufiventris* females between arms carrying VOCs of *Spodoptera*-induced maize seedlings that were either incubated in water (control plants) or in a 1 mM glyphosate solution (inhibited plants). Pretreatment of the wasps (=type of experience) is indicated on the left. Pie charts indicate overall responsiveness (=number of wasps entering the different types of arms). GLMs were performed in order to test for differences between the two odor arms within one group of wasps as well as to compare the types of experiences. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s. = no significant difference, $P > 0.05$

26.19, $P < 0.001$; experienced on control blend: $F_{1,15} = 6.19$, $P = 0.025$). As with *C. marginiventris*, the type of experience had an effect on the choice of the wasps ($F_{1,60} = 7.073$, $P = 0.010$). The responsiveness was high, and none of the *M. rufiventris* wasps entered an empty arm.

Role of Indole Indole was the major shikimic acid derived VOC released by *Spodoptera*-induced maize seedlings (Fig. 2). We tested its role in the attraction of the two parasitoid

species by comparing the attractiveness of HIPV blends released by two inhibited plants, whereby we added synthetic indole back to one of the blends. The amounts of indole added (192.77 ± 11.46 ng/3 hr) fell within the lower ranges of indole detected in a natural induced maize blend (see above). *C. marginiventris* did not distinguish between the two blends (Fig. 4a, GLM: $F_{1,47}=0.87$, $P=0.36$). In contrast, *M. rufiventris* significantly preferred the inhibited blend without synthetic indole (Fig. 4b, $F_{1,47}=43.16$, $P<0.001$). Results for both wasps are consistent with those from the previous experiment, showing an insignificant role of shikimate-derived compounds for *C. marginiventris* attraction, whereas they have a repellent effect on *M. rufiventris*.

Learning of Indole When *C. marginiventris* females were given a choice between one arm with synthetic indole (192.77 ± 11.46 ng/3 hr) and three arms with clean air only, they neither showed an innate (naive wasps) attraction towards indole (Fig. 5a, GLM: $F_{1,47}=0.60$, $P=0.44$), nor were they attracted to indole after having experienced a natural blend that contained similar amounts of indole ($F_{1,47}=0.29$, $P=0.60$). However, if they were exposed to pure indole during oviposition experiences they preferred an arm carrying indole over arms with clean air ($F_{1,47}=11.62$, $P=0.001$), although the type of experience had only a marginal effect on their choices ($F_{1,124}=2.919$, $P=0.090$) and the overall responsiveness was rather low (Fig. 5a).

In an additional experiment, we tested whether exposing the wasps to indole during ovipositions increases the attraction towards an induced maize blend containing indole (control plant) compared to a blend with only trace amounts of indole (inhibited plant). As

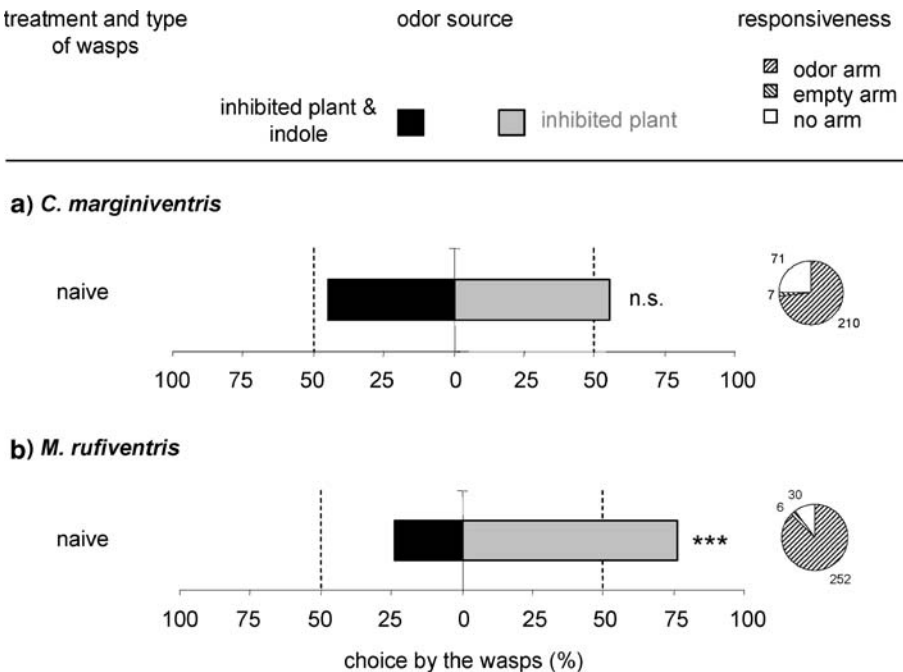


Fig. 4 Importance of indole for the attraction of two parasitoid species. (a) Choice of naïve *C. marginiventris* females and (b) naïve *M. rufiventris* females between arms carrying *Spodoptera*-induced maize VOCs of inhibited plants and of inhibited plants to which synthetic indole was added. See Fig. 3 for further explanations

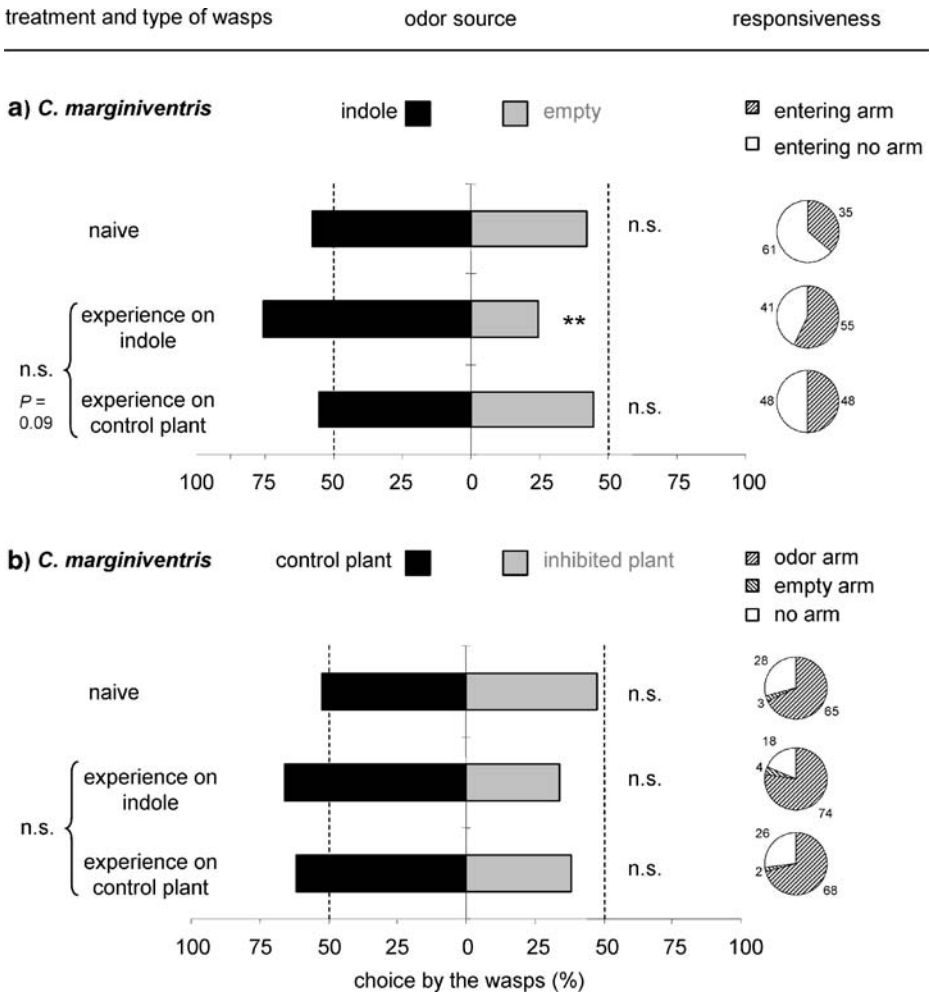


Fig. 5 Importance of indole in learning experiments by *C. marginiventris*. (a) Choice of females with different learning experiences between arms carrying synthetic indole and empty arms with clean air only. (b) Choice of females with different learning experiences between arms carrying *Spodoptera*-induced maize VOCs of control and inhibited plants. See Fig. 3 for further explanations

in the experiments above, naive wasps and wasps that had experienced the control blend did not distinguish between the two blends (Fig. 5b, GLM: $F_{1,15}=0.062$, $P=0.81$; $F_{1,15}=1.92$, $P=0.19$, respectively). Interestingly, even an experience with pure synthetic indole did not result in a change in preference ($F_{1,15}=3.70$, $P=0.074$), and the type of experience did not have a significant effect on the wasps’ choice ($F_{1,60}=0.214$, $P=0.646$).

Discussion

One way of studying the importance of individual VOCs for the attraction of natural enemies is to compare the attractiveness of an incomplete with a normal blend of HIPVs (D’Alessandro and Turlings, 2005). By restoring the incomplete blend with synthetic

compounds that are missing, the importance of the added compounds can be confirmed (de Boer and Dicke, 2004). Here, we used glyphosate, an inhibitor of the enzyme EPSP synthase (Haslam, 1993; Schönbrunn et al., 2001), to inhibit the production of shikimic acid derived HIPVs. As expected, this inhibitor treatment resulted in a strong reduction of the emission of indole, methyl anthranilate, phenethyl acetate, and benzyl acetate, whereas the amounts of most other compounds, except two green-leaf volatiles, were not affected by the inhibitor treatment (Fig. 2). Treating plants with glyphosate normally results in lower amounts of chorismate, a precursor for the production of salicylic acid (SA) (Shah, 2003). SA has been shown to synergistically or antagonistically interact with jasmonic acid (Thaler et al., 2002; Bostock, 2005), an important hormone involved in the induction of maize VOCs (Schmelz et al., 2003c). We speculate that SA does not play a major role in induced volatile emissions in this maize variety. Indeed, MeSA, the volatile form of SA, was only occasionally detected in trace amounts in our control plants.

Glyphosate-treated plants are also expected to contain lower levels of phenolic compounds, which should have a positive effect on herbivores. However, we did not observe any difference in the amount of leaf damage inflicted by *Spodoptera* larvae between glyphosate-treated and glyphosate-untreated plants. A possible negative effect of glyphosate through direct toxicity has been ruled out in ecotoxicological risk assessment studies with arthropods (Giesy et al., 2000). Furthermore, glyphosate is not readily volatilized and is primarily degraded by microbial metabolism in the soil (Tu et al., 2001). Indeed, we did not detect additional VOCs from glyphosate-treated seedlings, making glyphosate a suitable inhibitor to study the attractiveness of shikimic acid derived VOCs.

Attractiveness of Shikimic Acid Derived VOCs It is generally assumed that HIPVs are exploited by natural enemies of the herbivores in order to locate their host or prey (Dicke, 1999; Turlings and Wäckers, 2004), but which compounds of a volatile blend are actually important in the foraging behavior of natural enemies is not yet known for most tritrophic systems (Dicke and van Loon, 2000). Previous studies on caterpillar-induced maize volatiles showed that qualitative differences in the odor blend may be more important for the attraction of parasitic wasps than quantitative differences (Fritzsche Hoballah et al., 2002). Indeed, a blend of induced maize volatiles with reduced amounts of the three major sesquiterpenoids, (*E*)- β -caryophyllene, (*E*)- α -bergamotene, and (*E*)- β -farnesene, was equally attractive to naive *C. marginiventris* as control blends with high amounts of these compounds, whereas removing some minor, polar compounds from the blend rendered it completely unattractive to *C. marginiventris* (D'Alessandro and Turlings, 2005). Here, we provide another example showing that some common HIPVs, i.e., shikimic acid derived volatiles, are not involved in the initial attraction of two parasitoids to host infested plants (Figs. 3 and 4). Indole, which is one of the dominating compounds in *Spodoptera*-induced maize volatiles (Turlings et al., 1998; D'Alessandro and Turlings, 2005), might even be repellent or might mask the attractiveness of compounds used for host location. Interestingly, several studies on maize volatiles indicate specific induction of volatile indole by herbivore-derived elicitors and not by excision stress or mechanical damage (Frey et al., 2000; Schmelz et al., 2003b). However, in other studies, many of the common herbivore-induced VOCs, including indole and several terpenoids, have been detected in analyses of VOCs released by plants exposed to other forms of stresses, as for example mechanical wounding (van den Boom et al., 2004), exposure to other VOCs (Ruther and Fürstenau, 2005), or infection by microorganism (Huang et al., 2003). Hence, the emission of various volatiles can be induced by a number of enemies and stresses, yet natural enemies are able to discriminate between different forms of stresses (Takabayashi et al.,

1995; De Moraes et al., 1998; de Boer et al., 2004; Vuorinen et al., 2004). Selection must have favored parasitoids with an ability to distinguish between host- and nonhost-related compounds and an innate response to compounds that are specifically correlated with host presence is the most likely mechanism that allows them to make such distinctions (Vet and Dicke, 1992). We hypothesize that naïve females of generalist parasitic wasps are attracted only to a few key compounds within a complex blend of volatiles, and that most other compounds within such a blend contribute little to its initial attractiveness. They may mask the attractive compounds, or may even be repellent. Still, these compounds may become attractants when the wasps have associated them with host presence.

Importance of Learning One way for parasitoids to deal with highly complex and variable blends is their ability to learn by association (Turlings et al., 1993; Vet et al., 1995). It is assumed that such learning processes are specifically important for generalist wasps such as *C. marginiventris* and *M. rufiventris*, parasitizing various host, feeding on different plant species (Vet and Dicke, 1992; Steidle and van Loon, 2003). Indeed, *C. marginiventris* shows a keen ability for associative learning, whereas this form of learning is less clear for *M. rufiventris* (D'Alessandro and Turlings, 2005; Hoballah and Turlings, 2005; Tamò et al., 2006). Here again, *C. marginiventris* showed a significant shift in its preference in favor of the blend that it had experienced during multiple ovipositions (Fig. 3a). The response of *M. rufiventris* also changed significantly after experience, but it maintained a significant preference for the odor of inhibited plants even after having experienced the odor of control plants (Fig. 3b).

The predatory mites (*Phytoseiulus persimilis*) have been found to strongly associate MeSA with the presence of their prey if they are reared in the presence of a complex blend of herbivore-induced VOCs that includes MeSA (de Boer et al., 2004). Similarly, Vet et al. (1998) found that the *Drosophila* parasitoid *Leptopilina heterotoma* learned to discriminate between odors from substrates that were qualitatively different, but failed to discriminate when differences were small, unless unrewarding experiences provided evidence of the absence of hosts in one of the substrates. That some compounds are more important than others for associative learning was found for the parasitoid *M. croceipes*. After conditioning to a complex mixture, females of this species established a hierarchy among various components, with some of them accounting for a major part of the behavioral activity evoked by the mixture (Meiners et al., 2003). In our study, *C. marginiventris* was able to learn and subsequently respond to pure synthetic indole; however, this learning of indole had no effect on the females' responses to natural, complex blends with or without indole (Fig. 5). Apparently, indole is not a compound that is strongly associated during learning processes, especially if it is not offered in a complex volatile environment. Although indole is strongly induced after *Spodoptera* infestation on maize plants, it is also found in a variety of other stress-induced plant volatile blends (see above), and may not provide foraging parasitic wasps with specific information on the presence or absence of hosts.

In summary, we studied the role of shikimic acid derived VOCs, in particular indole, in the host searching behavior of two parasitoid species, *C. marginiventris* and *M. rufiventris*. This group of VOCs forms a substantial part of the volatile blend released by maize seedlings in response to feeding by lepidopteran larvae, but the results show that they are not important for the attraction of the two wasp species tested here. Attraction of *C. marginiventris* was not affected by the presence or absence of indole, the major shikimic acid derived VOC, whereas this compound was repellent rather than attractive to *M. rufiventris*. Learning of indole during oviposition experiences did not greatly alter these

responses. Hence, this study suggests that parasitoids do not use all herbivore-induced VOCs for habitat and host location to a similar degree, but rather pay selective attention to a few compounds. Identifying these key compounds seems crucial for a good understanding of the host searching process in parasitoids and for the development of strategies to increase the efficiency of natural enemies for the control of pest insects (Turlings and Ton, 2006).

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controls. The absolute configuration of the insect-produced pheromonal components remains to be elucidated, but the 1'S stereochemistry was established for at least one of the isomers.

Key words Semiochemical · zingiberenol · (1RS, 4RS, 1'S)-4-(1', 5'-dimethylhex-4'-enyl)-1-methylcyclohex-2-en-1-ol · allomone

Introduction

The rice stalk stink bug, *Tibraca limbativentris* Stal, 1860 (Hemiptera: Pentatomidae), is a neotropical pest of paddy rice in Brazil, Argentina, and Uruguay (Panizzi et al., 2000). Recently, it was reported in the Dominican Republic (Jorge, 1999) and also in Colombia, Ecuador, Panama, Costa Rica, and Suriname, and is a potential threat to the United States. Introduction into North America may result in serious losses to U.S. and Mexican rice production. Other economically important crops may serve as host plants as well (North American Plant Protection Organization-Phytosanitary Alert System; <http://nappo.org/>).

Tibraca limbativentris is a damaging insect to rice in Brazil. Feeding of the insects at the base of the plants in the vegetative stage causes the phenomenon known as “dead heart”, and feeding during the reproductive stage causes the plants to exhibit damage known as “white panicle” (Ferreira et al., 1997). Bug populations have been high for several consecutive years (up to 200 individuals/m²), causing production losses varying from 5% to 80% (Ferreira et al., 1997 and references therein). This species occurs in almost all states of Brazil and is sometimes found on alternative hosts such as soybean, tomato, wheat, and native Gramineae (Panizzi et al., 2000 and references therein).

The control of *T. limbativentris* is heavily based on chemical pesticides (Usta et al., 1994; Ferreira et al., 1997; Costa and Link, 1998/99; Panizzi et al., 2000). For example, in Colombia, stink bugs in rice require up to three insecticide applications per season (Pantoja et al., 1995).

Because of the habit of *T. limbativentris* feeding in the upside-down position at the base of the plants, when the damage is perceived by growers, the economic threshold has already been reached. Thus, pheromone-baited traps might be a useful tool for monitoring these pests. The objectives of the research described herein were to determine whether *T. limbativentris* produces a male-specific sex pheromone, as reported for other phytophagous stink bug species, and, if so, to identify the chemical structure(s) of the biologically active compound(s).

Methods and Materials

Insects

Tibraca limbativentris adults were from a colony started with adults collected near Embrapa Rice and Beans, Goiania-Goiás (16°4'S and 49°14'W). Insects were reared on rice plants, *Oryza sativa*, potted in a large (5 kg) pot in a green house and kept as a stock colony. In the laboratory, where the knowledge of sexual maturity (age) was necessary for bioassays and collection of volatiles (aeration experiments), bugs were reared in 5-l plastic containers with the food supply being renewed 3 times per wk (Cavalcante et al., 2004), at 26 ± 1°C and 65 ± 10% relative humidity under 14:10 light/dark photoperiod (from 06:00 to 20:00 hr).

Most of the egg masses were laid on the rice plants, and 3 times a week these plants were transferred to new plastic containers where the nymphs were reared as above.

To prevent interactions between the sexes, males were separated from females after their imaginal molt and cuticular hardening (ca. 24 hr). For all experiments, sexually mature adults (>15-d-old) were used (Cavalcante et al., 2004).

Collection of Volatiles

Volatiles were collected from individual groups of 15 male or female *T. limbativentris* ($N=50$). The insects were gently introduced into 1-l glass containers (Zhang et al., 2003) to minimize emission of defensive secretions. Air was drawn into the container through 4–12 mesh-activated charcoal (Fisher Scientific, Pittsburgh, PA, USA), and out of the container through two traps (15×1.5 cm OD) containing Super Q (200 mg each; Alltech Associates, Inc., Deerfield, IL, USA) by vacuum pump (~ 1 l/min). The adsorbent traps were eluted with hexane (4×0.5 ml per sample) and each sample was concentrated to approximately 100 μ l under a gentle flow of nitrogen for further analysis. Insects were fed with fresh green beans (*Phaseolus vulgaris*; replaced three times a week) and aerated continuously for 15 d, changing the adsorbent traps daily, to get enough material for bioassays and chemical analysis. To check the emanation of any volatiles from the green beans that would contaminate the airborne collection from the above experiment, the same procedure was carried out with three green beans inside the glass chambers and the volatiles were collected for 24 hr for 4 d ($N=12$).

Olfactometer Bioassays

A two-choice olfactometer modified from Borges and Aldrich (1994) was used to test the biological activity of live insects, insect aeration extracts of *T. limbativentris*, and synthetic compounds. The olfactometer release chamber was a 500-ml, three-neck, round-bottomed flask (all 24/40 joints, Kontes, Vineland, NJ, USA). Two 250-ml rotary evaporator trap adapters (24/40 joints) were attached to each side arm of the release flask (the treatment and control arms). A charcoal (20/40 mesh) filter (130×10 mm ID) was attached to the side arms using two 40-cm-long pieces of a Silastic tubing (3/16 inch ID \times 5/16 inch OD; VWR Scientific Corporation, Darmstadt, Germany), inserted in a “Y” connector of the same diameter and connected to adapters (24/40 joint) on each side arm of the olfactometer. The air was humidified by passage through a container of distilled water between the charcoal filter and the arms of the olfactometer. The middle neck of the flask was connected to the vacuum pump with an adapter and the air flow was adjusted with a “Clear Flow Rotameter” (Accura Flow Products, Warminster, PA, USA) to a flow of 0.8 l/min. The apparatus was positioned horizontally on a countertop in a room with bright fluorescent lights (4×40 W) during daytime conditions, and lighted with red lamps (4×60 W) during scotophase. The temperature in the bioassay room was maintained at $26.0 \pm 1.0^\circ\text{C}$. The positions of the olfactometer arms were inverted between control and treatments after each three repetitions to avoid any positional bias. The apparatus was cleaned with fragrance-free liquid soap, rinsed thoroughly with water, and dried at 80°C , after each five replicates. The insects were placed in the round-bottom flask (release chamber), and the treatments were placed at the end of the reducing adapter chamber (treatment arms).

A single *T. limbativentris* adult was gently introduced into the release chamber of the Y-tube olfactometer with the aid of an artist’s paint brush (Camel Hair, number 1), and its pattern of behavior (response) was recorded for 15 min/replicate. The duration of each

bioassay replicate was monitored using a stopwatch. Before testing, the insects were allowed to acclimatize for a short period (ca. 3 min) in the release chamber while assembling the treatment chambers. The first choice of the insect was recorded, i.e., the first arm of the olfactometer that the insect chose and remained in for at least 100 sec. The test insects were used only once during the bioassays.

Determination of Period of Maximum Activity

To test if *T. limbativentris* had a daily cycle of sexual activity, bioassays were carried out to determine the cycle of maximum response by testing insect responses to volatiles from live bugs used as the pheromone source. For this experiment, the following periods were designated: morning (M=06:00–12:00), afternoon (A=12:00–18:00), and night (N=18:00–24:00). Fifty replicates were carried out for each time period and the number of insects attracted to the pheromone source was recorded.

Bioassays with Living Insects

Experiments were carried out to determine whether males were the pheromone-producing sex, analogous to other phytophagous stink bug species (Borges et al., 1987; Aldrich et al., 1987, 1994; Borges and Aldrich, 1994; Borges, 1995; McBrien and Millar, 1999; Millar, 2005; Moraes et al., 2005). The attraction of insects of both sexes to odors from males or females was tested, respectively, by offering test individuals a choice between odors from live insects and a clean air control ($N=50$ for each combination). Because observations during rearing indicated peak mating activity occurred at night, bioassays were performed in the evening and scotophase.

Bioassays with Insect Extracts and Synthetic Compounds

The same bioassay procedures described above were used to compare the biological activity of aeration extracts and authentic synthetic standards. The solution of test stimulus was one individual equivalent (IE) of aeration extract spotted on a strip of filter paper (1.5 cm long and 0.5 cm wide). Controls consisted of filter papers treated with hexane. Because females were attracted to male odor, and all other combinations with live insects did not show a positive response, only females were used as responders in all subsequent bioassays. Bioassays ($N=15–31$) with an aeration extract from males or females were carried out using an extract obtained from a 24-hr aeration of 15 bugs.

Based on experimental findings with 0.01, 0.1, and 1 mg/ml, bioassays were conducted with 10 μ l of 0.1 mg/ml, (i.e., 1000 ng of the synthetic compound) loaded on a strip of filter paper (1.5 cm long and 0.5 cm wide). The responses of *T. limbativentris* females to two sets of zingiberenol isomers (zingiberenol I comprising the four stereoisomers of the group (1*RS*, 4*RS*, 1'*R*)-4-(1',5'-dimethylhex-4'-enyl)-1-methylcyclohex-2-en-1-ol ($N=56$) and zingiberenol II comprising the four stereoisomers of the group (1*RS*, 4*RS*, 1'*S*)-4-(1',5'-dimethylhex-4'-enyl)-1-methylcyclohex-2-en-1-ol ($N=71$) were compared to a hexane control. In addition, zingiberenol I was compared against zingiberenol II ($N=41$).

Chemical Analysis

Extracts were analyzed by gas chromatography (GC; HP-6890, HP-1 column, 50 m \times 0.25 mm ID, 0.25 μ m film, J&W Scientific, Folsom, CA, USA), by using a

temperature program of 30°C for 1 min, 5°C/min to 150°C for 0.1 min, 10°C/min to 250°C for 20 min. Samples (1 µl) were injected in cool on-column mode with hydrogen carrier gas, and a flame ionization detector (FID) at 270°C.

Extracts were analyzed by coupled GC–mass spectrometry (GC–MS) by using a Thermo-Finnigan MAT95XP magnetic sector mass spectrometer, which was directly coupled to a TRACE 2000 GC. The GC was equipped with a DB-1 column (50 m × 0.25 mm ID, 0.25 µm film, J&W Scientific) and a cool on-column injector. The carrier gas was helium. Ionization was by electron impact (70 eV, source temperature 200°C). Data were collected and analyzed with Xcalibur software.

The components of aeration extracts from both sexes of *T. limbativentris* were tentatively identified by comparison of GC retention times with those of synthetic standards using polar and nonpolar columns (HP-Wax column, 30 m × 0.3 mm ID, 0.45 µm film or HP-1 column, 50 m × 0.25 mm ID, 0.25 µm film, both from J&W Scientific) with the temperature programs described above. Chemical identifications were confirmed by comparison of retention time and MS data with authentic standards and with MS library data (NIST, Saturn library 2000).

To confirm the presence of an alcohol group in the putative pheromone components, microsilylation was carried out using *N*-methyl-(trimethylsilyl)-trifluoroacetamide (MSTFA) in a 1-ml glass vial. An aliquot of 50 µl was taken from an extract of volatiles of 20 males of *T. limbativentris*, concentrated under a stream of nitrogen flow almost to dryness, and redissolved in 100 µl of MSTFA. The sample was heated in a water bath at 60°C for 1 hr, and analyzed directly by GC–MS using the conditions described above. The same reaction was conducted with a standard solution of zingiberenol (2 µl of 100 mg/ml), prepared as described below.

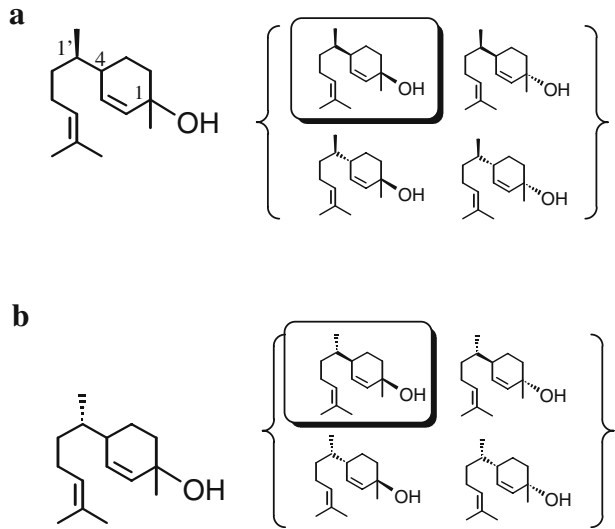
Synthesis of 4-[(1'*R*)-1,5-Dimethylhex-4-enyl]-1-Methylcyclohex-2-en-1-ol

Zingiberenol mixtures were prepared by Fuji Flavor Co., Ltd. (Tokyo, Japan), with the help of Dr. Tatsuji Chuman. The eight stereoisomers of zingiberenol were prepared in two samples, with each of the samples containing four diastereoisomers. The zingiberenol I mixture contained the (1*R*,4*R*,1'*R*), (1*S*,4*R*,1'*R*) (1*R*,4*S*,1'*R*) and (1*S*,4*S*,1'*R*) stereoisomers, whereas Zingiberenol II contained the (1*R*,4*R*,1'*S*), (1*S*,4*R*,1'*S*), (1*R*,4*S*,1'*S*), and (1*S*,4*S*,1'*S*) stereoisomers (Figure 1). The syntheses of zingiberenols I and II were conducted following method II from Hagiwara et al. (2002). In brief, the 4-[(1'*R*)-1,5-dimethylhex-4-enyl]-1-methylcyclohex-2-en-1-ols were synthesized from (*R*)-citronellal. GC–MS *m/z* 222, 207, 204, 189, 179, 161, 151, 137, 123, 119, 109, 95, 77, 69, 55. IR (KBr): 3352, 2927, 2858, 1450, 1377, 1122, 980 cm⁻¹. ¹H-NMR (300 MHz, CDCl₃), δ: 0.81 (d, 1.2 H, *J*=6.9 Hz), 0.84 (d, 0.3 H, *J*=7.5 Hz), 0.86 (d, 1.2 H, *J*=6.9 Hz), 0.88 (d, 0.3 H, *J*=7.5 Hz), 1.10–2.14 (m, 11 H), 1.27 (s, 3 H), 1.60 (s, 3 H), 1.69 (broad s, 3 H), 5.06–5.12 (m, 1 H), 5.50–5.65 (m, 2 H). The compounds 4-[(1'*S*)-1,5-dimethylhex-4-enyl]-1-methylcyclohex-2-en-1-ol were synthesized using the same procedure starting from *S*-citronellal, and gave analogous spectra.

Chemicals

Decanal, nonanal, limonene, 6-methyl-5-hepten-2-one, decane, (*E*)-2-octenal, tridecane, tetradecane, and pentadecane were purchased from Sigma Aldrich (Gillingham, Dorset, UK). α-Copaene, α-pinene, β-pinene, and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were purchased from Fluka (Steinheim, Germany).

Fig. 1 (a) Zingiberenol I, containing the four isomers, (1*RS*,4*RS*,1*R*)-4-(1',5'-dimethylhex-4'-enyl)-1-methylcyclohex-2-en-1-ol. (b) Zingiberenol II, containing the four isomers (1*RS*,4*RS*,1'*S*)-4-(1',5'-dimethylhex-4'-enyl)-1-methylcyclohex-2-en-1-ol



Statistical Analysis

Data on the responses of males and females to different treatments (live insects, aeration extracts, and synthetic compounds vs. control) were analyzed using χ^2 tests, for first choice. Only the insects that left the release chambers of the olfactometer were considered for the analyses. Treatments in which the “nonresponders” (insects that fail to respond in each of the treatments) were >70% were not statistically analyzed.

Results

Determination of Period of Maximum Activity

Bioassays with virgin sexually mature females showed that the attraction to males was strongest during the night period (18:00–24:00 hr). During the morning and afternoon period, >90% of the insects tested did not leave the release chamber of the olfactometer,

Fig. 2 Number of female *T. limbativentris* responding to live males during different periods of the day and night in laboratory bioassays. Morning; 8:00–12:00 hr; afternoon, 12:00–18:00 hr; and night, 18:00–24:00 hr ($\chi^2 = 14.44$, $P < 0.001$, $N = 50$). Left-hand side: Number of insects that failed to respond in each of the treatments. $**P < 0.01$

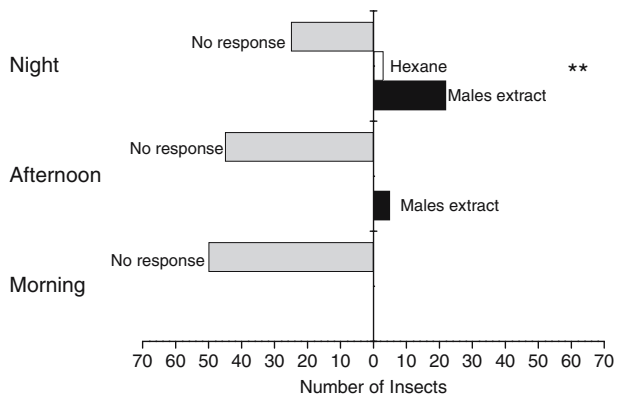
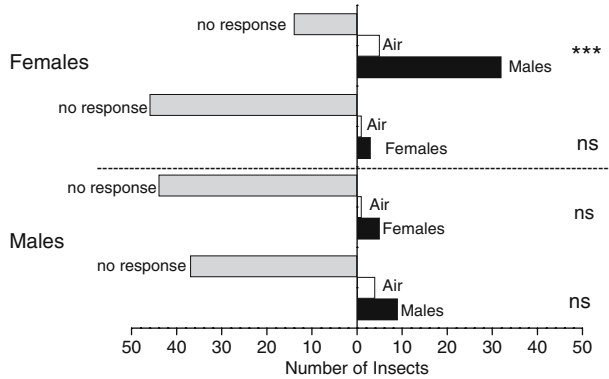


Fig. 3 Response of *T. limbativentris* (first choice) males and females in olfactometer arms responding to different treatments: females attracted by males ($\chi^2 = 19.70$, $P < 0.001$, $N = 50$), females attracted by females, males attracted by females, and males attracted by males, recorded during evening and scotophase under laboratory conditions $***P < 0.001$



indicating that this species has a crepuscular/nocturnal sexual activity cycle (Figure 2). Therefore, all subsequent bioassays were performed during this period.

Bioassays with Live Insects

In the Y-tube olfactometer bioassays, female bugs were attracted to odors of live males, but not to odors of live females (Figure 3). Males were not attracted to odors of either sex (Figure 3). These results, in concordance with the analyses of insect extracts, show that males produce a sex pheromone.

Bioassays with Insect Extracts and Synthesized Compounds

Tibraca limbativentris females were more attracted to male extract (treatment arm) than to the hexane control (Figure 4). Male extract did not attract males, and extracts of females were not attractive to either sex (Figure 4). Following results with live insects and extracts, and chemical analyses, the responses of *T. limbativentris* females to zingiberenol I comprising the four stereoisomers, (1*RS*, 4*RS*, 1'*R*)-4-(1',5'-dimethylhex-4'-enyl)-1-methylcyclohex-2-en-1-ol, and to zingiberenol II comprising the four stereoisomers, (1*RS*, 4*RS*, 1'*S*)-4-(1',5'-dimethylhex-4'-enyl)-1-methylcyclohex-2-en-1-ol, were tested in olfactometer bioassays. First, zingiberenols I and II were tested against male extract and in both bioassays the synthetic compounds were as attractive as extracts from males (Figure 5). When zingiberenols I and II were compared against each other, the two were equally

Fig. 4 Number of *T. limbativentris* (first choice) males and females in olfactometer arms responding to different treatments: females attracted by extracts from males ($\chi^2 = 13.37$, $P < 0.001$, $N = 27$), females attracted by extracts from females, males attracted extracts from males, and males attracted by extracts from females $***P < 0.001$

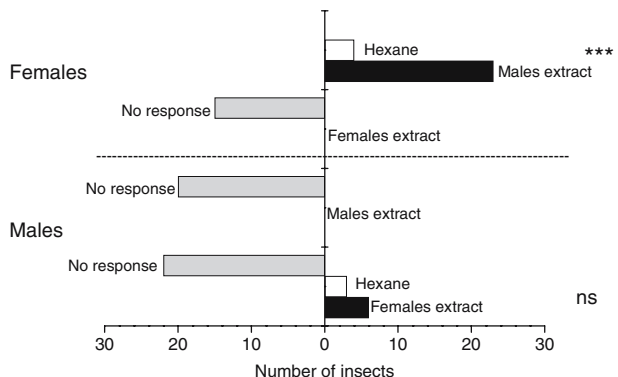
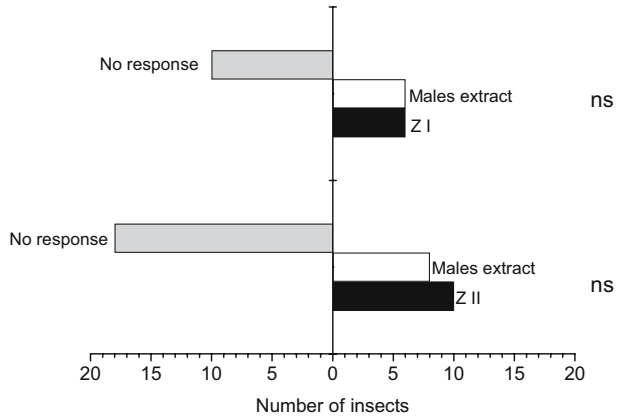


Fig. 5 Number of *T. limbativentris* (first choice) females in olfactometer arms responding to different treatments: females tested to extracts from males (1 IE) and zingiberenol I (Z I; 1000 ng), and females tested to extracts from males (1 IE) and zingiberenol II (Z II; 1000 ng). ns: not significant

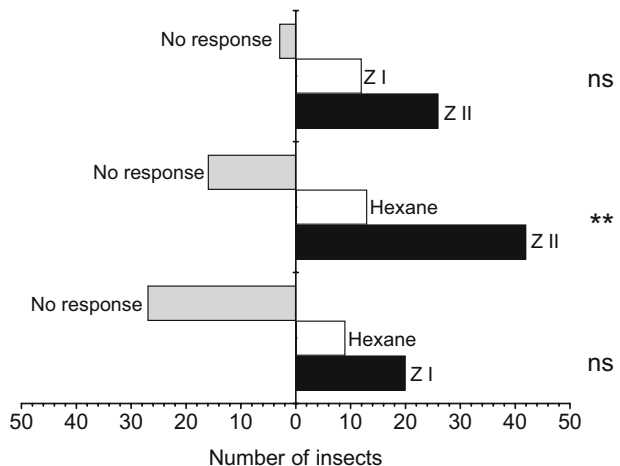


attractive (Figure 6). However, when zingiberenols I and II were compared against neutral controls, only zingiberenol II was attractive (Figure 6). These results suggest that at least one of the stereoisomers in the zingiberenol II blend is a sex pheromone component for this species.

Chemical Analysis

Comparison of aeration extracts collected from both sexes of *T. limbativentris* by GC and coupled GC–MS showed that aeration extracts from males contained at least two male-specific compounds, in addition to several compounds that were common to both sexes (Figure 7). Compounds found in both sexes were identified by comparison with authentic standards as α -pinene (1), β -pinene (2), 6-methyl-5-hepten-2-one (3), decane (4), limonene (5), (*E*)-2-octenal (6), nonanal (7), dodecane (8), decanal (9), tridecane (10), α -copaene (11), tetradecane (12), pentadecane (13), and hexadecane (14), by using both polar and apolar columns. The GC analysis of control extracts obtained from aerations of green beans for 4 d did not show any compound detectable by FID.

Fig. 6 Number of *T. limbativentris* (first choice) females in olfactometer arms responding to two isomer groups of zingiberenol: 1000 ng of the zingiberenol I blend ($\chi^2=4.17$, $P=0.041$, $N=29$) versus hexane as a control and to 1000 ng of zingiberenol II versus hexane as a control ($\chi^2=7.64$, $P<0.01$, $N=55$), and zingiberenol I versus zingiberenol II. ** $P<0.01$



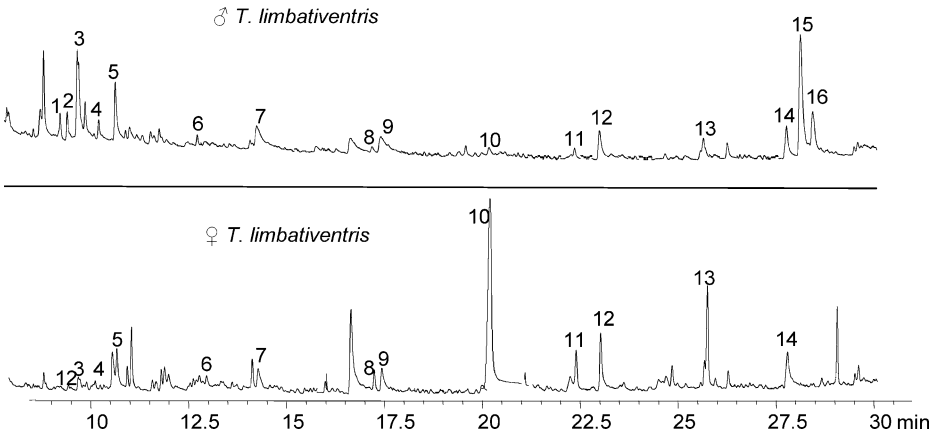


Fig. 7 Gas chromatograms of the volatile chemicals collected from live male (top) and female (bottom) *T. limbativentris*. 1, α -pinene; 2, β -pinene; 3, 6-methyl-5-hepten-2-one; 4, decane; 5, limonene; 6, (*E*)-2-octenal; 7, nonanal; 8, dodecanal; 9, decanal; 10, tridecane; 11, α -copaene; 12, tetradecane; 13, pentadecane; 14, hexadecane; 15 and 16, isomers of (1*RS*,4*RS*,1'*S*)-zingiberenol II

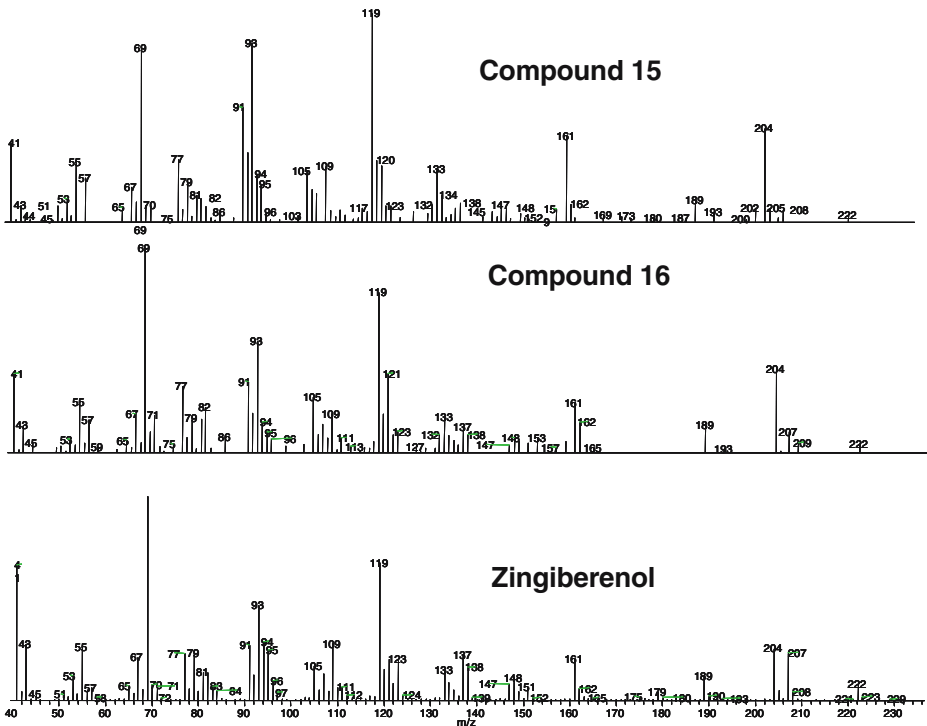


Fig. 8 Mass spectral data of compounds 15 (top) and 16 (center) obtained from volatiles collected from live males, and mass spectra from synthetic zingiberenol (bottom)

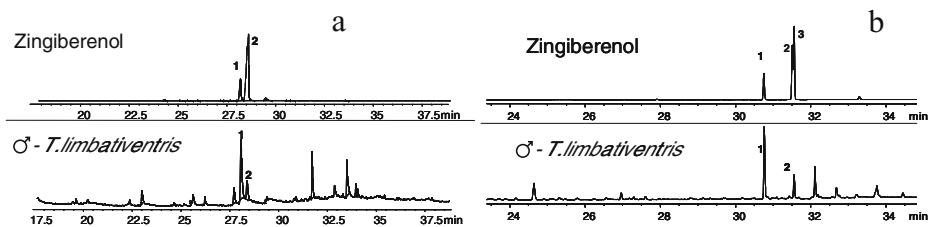


Fig. 9 Gas chromatogram of volatile chemicals collected from live male *T. limbativentris* and of a standard solution of synthetic zingiberenol containing the four diastereoisomers. (a) Polar column, DB-WAX. (b) Nonpolar column, HP-1. Numbers 1 and 2 indicate isomers of zingiberenol. Two diastereoisomers were resolved on the HP-1 column and three diastereoisomers were resolved on the DB-WAX column

The fragmentation patterns for compounds 15 and 16 suggested a sesquiterpene-type structure with an alcohol group, with a possible molecular ion at m/z 222, and an ion at m/z 204 (M-18), arising from loss of water (Figure 8). The NIST library matched the fragmentation pattern of compounds 15 and 16 with the fragmentation pattern of sesquisabinene hydrate or zingiberenol (Figure 8). Comparison of the GC retention times of compounds 15 and 16, by using polar and nonpolar columns, with synthetic zingiberenol isomers (I and II) containing the four diastereoisomers, was carried out (Figure 9a and b). Compounds 15 and 16 coeluted with the two isomers of zingiberenol solution II that were resolved on the HP-1 column. Because zingiberenol has three chiral centers (positions 1', 1, and 4), there are eight isomers (four enantiomeric pairs of diastereomers). Zingiberenols I and II contained four diastereoisomers each and are “enantiomers.” Although diastereoisomers frequently can be separated on an achiral column, the zingiberenol I and II mixtures separated into only two peaks on the HP-1 column. Therefore, zingiberenols I and II were silylated to obtain a better separation of the four diastereoisomers on an achiral column. The derivatization was carried out using *N*-methyl-(trimethylsilyl) trifluoroacetamide (MSFTA) with the male aeration extract and the zingiberenol (I and II) mixtures. The four derivatized diastereoisomers in zingiberenols I and II were indeed separated on the HP-1 column (Figure 10a). For the derivatized aeration extract of male *T. limbativentris*, compounds 15 and 16 disappeared and three new compounds were generated, which

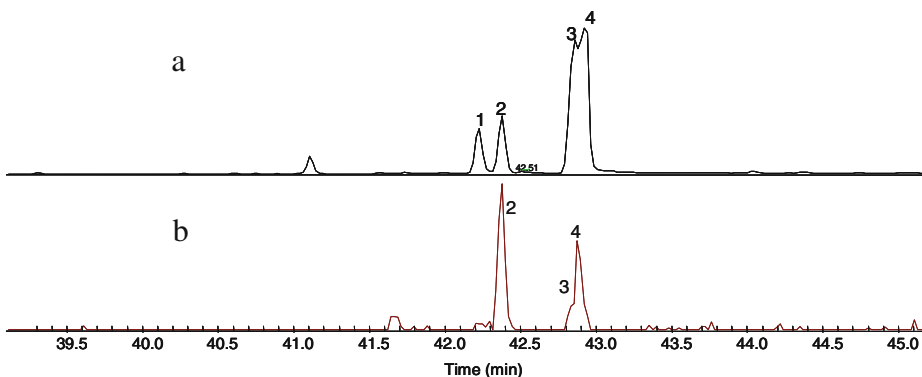


Fig. 10 Gas chromatogram showing four isomers of silylated zingiberenol ($C_{15}H_{28}O-TMS$) (top) and the three peaks obtained from derivatization of the sample obtained from airborne collection of volatiles of male *T. limbativentris* (bottom)

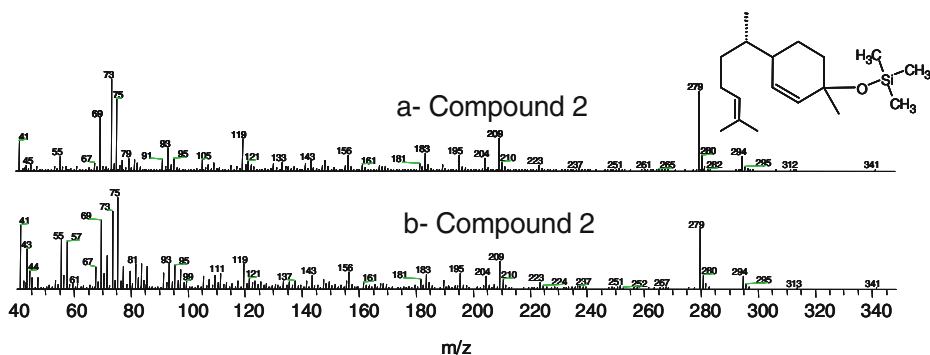


Fig. 11 Mass spectral data showing the pattern of fragmentation. (a) Compound 2: silanized zingiberenol (from Fig. 5a) ($C_{15}H_{28}O-TMS$). (b) Compound 2 (from Fig. 5b), obtained from derivatization of samples obtained from collection of volatiles of male *T. limbativentris*

matched the retention times of three of the four derivatized isomers in zingiberenols I and II (Figure 10b). Coinjection of MSFTA-derivatized zingiberenols I and II confirmed that each solution contained only the four diastereoisomers and all peaks matched each other. GC–MS analysis of the derivatized zingiberenol I and II mixtures and the male *T. limbativentris* aeration extract showed that the four derivatized compounds in zingiberenols I and II closely matched fragmentation patterns to the three derivatized compounds in the male extract. Figure 11 shows the fragmentation pattern of compound 2 of zingiberenol II mixture, identical to compound 2 of the male extract.

Discussion

Some of the compounds identified in the aeration extracts from *T. limbativentris* are common in stink bugs, such as (*E*)-2-octenal, (*E*)-2-decenal, nonanal, tridecane, and dodecane (Borges and Aldrich, 1992; Aldrich et al., 1993, 1995; Ho and Millar, 2001; McBrien et al., 2002; Moraes et al., 2005). In addition, males produced at least three isomers of zingiberenol, one or more of which appear to be sex pheromone components for this species.

In a direct comparison, female *T. limbativentris* were equally attracted by the zingiberenol I and II blends. However, fewer insects responded to zingiberenol I than to zingiberenol II when each blend was tested versus a control. This suggests that one or more of the isomers in zingiberenol II constitute the natural pheromone. Although the stereoisomeric mixture of zingiberenol II was attractive to females in this study, further electrophysiological and behavioral bioassays with individual isomers and specific blends need to be carried out to determine the precise configurations of the male-produced *T. limbativentris* sex pheromone component(s). There are several examples known of stink bug species that use different proportions of stereoisomers to produce a unique signal, such as *Acrosternum hilare* (McBrien et al., 2001) and *Nezara viridula* (Brézot et al., 1994).

When tested during different times of the day, females responded best during the period between 18:00 and 24:00 hr. This is similar to the behavior patterns found for other pentatomid species, such as *Euschistus heros* and *Thyanta perditor*, where the strongest responses were recorded during the afternoon to evening (Borges et al., 1998a,b; Moraes et al., 2005). However, this pattern differs from that found in other pentatomids such as

Nezara viridula (Borges et al., 1987) and *E. obscurus* (Borges and Aldrich, 1994), which show no variation in the level of responsiveness of females to males throughout the day.

In this study, both genders released some compounds that are seldom found or reported from stink bug airborne collections, such as α -pinene, β -pinene, and 6-methyl-5-hepten-2-one. The specific functions of these compounds, as well as the impact of factors such as different rearing conditions and diets upon their production, remain to be studied. The compound 6-methyl-5-hepten-2-one is clearly insect-produced and not from diet, because extracts of the metathoracic glands of both sexes contain this compound. However, some of the other compounds, such as α -pinene, β -pinene, limonene, and α -copaene, were not found in metathoracic gland extracts. Additionally, the green bean control aerations did not show the presence of any of these compounds. Thus, all these compounds appear to be released by *T. limbativentris*.

In summary, this work demonstrated that female *T. limbativentris* were attracted to a pheromone blend released by conspecific males and that females were attracted to a blend of synthetic zingiberenol isomers. The biological role of zingiberenol in the *T. limbativentris* male-specific emissions still needs to be determined under natural conditions. Furthermore, the role and absolute configuration of each of the zingiberenol isomers produced by males remain to be determined.

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Introduction

Hymenoptera are impressive with regard to the diversity of species and levels of social organization. According to their different lifestyles and ecological niches, they possess a huge variety of exocrine glands with diverse functions. In solitary Hymenoptera, these glands contain secretions that serve functions in the context of mate attraction, courtship, prey paralysis, and defense (e.g., Rathmayer, 1962; Wilson, 1972; Quicke, 1997; Gnatzy et al., 2004). Because of the increased requirements for communication and defense of social species, many of these glands have gained various new functions in bees, wasps, and ants (e.g., alarm, trail establishment, recruitment, or reproductive dominance; see Hölldobler and Wilson, 1990; Blum, 1992). One of these “social” glands, the postpharyngeal gland (PPG), has long been thought to be idiosyncratic to ants (Formicidae) and evolved in response to the requirements of the social life (e.g., Hölldobler and Wilson, 1990; Schoeters and Billen, 1997; Lenoir et al., 1999). The PPG of ants is a large cephalic reservoir that is involved in species (Oldham et al., 1999) and nestmate recognition (Soroker et al., 1994; Hefetz et al., 1996), as well as in task discrimination (Kaib et al., 2000). However, several other functions of the PPG such as digestion, caste determination, or a storage organ for nutrients for queens or larvae have been discussed. Not all of these roles have been well corroborated, but it can be assumed that in ants the PPG serves several functions depending on species, age, sex, caste, and mating status (Eelen et al., 2006).

Recently, a PPG was found in females of the European beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae; formerly Sphecidae, Melo, 1999), a solitary hunting wasp (Strohm et al., *in press*). Based on its morphology and the chemistry of its contents, the gland is considered to be homologous to the PPG known in the Formicidae (Strohm et al., *in press*). The function of the PPG in beewolf females is different from that in ants. Beewolf females hunt honeybee workers as prey for their offspring and store the paralyzed bees as provisions for the larvae in brood cells in the soil (Strohm, 1995; Strohm and Linsenmair, 1999). Females apparently apply the secretion of their PPG to the surface of their honeybee prey and this delays fungal infestation in the warm and humid underground nests (Strohm and Linsenmair, 2001; Herzner et al., unpublished data).

Unexpectedly, *P. triangulum* males also possess PPGs that are even larger than those in females (Herzner et al., *in press*). Beewolf males establish and scent-mark territories with a marking pheromone that attracts conspecific females for mating (Simon Thomas and Poorter, 1972; Evans and O’Neill, 1988; Strohm, 1995; Strohm and Lechner, 2000; Schmitt et al., 2003). The marking pheromone of *P. triangulum* has been characterized for three different populations (Borg-Karlson and Tengö, 1980; Schmidt et al., 1990; Schmitt et al., 2003) and was thought to be produced and stored in the mandibular gland (Gwynne, 1978; Schmidt et al., 1985; Evans and O’Neill, 1988; Schmidt et al., 1990). However, because the mandibular gland does not have a large reservoir, we hypothesized that the marking pheromone might be stored in the large PPG. Therefore, we took direct samples from dissected PPG reservoirs and analyzed these by combined gas chromatography-mass spectrometry (GC-MS). We compared our findings with a previous description of the pheromone in the same population (Schmitt et al., 2003).

The PPG content of female beewolves shows a marked dimorphism. The main component of the hydrocarbon mixture is either (*Z*)-9-pentacosene or (*Z*)-9-heptacosene (Strohm et al., unpublished). Because these compounds also occur in the male marking pheromone and such a variation in the male pheromone might have important implications for mate choice (Herzner et al., 2006), we looked for such a dimorphism in males. Finally,

we examined whether total-head extracts are sufficiently similar to samples directly taken from the PPG to allow for analyses of the pheromone by extracting heads instead of dissecting individual glands.

Methods and Materials

Specimens

Adult male beeswolves were obtained from a laboratory population (see, e.g., Strohm and Linsenmair, 1997). Freshly eclosed males were individually marked and kept as previously described (Herzner et al., 2006). Because age has been shown to influence pheromone composition (Kaltenpoth and Strohm, 2006), all males used for the analyses were the same age. Twelve to 14 d after emergence, males were caught and stored individually in small polystyrene vials (35 mm diam, 82 mm length, filled with 2 cm of moist sand) with rubber foam plugs for 2 d, so that they could replenish the reservoirs of their marking pheromone. They were then anesthetized with CO₂ and individually frozen at -18°C until chemical analyses were conducted.

Extracts

For the analyses, males were thawed and decapitated, and the heads were dissected under a stereo microscope. The PPG was carefully uncovered by removing the frontal part of the head capsule by cutting the cuticle between the eyes, toruli, and lateral ocelli very shallowly with a razor blade. If the reservoir is not damaged, it bulges out. A sample of the PPG content was taken by inserting the tip of a fine glass pipette directly into the gland reservoir. The sample was then dissolved in redistilled hexane.

For the complete inventory and characterization of the contents of the PPG, samples of three males were combined and reduced in volume to approximately 100 µl via a stream of nitrogen at ambient temperature. An aliquot of 1 µl was analyzed by combined GC-MS (setup 1, manual injection). To check for any nonpolar substances hidden under the peaks of polar pheromone compounds, the extract was fractionated on a conditioned SiOH glass column (Chromabond, 500 mg; Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions, and 1 µl of both the hexane and the dichloromethane fraction were analyzed by combined GC-MS (setup 1, manual injection).

Some components that were detected and characterized in the pooled samples could not be satisfactorily identified in samples of individual males because of the smaller amounts of secretion. Therefore, the number of peaks included in the respective analyses varies somewhat.

To compare samples directly taken from the PPG with those obtained from total head extracts, 15 males were dissected as described above. After a sample of the PPG content had been taken, the remaining head capsule still containing the PPG and most of its contents was extracted in redistilled hexane for 3 hr. The samples were reduced in volume to approximately 100 µl, and 1 µl of each solution was analyzed by GC-MS (setup 1, automatic injection).

To investigate possible dimorphism in the composition of the PPG content, we analyzed a large sample of 45 males in order to be able to detect a low frequency of one morph. Males were obtained and treated as described above. For this second analysis, we extracted whole heads that were incised to open the glands. The heads were individually extracted in

redistilled hexane for 3 hr, the sample volume was reduced to approximately 100 μl , and 1 μl of each sample was analyzed by GC-MS (setup 2, automatic injection).

Capillary Gas Chromatography-Mass Spectrometry. Setup 1

GC-MS analysis was performed with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector. The GC was equipped with an RH-5ms+ column (30 m \times 0.25 mm ID; $df=0.25\ \mu\text{m}$), and the temperature program ramped from 60°C for 1 min to 300°C at 5°C/min, hold 10 min. Helium was used as carrier gas (constant flow 1 ml/min). A split/splitless injector was used (250°C) with the purge valve opened after 1 min. Electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV, a source temperature of 230°C, and an interface temperature of 315°C. MSD ChemStation Software (Agilent Technologies, Palo Alto, CA, USA) for Windows was used for data acquisition.

Capillary Gas Chromatography-Mass Spectrometry. Setup 2

GC-MS analysis was performed with a Fisons Instruments (Fisons, Egelsbach, Germany) GC 8000 Series coupled to a Fisons Instruments MD800 quadrupole mass detector. The GC was equipped with a DB-5 column (30 m \times 0.25 mm ID; $df=0.25\ \mu\text{m}$; J&W, Folsom, CA, USA), and the temperature program ramped from 60°C to 310°C with 5°C/min, hold 10 min. Helium was used as carrier gas (constant pressure, 90 mbar). A split/splitless injector (240°C) was used with purge valve opened after 60 sec. Electron impact mass spectra were recorded with an ionization voltage of 70 eV, a source temperature of 220°C and an interface temperature of 315°C. Xcalibur software (ThermoFinnigan, Egelsbach, Germany) for Windows was used for data acquisition.

Chemicals and Pheromone Compound Identification

Solvents (Fluka, Deisendorf, Germany) were distilled and checked for purity by GC-MS prior to use. (*Z*)-9-Octadecen-1-ol and 1-eicosanol were identified by comparing retention times and mass spectra of the PPG extracts with those of synthetic standards [(*Z*)-9-octadecen-1-ol: Merck Schuchard OHG, Hohenbrunn, Germany; 1-eicosanol: Dr. Ehrens-dorfer GmbH, Augsburg, Germany]. *n*-Alkanes were identified by comparing retention times and mass spectra of beewolf gland extracts with data from earlier analyses (Schmitt et al., 2003; Strohm et al., *in press*) and with data from a commercial MS library (NIST, Gaithersburg, MD, USA). The corresponding alkenes were identified by their typical mass spectra and retention times. The positions and geometries of the double bonds were inferred by comparison with earlier analyses on the chemistry of the pheromone (Schmitt et al., 2003) and of the PPG content or cuticular hydrocarbons of conspecific females that had been characterized by Fourier transform infrared (FTIR) spectrometry and dimethyl disulfide (DMDS) derivatization (Strohm et al., *in press*); peaks with identical retention times were considered to be the same isomers. Some alkenes were present only in threshold amounts in all our earlier and the current analyses and could thus not be characterized completely. The alkadienes were characterized by their typical mass spectra and their retention times. Due to their very small amounts, the position and geometry of the double bonds could not be determined. Methyl alkanes were identified by diagnostic ions, standard MS databases (see above), and by determining Kovats indices by the method of Carlson et

al. (1998). The ketone Δ -16-pentacosen-8-one was identified by comparing the retention time and mass spectrum of the peak with Δ -16-pentacosen-8-one present in the female PPG (Strohm et al., *in press*).

Statistics. Head Extracts vs. PPG Content

Peak areas were obtained by manual integration (MSD ChemStation) and relative peak areas were transformed to logcontrasts (Aitchison, 1986; Reyment, 1989) before analysis. Mean values of individual peaks were normalized by log transformation. To test for a chemical congruency between the substances found in the PPGs and in the head extracts, we conducted a regression analysis between the proportions of components (Aitchison and log transformed) in the samples directly obtained from the PPG and in the head extracts [reduced major axis regression (RMA); Legendre and Legendre, 1998] using RMA Software for Reduced Major Axis Regression v.1.17 (A. J. Bohonak, San Diego University, USA; freely available at <http://bio.sdsu.edu/pub/andy/RMA.html>). To assess the chemical similarity between the PPG samples and the head extracts, we tested whether there was a direct proportionality, i.e., the slope of the resulting regression line should not deviate significantly from 1 and the y -intercept should not deviate significantly from 0.

Statistics. Chemical Dimorphism

Peak areas were obtained by automatic integration (Xcalibur). The relative peak areas were transformed to logcontrasts (Aitchison, 1986; Reyment, 1989). Since in females the dimorphism is most striking for the compounds (*Z*)-9-pentacosene and (*Z*)-9-heptacosene (Strohm et al., *in press*), we first focused on these peaks. The different isomers of the unsaturated hydrocarbons were not well separated in the chromatograms (see below). Therefore, in the following “pentacosene” and “heptacosene” refer to the mixture of isomers. However, in all cases the (*Z*)-9 isomers were dominant.

Inspection of the chromatograms revealed that based on the proportions of pentacosene and heptacosene in their pheromone blends, males could be classified into two distinct types: those with pentacosene as the dominant hydrocarbon (C_{25} -type males) and those with comparable proportions of pentacosene and heptacosene (C_{25}/C_{27} -type males). To test for a bimodal distribution, we analyzed the frequency distribution of the proportion of heptacosene via a histogram plot (see results). The histogram revealed that the males could be assigned to two groups according to their proportion of heptacosene. We compared the proportions (relative peak areas, Aitchison transformed) of all substances between these two groups with exact tests for two independent samples (using SPSS 13.0 for Windows, SPSS Inc., Chicago, IL, USA).

Results

PPG Content

The substances found in the samples taken directly from the PPGs of three males are shown in Table 1 (the assignment of numbers to the different compounds in Table 1 is consistent throughout the manuscript). Apart from the previously described 11 substances (Schmitt et al., 2003), we characterize another 42 compounds, 33 of which were hydrocarbons and nine of which contained functional groups. Two new peaks were not identified.

Table 1 Compounds detected in the postpharyngeal gland of male *P. triangulum* arranged in the order of elution on our gc-ms setup 1

Compound	Compound Name
1	Hexanoic acid
2	Nonanal
3	Octanoic acid
4	Nonanoic acid
5	Pentadecane
6	(<i>S</i>)-2,3-Dihydrofarnesoic acid
7	(<i>Z</i>)-9-Octadecen-1-ol
8	(<i>Z</i>)-10-Nonadecen-2-one
9	1-Octadecanol
10	Heneicosane
11	Unidentified substance 1
12	Docosane
13	(<i>Z</i>)-11-Eicosen-1-ol
14	$\Delta_{x,y}$ -Tricosadiene
15	(<i>Z</i>)-9-Tricosene
16	(<i>Z</i>)-7-Tricosene
17	1-Eicosanol
18	Δ_x -Tricosene
19	Tricosane
20	5-Methyl tricosane
21	7-Methyl tricosane
22	9-Methyl tricosane
23	$\Delta_{x,y}$ -Tetracosadiene
24	(<i>Z</i>)-9-Tetracosene
25	(<i>Z</i>)-7-Tetracosene
26	Tetracosane
27	Unidentified substance 2
28	$\Delta_{x,y}$ -Pentacosadiene
29	(<i>Z</i>)-9-Pentacosene
30	(<i>Z</i>)-7-Pentacosene
31	Δ_x -Pentacosene
32	Δ_x -Docosenol
33	Pentacosane
34	13-Methyl pentacosane
35	11-Methyl pentacosane
36	7-Methyl pentacosane
37	(<i>Z</i>)-9-Hexacosene
38	Hexacosane
39	Δ -16-Pentacosen-8-one
40	$\Delta_{x,y}$ -Heptacosadiene
41	(<i>Z</i>)-9-Heptacosene
42	(<i>Z</i>)-7-Heptacosene
43	Heptacosane
44	13-Methyl heptacosane
45	11-Methyl heptacosane
46	Octacosane
47	Δ -18-Heptacosen-10-one
48	(<i>Z</i>)-9-Nonacosene
49	Nonacosane

Table 1 (continued)

Compound	Compound Name
50	15-Methyl nonacosane
51	13-Methyl nonacosane
52	Triacontane
53	(Z)-9-Hentriacontene
54	(Z)-7-Hentriacontene
55	Hentriacontane

Head Extracts vs. PPG Content

We detected 30 compounds in the samples directly taken from the PPG and in the head extracts of the 15 individual males. Several substances had to be combined for the analysis, because peaks were not always well separated (Fig. 1). A total of 20 peaks (Aitchison and log transformed) were subjected to the correlation and regression analyses. All substances found in the PPG samples were also present in the total-head extracts and *vice versa*. Furthermore, the relative amounts of substances in the heads showed a strong linear correlation with the corresponding substances in the PPG that did not deviate significantly from direct proportionality (Fig. 1) [slope of the RMA regression line: 0.960 (95% confidence intervals, 0.797–1.123), *y*-intercept -0.1247 (95% confidence intervals, -0.2391 to -0.0104)]. The only substance deviating conspicuously from the regression line was (*S*)-2,3-dihydrofarnesoic acid, which was always more abundant in the head extracts.

Chemical Dimorphism

Twenty-three peaks were reliably identified in all 45 samples and were included in this analysis. As noted above, several peaks had to be combined for the analysis (Fig. 4). The

Fig. 1 Correlation between the relative amount (relative peak area Aitchison- and logtransformed, see text) of a particular component in the postpharyngeal gland (PPG) and in head extracts of males of the European beewolf ($r^2=0.88$, $N=20$ compounds, $P<0.001$). The regression line was generated by using reduced major axis regression ($y = 0.9600x - 0.1247$). The data are based on extracts of 15 males. The numbers correspond to the numbers in the peak list (Table 1)

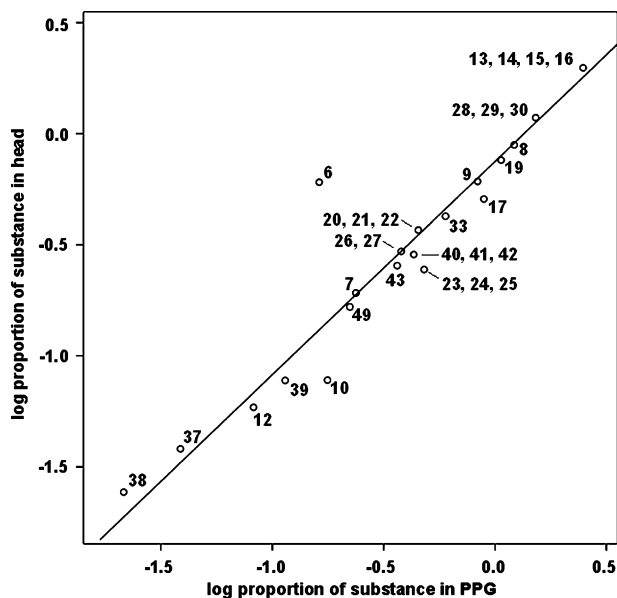
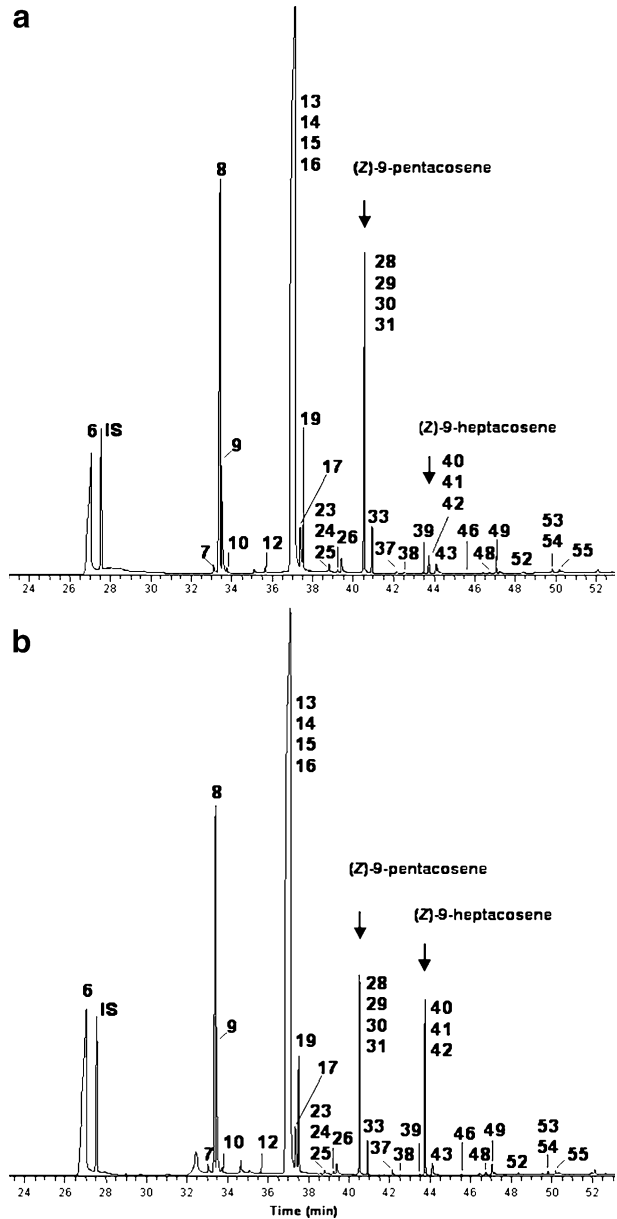
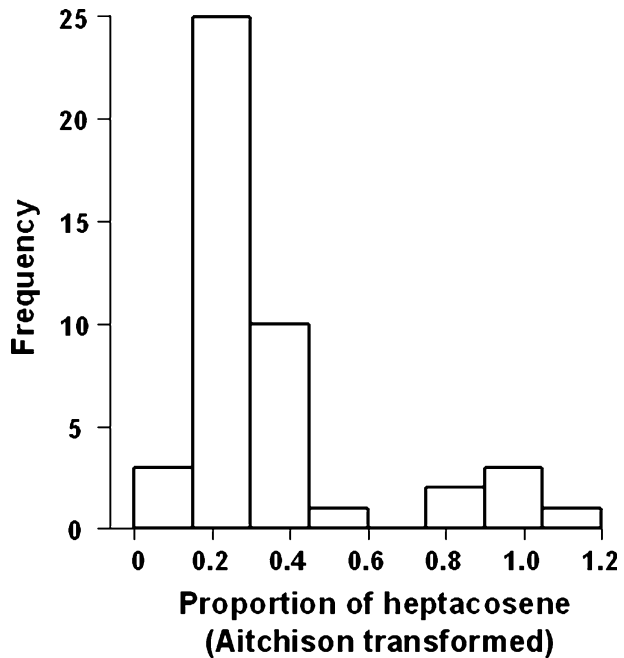


Fig. 2 Total ion chromatograms of the head extracts of an individual male with pentacosene as the predominant hydrocarbon peak (C_{25} -type, a) and an individual male with similar proportions of pentacosene and heptacosene (C_{25}/C_{27} -type, b). The numbers correspond to the numbers in the peak list (Table 1). IS: internal standard (octadecane)



chromatograms and the histogram revealed that among the 45 males analyzed, 39 individuals (87%) belonged to the C_{25} -type with pentacosene as the predominant hydrocarbon, whereas six individuals belonged to the C_{25}/C_{27} -type with pentacosene and heptacosene in approximately equal proportions (Figs. 2 and 3). Besides the differences in the proportions of pentacosene and heptacosene, the two types significantly differed in five other peaks (Fig. 4).

Fig. 3 Frequency distribution of the proportion of heptacosene (mixture of isomers, see text) in the head extracts of 45 males. The males can clearly be separated into two groups with no overlap



Discussion

Fifty-five compounds were identified from samples directly taken from the PPG of male *P. triangulum*, including 11 compounds previously described as components of the marking pheromone of *P. triangulum* males (Schmidt et al., 1990, 2003). These results, together with our observation that (as described for other *Philanthus* species; Schmidt et al., 1985) the compounds isolated from heads or PPGs of male *P. triangulum* can also be found on freshly marked territories of beewolf males (Herzner et al., unpublished data), support our hypothesis that the PPG of male European beewolves functions as the marking pheromone reservoir.

The 44 substances that had not been described by Schmitt et al. (2003) were included here for several reasons. In the earlier study, most of the minor hydrocarbons were omitted because they were also found in cuticle extracts and we concluded that they might not be part of the marking pheromone (see also Schmidt et al., 1990). Here, we show that the hydrocarbons are present in the PPG and therefore, we consider them to be part of the marking secretion. Most importantly, for the current study we pooled the gland extracts of three large males, concentrated the resulting sample and injected it manually, permitting the detection of trace components in the extracts.

One of the compounds that is new to the cephalic secretion of male European beewolves is (*Z*)-9-octadecen-1-ol. It had been previously isolated from glands and cuticle of several apid species (see, e.g., Andersen et al., 1988; Bergström, 1985) and from jojoba (*Simmondsia chinensis*) seeds (Tobares et al., 2003). 1-Eicosanol has been described as a component of the marking secretion of males of a cuckoo bumblebee (Kullenberg et al., 1970) and is present in the female sex pheromone gland of the European grapevine moth *Lobesia botrana* (Arn et al., 1988). It can also be found on cocoons and on the cuticles of different developmental stages of the honeybee *Apis mellifera* (Donzé et al., 1998). The

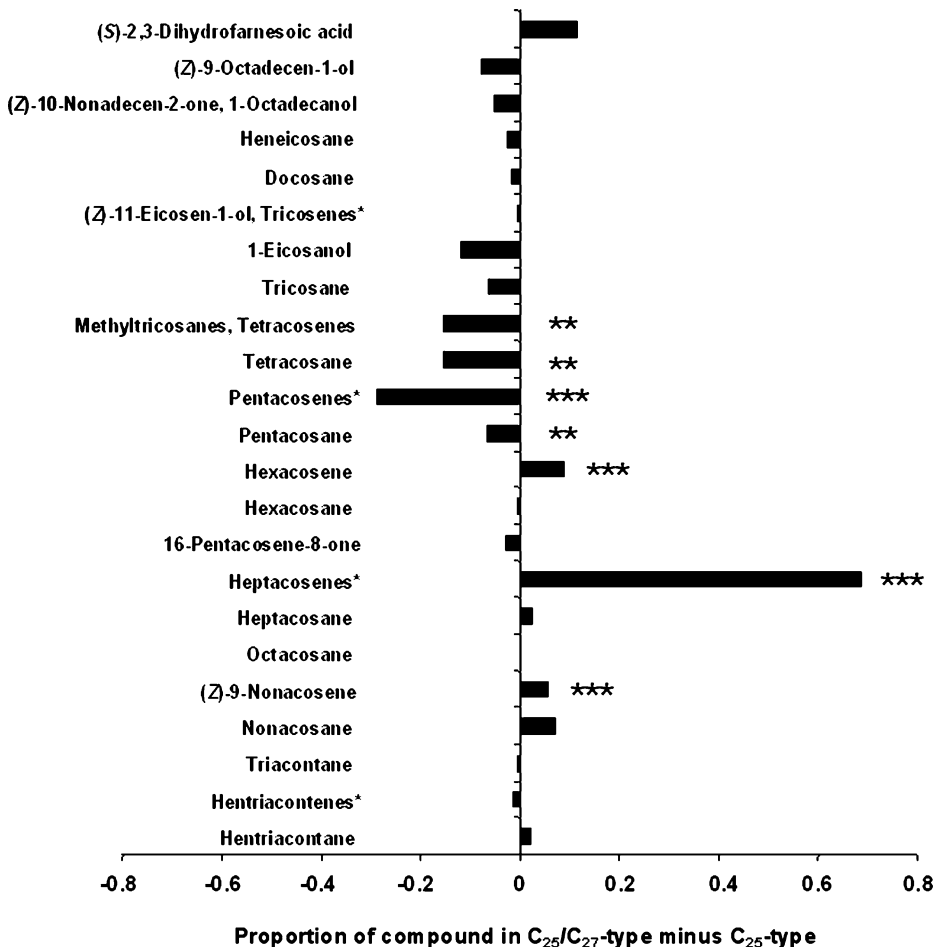


Fig. 4 Differences between the C₂₅-type and the C₂₅/C₂₇-type in the mean proportions of individual components of head extracts (Aitchison-transformed, $N=45$ males). Bars to the right of the line (positive values) indicate that the proportion of the given substance is higher in the C₂₅/C₂₇-type; bars to the left of the line (negative values) indicate that the proportion of the substance is higher in the C₂₅-type. Asterisks indicate significant differences (exact tests for independent samples; *** $P<0.0001$; ** $P<0.001$)

ketone Δ -16-pentacosen-8-one is also present in the PPG of *P. triangulum* females (Strohm et al., *in press*). Δ -18-Pentacosen-10-one has been found to be a contact pheromone component of the white-spotted longicorn beetle *Anoplophora malasiaca* (Yasui et al., 2003).

Based on descriptions in the literature (Gwynne, 1978; Borg-Karlson and Tengö, 1980; Schmidt et al., 1985, 1990; Evans and O'Neill, 1988; McDaniel et al., 1992), we had initially assumed that the marking pheromone in *P. triangulum* was secreted from the mandibular glands. Owing to its delicate structure (Herzner et al., *in press*), the PPG of male beeswolves is destroyed when dissecting heads to remove and extract the mandibular glands. When the PPG is damaged, its contents spill out and disperse in the head capsule contaminating the mandibular gland with the PPG contents. This was possibly the case in

previous studies on beewolf male pheromones (Borg-Karlson and Tengö, 1980; Schmidt et al., 1985, 1990, 2003).

When taking extreme care during dissection, it is possible to remove the mandibular glands without damaging the PPG. When we extracted mandibular glands that were not contaminated with the contents of the PPG, we detect only minor amounts of some of the PPG gland components (Herzner and Strohm, unpublished data). Therefore, in total head extracts, the vast majority of the compounds detected seem to originate from the PPG and not from the mandibular glands.

In our analyses, all components of the PPG were also present in total head extracts and *vice versa*. Furthermore, the proportions of all compounds were similar between the two types of extracts. The only component that showed a marked discrepancy in its relative abundance between the PPG and the head extracts was (*S*)-2,3-dihydrofarnesoic acid, which was always more abundant in the head extracts. The reason for this difference is not yet clear. Owing to the extraordinary size of the PPG (Herzner et al., *in press*), its contents dominate head extracts, and the contribution from other tissues or the cuticle to the extract is minimal. The high congruence between direct samples of the PPG and total head extracts of male European beewolves shows that the latter yield valid samples of the marking secretion. Therefore, it seems justified and far more practical to use total-head extracts for studies on the male beewolf marking pheromone. In particular, when doing quantitative analyses, the risk of losing some secretion due to improper dissection can be avoided.

The PPG has long been considered idiosyncratic to ants. In ant workers, it typically contains a blend of straight and methyl-branched hydrocarbons (Soroker et al., 1995; Cabrera et al., 2004; Lucas et al., 2004) that mainly play a role in nestmate recognition (Crozier and Dix, 1979; Soroker et al., 1998; Vienne et al., 1995; Lenoir et al., 1999). Female *P. triangulum* also possess large PPGs (Strohm et al., *in press*) that contain a blend of mainly straight-chain unsaturated and saturated hydrocarbons, as well as minor proportions of methyl-branched long-chain hydrocarbons and long-chain unsaturated ketones (Strohm et al., *in press*). Many of the hydrocarbons, as well as the ketones Δ -16-pentacosen-8-one and Δ -18-heptacosen-10-one, that were present in the male PPG are also found in the female PPG. The female PPG is morphologically different from the male gland and contains, besides the ketones, no substances with functional groups. The PPG secretion of female beewolves is used for prey preservation (Strohm and Linsenmair, 2001) and has a completely different function from the PPG content of beewolf males.

Thus, in beewolf females the PPG may be subject to natural selection to increase the protection against fungal infestation of their honeybee prey and larvae (Strohm and Linsenmair, 2001). In contrast, the PPG of males is subject to strong sexual selection (Herzner, 2004; Herzner et al., 2005, 2006; Kaltenpoth and Strohm, 2006). Such a change in function and sexual dimorphism regarding glands and their contents is not unusual (e.g., the methathoracic and dorsal abdominal glands in some Heteroptera (Aldrich, 1988; Aldrich et al., 1996; Ho et al., 2003; Zhang and Aldrich, 2003)). The significance of this sexual dimorphism remains mostly unclear, however. Even in ants, the morphology and function of the PPG seem to exhibit inter- and intraspecific variability (Schoeters and Billen, 1997; Eelen et al., 2006).

In addition to the morphological and chemical differences in PPG between the sexes, there is a dimorphism with regard to the composition of the gland content within each sex of *P. triangulum*. The majority of males (87%) had pentacosene as the dominant hydrocarbon (C_{25} -type). A minority of males (13%), however, possessed almost equal proportions of pentacosene and heptacosene (C_{25}/C_{27} -type) with a bimodal and non-

overlapping distribution of the two types with regard to the proportion of heptacosene. The chemical dimorphism in the PPG content of beewolf females is much more pronounced than in males. In females, the main compound, accounting for 70–80% of the blend, is either (*Z*)-9-pentacosene or (*Z*)-9-heptacosene, and there are no intermediate types with similar amounts of both compounds such as the C_{25}/C_{27} -type in males (Strohm et al., *in press*). The proportions of the pentacosene-morph and the heptacosene-morph in females are 80% and 20%, and similar to the proportions of the C_{25} -type and the C_{25}/C_{27} -type in males (87% vs. 13%). The meaning of this chemical dimorphism is not yet clear. The dimorphism is only exhibited by hydrocarbons without functional groups. Moreover, if the dimorphism had a genetic basis, recognition of relatives—in particular with regard to the avoidance of inbreeding (Herzner et al., 2006)—would be facilitated.

Our findings might have important implications for the evolution of the PPG within the Hymenoptera. If the PPG existed only in ants, it would be tempting to assume that it had evolved in response to the requirements of the social communication system (Hölldobler and Wilson, 1990; Lenoir et al., 1999; Eelen et al., 2006). The existence of a PPG in beewolf females suggested that this gland might have evolved in some early Aculeates for the purpose of prey preservation (Strohm and Linsenmair, 2001; Strohm et al., *in press*). We now know that the PPG in male beewolves is a marking pheromone reservoir. This beewolf marking pheromone probably represents the male sex pheromone, because females are attracted to males territories by the windborne pheromone, and most matings occur in the males' territories or in the nearby vegetation (Simon Thomas and Poorter, 1972; Alcock, 1975; Gwynne, 1978; O'Neill, 1979, 1983; Evans and O'Neill, 1988; Strohm, 1995; Strohm and Lechner, 2000; Schmitt et al., 2003; Herzner et al. 2005, 2006). Therefore, the pheromone should be subject to sexual selection with regard to both its composition and amount (Herzner, 2004, 2005, *in press*, 2006; Kaltenpoth and Strohm, 2006). Because sexual selection can be a strong evolutionary force (Kaneshiro and Boake, 1987; Lande, 1981; Higashi et al., 1999), the evolution of the gland might have been influenced by the requirements of males to store large amounts of pheromones to attract females. Regardless of the evolutionary origin of the PPG, our findings show that this gland, which was assumed to have a "social" function in ants, serves an entirely different function as a reservoir for a male sex pheromone in a solitary wasp.

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then sexuals at the end of the summer. In Central Europe, the first drones usually start emerging at the end of August, and gynes from the beginning of September. After several days, the sexuals leave the nest individually without orientation flights (Matsuura and Yamane, 1990) at which time most gynes mate only once, with double or triple mating occurring rarely (Foster et al., 1999; Takahashi et al., 2003, 2004). Details about the rendezvous sites of *Vespa* drones and gynes, as well as the male seeking strategy, are vague. Batra (1980) reported anecdotal observations of males entering nests or flying at specific places near the nests. She observed several matings on the nest envelope, and suggested that mating occurs mainly inside the nesting cavity, with colony feces acting as male attractant. In contrast, Matsuura and Yamane (1990) reported matings at the nest entrance only for *V. mandarinia*, and suggested that mating in other *Vespa* species occurs at locations other than the nest.

Chemical communication is widespread among social wasps (Landolt et al., 1998) and in *Vespa* species alarm (Veith et al., 1984; Ono et al., 2003), marking (Ono et al., 1995), and queen (Ikan et al., 1969) pheromones are known. Furthermore, cuticular lipids are involved in nestmate recognition (Ruther et al., 1998, 2002). The use of pheromones in male attraction is common in social Hymenoptera (reviewed by Ayasse et al., 2001; Keeling et al., 2004), and although there is evidence for sex pheromones in some species of the Vespidae, not a single one has been identified (Ayasse et al., 2001). Ono and Sasaki (1987) demonstrated the presence of copulation releasing pheromones in six *Vespa* species, including *V. crabro*. Cuticular extracts applied to dead drones elicited mating attempts by conspecific males, suggesting that cuticular lipids are involved. Males also attempted to copulate with immobilized gynes of other species, indicating that the chemical signals are not species-specific. However, the study was conducted in the laboratory with large groups of walking drones, and it is unknown whether extracts from gynes attract flying males over longer distances under natural conditions.

The aim of the present study was to obtain a more detailed description of the male seeking strategy in *V. crabro* in their natural habitat and to investigate whether sex pheromones or fecal volatiles are involved in mate finding. We also compared the composition of the cuticular lipids from gynes, workers, and drones.

Methods and Materials

Experimental Sites Field experiments were carried out at three different garden sites (A, B, and C with 1, 3, and 3 nests, respectively) in Berlin, in September and October 2002 and 2003. The nest at site A was a naturally founded one, whereas those at B and C had been relocated from their natural nest sites at the beginning of the colony cycle and established at their new sites for at least 6 wk prior to the emerging of drones and gynes. General observations on the male mate seeking tactics were carried out by observing the habitat around the nests on eight consecutive days, at the end of September and the beginning of October 2002 for sites B and A, respectively. The maximum number of drones seen simultaneously at any spot was determined once a day for 1 hr starting at 8.30 A.M. Additionally, the presence of patrolling drones was verified several times a day until dusk. Observations were never carried out when other experiments were taking place.

Response to Caged Gynes We investigated the response of patrolling drones to living gynes held in wooden cages (5 × 6 × 1.5 cm), with each end covered in metal screen (1.5 mm

mesh width). This arrangement allowed for the release of volatile cues but removed any visual cues. Pairs of cages (one containing a gyne and an empty control, $N=7$) were hung in bushes, 1.5–2 m above the ground, at patrol sites. Each trial lasted for 8 min, with the position of the cages being switched after 4 min. The number of drones landing on the cages was counted, and repeated landings by a drone in the same behavioral sequence were counted as one single event.

Response to Gyne and Worker Extracts Gynes and workers ($N=8$ for each caste) were frozen, washed for 1 min in 1.5 ml dichloromethane, and the extracts were stored at -18°C until used. For the bioassays, the extract of each gyne was slowly applied to the body of a worker, which had already been extracted and thus cleaned of its own cuticular lipids, using an Eppendorf pipette. The solvent was allowed to evaporate for at least 10 min. Each pair ($N=8$), consisting of one treated and one untreated worker, was hung about 20 cm apart on the outer leaves of a sun lit bush at the rendezvous sites in the two garden colonies (sites A and C). The number of drone landings, copulation attempts (protruded genitalia), and actual copulations were counted for 20 min, with the positions of treated and untreated workers being exchanged after 10 min. In a control experiment, 5 pairs of workers were tested, with one being treated with worker extract to exclude the possibility that the response of the drones was due to the extraction and reapplication procedure *per se*.

Response to Fecal Volatiles As hornets defecate under their nests, the feces may serve as an orientation cue for drones seeking gynes still inside the nests. Before placing nests inside the nesting boxes, their bottoms were covered with untreated wood chip pet litter to absorb the feces. The litter was removed from one colony after 2 mo and a 2-cm-deep layer was spread in the bottom of three open wooden boxes ($22 \times 12 \times 12$ cm). The boxes were placed at sites with drones for 1 hr on 2 d, and the number of drones inspecting the boxes was recorded.

Chemical Analyses Aliquots (1 μl) of gyne ($N=4$), worker ($N=5$), and drone ($N=6$) extracts were analyzed by coupled gas chromatography-mass spectrometry (GC-MS). Analytical separations were performed on a Fisons 8060 GC, mass spectra were obtained on a MD800 quadrupole mass spectrometer (Thermo Finnigan, Egelsbach, Germany). We used a $30 \text{ m} \times 0.32 \text{ mm}$ ID DB-5ms fused silica column, film thickness 0.25 μm (J&W Scientific), with helium (inlet pressure 10 kPa) as the carrier gas. The temperature started at 150°C , increased at $2^{\circ}\text{C}/\text{min}$ up to 300°C . Identification of the straight-chain hydrocarbons was done by comparisons of mass spectra and retention times with those of authentic reference compounds. Branched hydrocarbons were identified by diagnostic ions resulting from the favored fragmentation at the branching points (Lockey, 1988; Nelson, 1993) and by comparing retention indices with compiled data for nonpolar columns (Carlson et al., 1998). The position of the double bonds of unsaturated hydrocarbons was determined by iodine-catalyzed methylthiolation using dimethyl disulfide (Francis and Velant, 1981; Howard, 1993). Peak areas for each compound were calculated, and related to the total peak area for each run. In cases where more than one compound coeluted, detected by a scan-by-scan analysis of diagnostic ion chromatogram, only one area was calculated.

Statistical Analyses The maximum numbers of drones observed simultaneously at sites A and C were compared by using a *t*-test. The differences in the number of drones landing near and attempting to copulate with treated and untreated workers, as well as the number

of landings on caged gynes and control cages, were analyzed by using the Wilcoxon matched-pair test. The relative abundance of lipids in cuticular extracts from gynes, workers, and drones were compared via a Kruskal–Wallis ANOVA and Mann–Whitney U tests. All tests were run with Statistica 5.1 scientific software (StatSoft, Tulsa, OK, USA).

Results

Male Seeking Strategies Patrolling drones were observed at all three nesting sites, at different spots up to 185 m away from the actual nests. We found 10 different spots at site A and 6 at site B. Drones flew with their oriented heads towards vegetation such as bushes, trees, and hedges, which were only visited if they were in direct sunlight. After patrolling a spot for a few seconds, they flew to another one. No substrate marking behavior was observed, and there was no evidence that drones preferred to alight on specific parts of the vegetation. During the whole period, they only alighted five times and never at the same site. The maximum number of drones observed simultaneously at one spot was 4 ± 1.4 ($N=19$ observations) with no difference between sites A and C ($t=-1.39$, $df=17$, $P=0.183$), and there was no evidence of aggression (e.g., chasing) between individuals. Males started patrolling the early morning when it was sunny, even when temperatures were as low as 7°C , but they were never encountered after 4 P.M. ($N=5$ observations). On three occasions, drones were observed alighting on colony nesting boxes, but were chased away by workers before they could enter the hive.

Response to Caged Gynes Drones alighted more often on cages with gynes than on the control cages ($Z=2.201$; $P=0.028$; Table 1). During each trial, 2–7 drones were patrolling the bushes with cages.

Response to Gyne and Worker Extracts Drones alighted beside or on ($Z=2.37$, $P=0.018$; Table 2), and attempted to copulate ($Z=2.20$, $P=0.028$) more often with gyne extract-treated than control workers. In three cases, drones succeeded in copulating with the dead workers (Fig. 1). Workers treated with their own cuticle extract did not elicit significantly more landings ($N=4$) of drones than the controls ($N=3$; $Z=0.53$; $P=0.60$), and no copulation attempts were observed.

Response to Fecal Volatiles The litter containing nest fecal matter was completely ignored by the drones at all sites, and none ever alighted on a box.

Table 1 Number of *Vespa crabro* drones landing on cages containing virgin gynes and empty control cages

Caged Gyne	Empty Cage
2	0
5	0
0	0
2	0
7	0
2	0
4	0
^a Median values [first and third quartiles].	2 [2; 2.5] ^a
	0 [0; 0] ^a

Table 2 Responses of *Vespa crabro* drones to workers treated with cuticular extracts from gynes and untreated control workers

Pair No.	Landings		Copulation Attempts		Copulations	
	Treatment	Control	Treatment	Control	Treatment	Control
1	3	0	2	0	1	0
2	3	1	2	0	2	0
3	6	1	3	0	0	0
4	11	1	1	0	0	0
5	3	3	0	0	0	0
6	4	2	4	0	0	0
7	4	2	2	0	0	0
8	5	1	1	0	1	0
Total	39	11	15	0	3	0
Median value	3.5	1	1.5	0	0	0
[first and third quartiles]	[3; 5.25]	[0.75; 1.25]	[0.75; 2.25]	[0; 0]	[0; 0.25]	[0; 0]

Chemical Analyses The chemical analyses of cuticular extracts from gynes, workers, and drones by GC-MS revealed 65 chromatographic peaks but as some compounds co-eluted, 86 chemicals were identified (Table 3, Fig. 2). No components were detectable within the first 15 min. Apart from the two terpenoid esters citronellyl citronellate and citronellyl geranylate, all compounds were typical cuticular hydrocarbons (saturated, monounsaturated, monomethylalkanes, and dimethylalkanes) with chain lengths between 21 and 33 carbon units. Extracts from workers and drones were similar with only the relative abundance of 15 peaks being significantly different between the two. In contrast, the hydrocarbon profiles from queens were more diverse, and 50 out of the 65 peaks occurred in significantly higher relative abundances in gynes than in workers and drones. The most striking differences between extracts from gynes and those of workers and drones were that (a) citronellyl citronellate (peak 1) and citronellyl geranylate (peak 4) were not found in gynes; (b) whereas all three had monoenes with a double bond in position 9 (peaks 5, 16, 30, 46), gynes also had considerable amounts of other monoenes, particularly with double bonds in position 7 and 5

Fig. 1 Copulation of a *V. crabro* drone with a dead worker treated with a cuticular extract from a gyne



Table 3 Mean relative peak areas of cuticular hydrocarbons (± 1 SE) identified in dichloromethane extracts from gynes, workers, and drones of the European hornet *V. crabro*^a

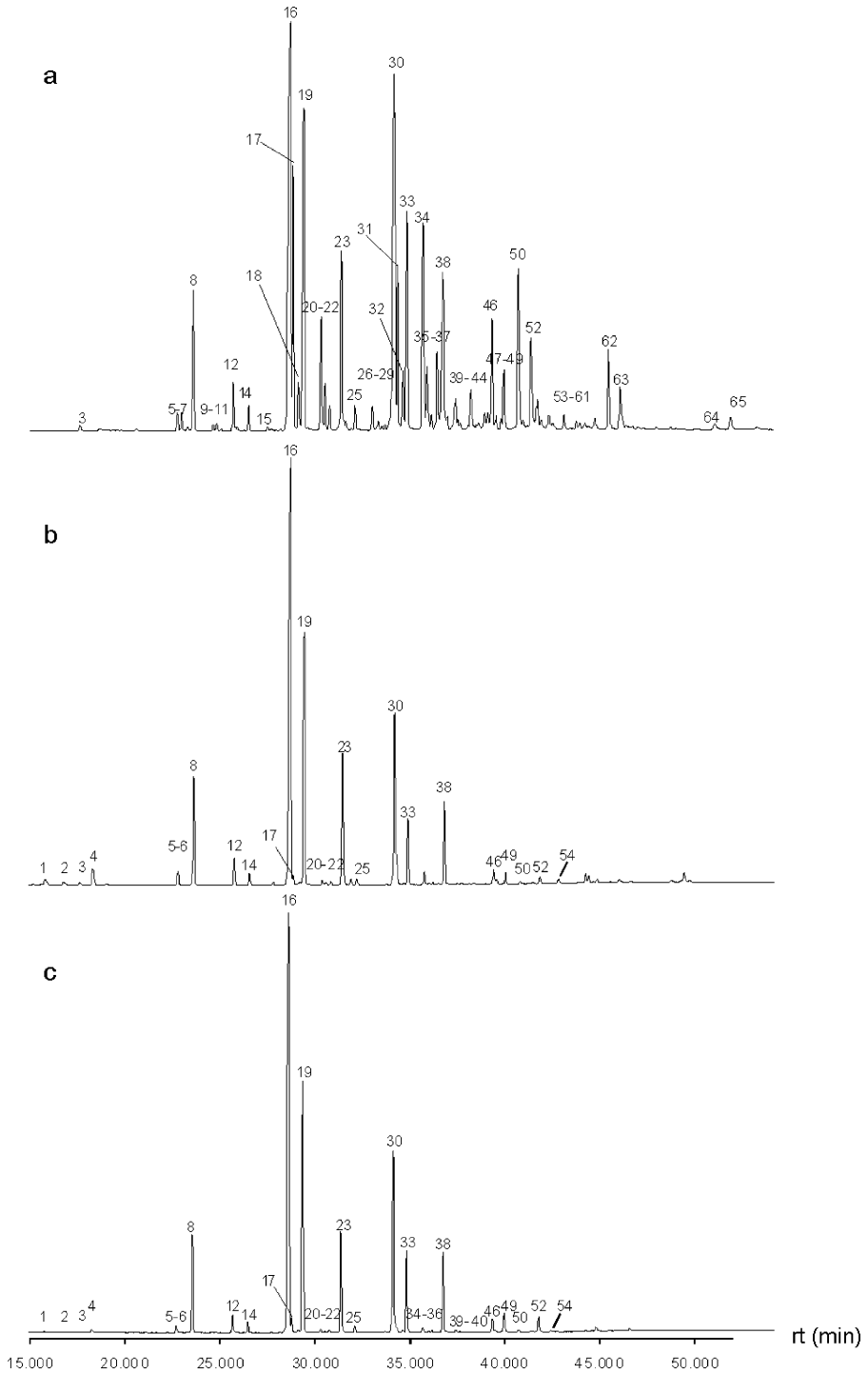
No.	RI	Compound	Gyne (n=4)	Worker (n=5)	Drone (n=6)	P value (Kruskal–Wallis)
1	2032	Citronellyl citronellate	0.00a	0.28 \pm 0.16b	0.07 \pm 0.01b	0.167
2	2071	C21:1(9)	0.00a	0.18 \pm 0.05b	0.05 \pm 0.01c	0.003
3	2100	C21	0.12 \pm 0.03a	0.21 \pm 0.01b	0.07 \pm 0.01a	0.007
4	2117	Citronellyl geranylate	0.00a	0.79 \pm 0.34b	0.16 \pm 0.03b	0.007
5	2272	C23:1(9)	0.32 \pm 0.07a	0.72 \pm 0.10b	0.48 \pm 0.02ab	0.028
6	2279	C23:1(7)	0.41 \pm 0.12a	0.06 \pm 0.01b	0.07 \pm 0.01b	0.010
7	2289	C23:1(5)	0.08 \pm 0.02a	0.00b	0.00b	0.002
8	2300	C23	4.1 \pm 0.6a	8.8 \pm 0.8b	7.3 \pm 0.4b	0.011
9	2336	11-MeC23	0.19 \pm 0.09a	0.00b	0.00b	0.002
10	2342	7-MeC23	0.09 \pm 0.02a	0.00b	0.00b	0.002
11	2351	5-MeC23	0.03 \pm 0.01a	0.00b	0.00b	0.002
12	2372	3-MeC23	1.1 \pm 0.2a	1.64 \pm 0.13b	1.4 \pm 0.1ab	0.042
13	2380	C23:1	0.05 \pm 0.01a	0.00b	0.00b	0.002
14	2400	C24	0.55 \pm 0.11	0.81 \pm 0.06	0.73 \pm 0.06	n.s.
15	2435	11-MeC24	0.07 \pm 0.03a	0.00b	0.00b	0.002
16	2476	C25:1(9)	15.6 \pm 1.0a	40.9 \pm 1.0b	36.9 \pm 1.2c	0.007
17	2481	C25:1(7)	4.7 \pm 0.5a	0.36 \pm 0.09b	0.67 \pm 0.04c	0.005
18	2490	C25:1(5)	0.80 \pm 0.10a	0.00b	0.11 \pm 0.02c	0.002
19	2500	C25	10.3 \pm 1.7a	17.6 \pm 0.7b	17.8 \pm 0.5b	0.018
20	2533	13-MeC25	2.4 \pm 0.3a	0.17 \pm 0.06b	0.42 \pm 0.08c	0.007
	2534	+11-MeC25				
21	2541	7-MeC25	0.76 \pm 0.09a	0.10 \pm 0.02b	0.14 \pm 0.02b	0.010
22	2550	5-MeC25	0.37 \pm 0.03a	0.15 \pm 0.02b	0.23 \pm 0.05ab	0.027
23	2574	3-MeC25	4.2 \pm 0.3a	7.2 \pm 0.3b	6.7 \pm 0.1b	0.011
24	2581	C25:1	0.2 \pm 0.01a	0.00b	0.00b	0.002
25	2600	C26	0.47 \pm 0.15	0.38 \pm 0.03	0.42 \pm 0.02	n.s.
26	2633	13-MeC26	0.37 \pm 0.05a	0.00b	0.00b	0.002
		+12-MeC26				
		+11-MeC26				
27	2645	C27:2	0.10 \pm 0.03a	0.00b	0.00b	0.002
28	2650	6-MeC26	0.03 \pm 0.01a	0.00b	0.00b	0.002
	2652	+C27:2	–	–	–	
29	2658	4-MeC26	0.11 \pm 0.01a	0.00b	0.00b	0.002
30	2676	C27:1(9)	9.9 \pm 1.3	10.1 \pm 1.2	11.6 \pm 0.6	n.s.
31	2682	C27:1(7)	3.0 \pm 0.8a	0.00b	0.28 \pm 0.04c	0.002
32	2692	C27:1(5)	1.1 \pm 0.3a	0.00b	0.10 \pm 0.01c	0.002
33	2700	C27	6.3 \pm 0.5a	3.3 \pm 0.3b	4.5 \pm 0.2c	0.004
34	2733	13-MeC27	5.2 \pm 0.8a	0.00b	0.47 \pm 0.09c	0.002
	2734	+11-MeC27				
	2737	+9-MeC27	–	–	–	
35	2742	7-MeC27	0.94 \pm 0.2a	0.00b	0.13 \pm 0.05c	0.002
36	2750	5-MeC27	0.29 \pm 0.03a	0.00b	0.16 \pm 0.01c	0.002
37	2762	11,15-DiMeC27	1.3 \pm 0.3a	0.00b	0.00b	0.002
38	2774	3-MeC27	4.7 \pm 0.5	4.5 \pm 0.24	5.3 \pm 0.2	n.s.
39	2783	7,x-DiMeC27	0.22 \pm 0.03a	0.00b	0.11 \pm 0.11ab	0.023
		+5,x-DiMeC27				
40	2806	3,15-DiMeC27	0.09 \pm 0.03a	0.00b	0.35 \pm 0.20c	0.042
		+3,13-DiMeC27	–	–	–	

Table 3 Continued

No.	RI	Compound	Gyne (<i>n</i> =4)	Worker (<i>n</i> =5)	Drone (<i>n</i> =6)	<i>P</i> value (Kruskal–Wallis)
41	2832	14-MeC28 +12-MeC28	0.57±0.15a	0.00b	0.00b	0.002
42	2843	C29:2	0.03±0.01a	0.00b	0.00b	0.013
43	2848	C29:2	0.06±0.03a	0.00b	0.00b	0.002
44	2860	12,16-DiMeC28 +11,15-DiMeC28 +4-MeC28	0.29±0.03a	0.00b	0.00b	0.002
45	2867	C29:1	–	–	–	0.002
46	2876	C29:1(9)	2.3±0.45a	0.63±0.13b	0.81±0.02b	0.017
47	2884	C29:1(7)	0.39±0.10a	0.00b	0.08±0.02c	0.004
48	2894	C29:1(5)	0.26±0.05a	0.00b	0.00b	0.002
49	2900	C29	1.9±0.6a	0.57±0.08b	1.1±0.05a	0.015
50	2932	15-MeC29	4.4±0.3a	0.13±0.04b	0.26±0.05b	0.010
	2933	+13-MeC29				
	2934	+11-MeC29				
	2936	+9-MeC29	–	–	–	
51	2943	7-MeC29	0.1±0.03a	0.00b	0.00b	0.002
52	2960	13,17-DiMeC29 +11,15-DiMeC29	2.4±0.3a	0.05±0.02b	0.15±0.05b	0.008
53	2972	7,15-DiMeC29	0.31±0.08a	0.00a	0.00a	0.002
54	2974	3-MeC29	0.89±0.18a	0.41±0.02b	0.98±0.02a	0.045
55	2981	5, x-DiMeC29	0.13±0.04a	0.00b	0.00b	0.002
56	3000	C30	0.31±0.12a	0.00b	0.00b	0.002
57	3032	15-MeC30 +14MeC30	0.32±0.07a	0.00b	0.00b	0.002
–	–					
58	3057	13,17-DiMeC30 3059 +4-MeC30	0.18±0.04a	0.00b	0.00b	0.002
59	3066	C31:1	0.11±0.03a	0.00b	0.00b	0.002
60	3078	C31:1(9)	0.21±0.04a	0.00b	0.00b	0.002
61	3100	C31	0.51±0.16a	0.00b	0.00b	0.002
62	3130	15-MeC31 3132 +13-MeC31 – 3133 +11-MeC31	1.9±0.4a	0.00b	0.00b	0.002
63	3157	13,17-DiMeC31 – +11,15-DiMeC31	1.2±0.4a	0.00b	0.00b	0.002
64	3329	15-MeC33 3331 +13-MeC33 3332 +11-MeC33	0.28±0.06a	0.00b	0.00b	0.002
65	3358	13,17-DiMeC33	0.51±0.18a	0.00b	0.00b	0.002

^a Numbers with different lowercase letters are significantly different at $P < 0.05$ (Mann–Whitney *U* test).

(peaks 6, 7, 17, 18, 31, 32, 47, 48); (c) 3-methyl derivatives (peaks 12, 23, 38) were common to all three morphs, but queens also had relative high amounts of alkanes with methyl branches at the more central positions 5, 7, 9, 11, 13, and 15 (peaks 9–11, 20–22, 34–36, 50, 51, 62); and (d) dimethylalkanes were generally more abundant in gyns (peaks 37, 39, 44, 52, 53, 55, 58, 63, 65).



Discussion

Our observations suggest that the mate finding strategy of *V. crabro* drones consists of nonaggressive patrolling behavior close to a nesting site, as reported for *V. maculifrons* (Post, 1980), without the establishment of substrate-based lek systems (Shelly and Whittier, 1997) seen in some paper wasps or hover wasps (Beani et al., 1992, 2002). This is supported by our observations, and those of Batra (1980) of drones patrolling at considerable distances from the nest. However, we rarely saw drones attempting to enter a nest, and when they tried, guard workers effectively drove them off. This is in contrast to the report of Batra (1980). However, one cannot rule out the possibility of the ability of *V. crabro* drones to enter nests under certain conditions, as intraspecific differences in mate seeking strategies have been reported for some bee species (Paxton, 2005). We did not observe marking behavior by *V. crabro* drones, as suggested by Batra (1980) or as been described in polistine wasps (Beani and Calloni, 1991). There are no examples of male marking behavior by vespine wasps in the literature (Downing, 1991).

The study demonstrates the involvement of a female sex pheromone in the mating system of *V. crabro*. The fact that drones alighted on cages containing gynes but never on control cages suggests that visual information is of minor importance. It is also unlikely that acoustic signals play a role in hornet mate finding, since they are neither able to detect airborne soundwaves nor able to produce sound with their folded wings. Hence, behavioral display by the gynes is not essential for male attraction. We found no evidence to support the hypothesis that volatiles of the feces from a hornet nest attract drones (Batra, 1980).

Summarizing these observations, the mating system of *V. crabro* fits into the conceptual framework about patterns in male-seeking tactics of Hymenoptera considering the rendezvous site, male sexual behavior, the use of sex pheromones, and the density and distribution of both sexes (Alcock et al., 1978; Ayasse et al., 2001). Since each nest produces several reproductives, this aggregation of gynes leads to a rendezvous site near the emerging site of the females, which are using sex pheromones to attract the males. Such a female-produced sex attractant is well known in ants (Hölldobler and Bartz, 1985) and other social wasps (Downing, 1991; Landolt et al., 1998), as well as in nonsocial parasitic wasps (Eller et al., 1984; Kaihno et al., 1991; Swedenborg and Jones, 1992; Swedenborg et al., 1993; McNeil and Brodeur, 1995).

Cuticular hydrocarbons are of importance for many social insect species (Howard and Blomquist, 2005), involved in nest (e.g., Singer and Espelie, 1992; Sumana et al., 2005) and nestmate (e.g., Ruther et al., 1998, 2002; Dani et al., 2001) recognition, as well as for marking trails between the actual nest and the entrance of the cavity housing a nest in a cavity breeding species (Steinmetz et al., 2002). In *Polistes dominulus* queens (Vespidae), there is a relationship between cuticular hydrocarbon profiles and the size of the corpora allata, the source of juvenile hormone that modulates ovarian development and sexual maturation (Sledge et al., 2001, 2004). A similar relationship could also exist in *V. crabro*, with males responding most to the cuticular profiles of sexually mature females. The active compounds in *V. crabro* gynes remain active, even when females are stored in separate glass vials at -18°C . This suggests that these bioactive hydrocarbons are stable and of low volatility, but clearly (as seen in this study) are sufficiently volatile to attract patrolling *V. crabro* males over some distance. A similar situation has been reported for

◀ **Fig. 2** Reconstructed total ion chromatograms of cuticle extracts from (a) gynes, (b) workers, and (c) drones of *V. crabro*. Peak numbers correspond to Table 3

deceptive orchids that mimic the sex pheromones of solitary bees, as flying *Andrena nigroaenea* males were attracted to dummies treated with a mixture of synthetic hydrocarbons with chain lengths between 21 and 29 carbon units (Schiestel et al., 1999, 2000).

Butts et al. (1995) reported that cuticular hydrocarbon profiles from *V. crabro* are colony-specific, with only quantitative differences between queens and workers. However, these authors identified only 13 compounds, possibly due to the use of suboptimal analytical techniques that could neither resolve the different monoenes nor detect numerous minor components. Another possible explanation for the difference between our results and those of the previous study is that the authors used old, mated queens from colonies rather than unmated gynes. As noted above, there is a relationship between cuticular profiles and ovarian development, and in addition the pheromone bouquet in some species of Hymenoptera can change after mating (Ayasse et al., 1990, 1999; Engels et al., 1993; Pankiw et al., 1996). Future studies are necessary to establish which of the gyne cuticular hydrocarbons are important in mate finding by *V. crabro* drones, probably by using GC-electroantennographic detection (EAD), as this approach has been successfully used for male hymenopterans (Schiestel et al., 1999).

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